AP Sites Are Not Significantly Involved in Mutagenesis by the (+)-*anti* Diol Epoxide of Benzo[*a*]pyrene: The Complexity of Its Mutagenic Specificity Is Likely To Arise from Adduct Conformational Polymorphism†

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Received November 30, 1992; Revised Manuscript Received April 5, 1993

ABSTRACT: In previous work, mutations induced by the (+)-*anti* diol epoxide of benzo[*a*]pyrene [(+)-*anti*-B[*a*]PDE] were scored in the *supF* gene of the *Escherichia coli* plasmid pUB3 [Rodriguez & Loechler (1993) *Biochemistry 32*, 1759]. pUB3 was reacted with (+)-*anti*-B[*a*]PDE and then either (1) transformed immediately into *E. coli* or (2) heated at 80 °C for 10 min prior to transformation. Heating only released a small fraction of adducts (~5%) and did not significantly affect the mutagenic pattern at most sites in *supF*. However, at the major base substitution hotspot, G_{115}, principally G→T mutations (87%) were obtained prior to heating, while after heating, G→T mutations decreased (45%) and G→A (21%) and G→C (33%) mutations became more prevalent. One model for this result is that prior to heating a heat-labile adduct at G_{115} causes one pattern of mutagenesis, but after heating the labile adduct is hydrolyzed to an apurinic site (AP site), which causes a second mutational pattern. To test this, a role for AP sites generated from labile adducts by heating at 80 °C for 10 min is investigated. It is shown that when plasmid pUB3 contains 22 (+)-*anti*-B[*a*]PDE adducts, 0.6% (or fewer) are converted to AP sites as determined in an assay based upon the action of an AP-endonuclease. In a separate line of investigation not involving (+)-*anti*-B[*a*]PDE adducts, mutation frequency (MF) per AP site is estimated. (In these experiments, AP sites were introduced into pUB3 by the classic procedure of heating at 70 °C/pH 5.0 to hydrolyze purines. In fact, the majority of mutants induced by this procedure probably arose via cytosine deamination to uracil and not via AP sites, based upon three criteria, including that ~75% of the mutations were GC→AT.) Given the number of AP sites formed from (+)-*anti*-B[*a*]PDE adducts and the estimate of MF/AP site, we conclude that <~2% of the 115 base substitution mutations induced in *supF* by (+)-*anti*-B[*a*]PDE can be attributed to AP sites when the adducted plasmid was heated at 80 °C for 10 min prior to transformation. (With the unheated adducted plasmid, this limit is <~1%). This makes it unlikely that AP sites are significantly involved in (+)-*anti*-B[*a*]PDE mutagenesis, including at G_{115}, where 29% of base substitution mutants were found. The most likely alternative model for the heat-induced changes in mutational pattern at G_{115}, as well as the complexity of the mutagenic spectra of (+)-*anti*-B[*a*]PDE in general, is that a single adduct can adopt multiple conformations, each of which can cause different kinds of mutations, and factors such as heating and DNA sequence context can influence adduct conformation.

We have been studying mechanisms of mutagenesis by benzo[*a*]pyrene (B[*a*]P)[1] (Benasutti *et al.*, 1988; Loechler, 1989; Loechler *et al.*, 1990; Mackay *et al.*, 1992; Rodriguez *et al.*, 1992; Rodriguez & Loechler, 1993a,b; Loechler, 1993), a well-known polycyclic aromatic hydrocarbon that is considered a bulky mutagen/carcinogen. B[*a*]P may be metabolized by a variety of pathways [e.g., Phillips *et al.* (1985), Marnett (1987), Cavalieri *et al.* (1990), and Devanesan *et al.* (1992)], but its corresponding (+)-*anti* diol epoxide [(+)-*anti*-B[*a*]PDE; Figure 1] is generally regarding to be the most important carcinogenic derivative (Conney, 1982; Phillips, 1983; Singer & Grunberger, 1983; Harvey, 1991) and the major DNA adduct is (+)-*anti*-B[*a*]P–N^2-Gua (Cheng *et al.*, 1989; Sayer *et al.*, 1991).

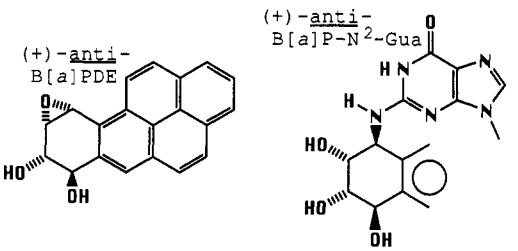

FIGURE 1: Structures.

In contrast to simple methylating agents, which principally cause a single kind of mutation [i.e., GC→AT mutations (references include Miller (1980, 1983), Burns *et al.* (1987), Richardson *et al.* (1987a,b), and Horsfall *et al.* (1990) via O^6-methylguanine [reviewed in Singer and Essigmann (1991)], the bulky mutagens/carcinogens typically have more complex mutational spectra [see Loechler (1993)]. For example, we have shown that (+)-*anti*-B[*a*]PDE induces a significant fraction of GC→TA (57%), GC→AT (23%), and GC→CG (20%) base substitution mutations, as well as deletions of a single G:C base pair in runs of G:C base pairs (Rodriguez & Loechler, 1993a,b). This kind of complexity has frequently been noted in mutational analyses of bulky mutagens/carcinogens, but explanations have remained elusive.

† This work was supported by grants from NIH (ES03775) and the American Cancer Society (CN-54).

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[1] Abbreviations: B[*a*]P, benzo[*a*]pyrene; (+)-*anti*-B[*a*]PDE, (+)-*r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (*anti*) (Figure 1); (+)-*anti*-B[*a*]P–N^2-Gua, the major adduct of (+)-*anti*-B[*a*]-PDE, which links the latter at its C10 position to Gua at the N^2-position (Figure 1); (+)-*anti*-B[*a*]PDE–pUB3, pUB3 adducted with ~22 adducts of (+)-*anti*-B[*a*]PDE/plasmid (Rodriguez & Loechler, 1993a,b); heated-(+)-*anti*-B[*a*]PDE, (+)-*anti*-B[*a*]PDE–pUB3 following 10 min at 80 °C (pH 6.8); MF, mutation frequency; AP sites, apurinic/apurimidinic sites.

0006-2960/93/0432-6555$04.00/0

Table I: Selected Data on the Mutagenic Specificity of both (+)-*anti*-B[*a*]PDE in the *supF* Gene of the *E. coli* Plasmid pUB3 and AP Sites

	G[a] →	T	A	C	MF[b]
5′-C<u>G</u>[115][c]	–heat[d]	13[e]	1	1	1.6
	+heat	15	7	11	1.4
5′-T<u>G</u>[f]	–heat	13	0	0	1.4
(all)	+heat	14	1	1	0.66
5′-G<u>G</u>[f]	–heat	16	5	2	2.5
(all)	+heat	15	6	8	1.2
AP site					
pattern 1		55%[g]	25%	20%	
pattern 2		81%	4%	15%	

[a] Assuming a G:C base pair, type of mutation observed at the Gua undergoing mutation. [b] Mutation frequency (×10[6]) as reported in Rodriguez and Loechler (1993b). [c] Sequence contexts with respect to the base on the 5′-side of a Gua undergoing mutation. [d] –Heat implies that pUB3 adducted with (+)-*anti*-B[*a*]PDE was not heated before transformation, while +heat implies the adducted DNA was heated for 10 min at 80 °C prior to transformation. [e] Number of mutants isolated of the indicated type; e.g., 13 G→T mutations were isolated in the absence of heat when Gua residues undergoing mutation had a Thy on their 5′-side. [f] "All" refers to the collection of all mutations at a Gua having a Thy (or Gua) on the 5′-side. [g] Percentage of G→T, A, and C mutations expected if an AP site is generated from a Gua residue based upon the analysis of Lawrence *et al.* (1991).

In previous work, we reacted the *Escherichia coli* plasmid pUB3 with (+)-*anti*-B[*a*]PDE *in vitro* [designated (+)-*anti*-B[*a*]PDE–pUB3], which was subsequently transformed immediately into *E. coli* (ES87) cells (Rodriguez & Loechler, 1993a). Mutations in a *supF* target gene in pUB3 were isolated and sequenced. In parallel, pUB3 adducted with (+)-*anti*-B[*a*]PDE was also heated at 80 °C for 10 min prior to transformation [designated heated (+)-*anti*-B[*a*]PDE–pUB3] (Rodriguez & Loechler, 1993b). Prior to heating, G→T mutations dominated at the major base substitution hotspot, G_{115}, while after heating G→A and G→C mutations became more prevalent (Table I). The probability that this difference was attributable to chance is slight ($P \sim 0.005$). Most other sites showed no such change in the pattern of mutagenesis, notably 5′-T<u>G</u>-3′ and 5′-G<u>G</u>-3′ sequence contexts, which changed marginally if at all (Table I). [Previous work (Rodriguez & Loechler, 1993a,b) revealed that the base on the 5′-side of the guanine undergoing mutation was important in defining the qualitative pattern of mutagenesis.]

One model for the results at G_{115} is that a heat-labile adduct (e.g., B[*a*]P–N7-Gua) causes one pattern of mutagenesis (i.e., principally G→T) prior to heating, while heating causes hydrolysis of the labile adduct, giving an apurinic site (AP site), which results in a different mutational pattern [model 2 in Rodriguez & Loechler (1993b)]. AP sites have previously been proposed as potential intermediates in the pathway of mutagenesis by bulky mutagens/carcinogens (Loeb, 1985), including by B[*a*]P (Eisenstadt *et al.*, 1982; Mazur & Glickman, 1988; Bernelot-Moens *et al.*, 1990). The pattern of mutations at G_{115} after heating is reasonably consistent with one of two patterns of AP site mutagenesis as described by Lawrence *et al.* (1991) based upon a site-directed study and a reanalysis of random mutagenesis studies on AP sites (Table I). [The two patterns of AP site mutagenesis may reflect the known structural complexity of AP sites, whose hydroxyl group can be alpha or beta, where the latter may adopt two conformations (Goljer *et al.*, 1992, and references cited therein).] Therefore, we wished to investigate AP site mutagenesis in our pUB3/*supF* system in relation to (+)-*anti*-B[*a*]PDE mutagenesis, especially vis-à-vis the change in mutational pattern at G_{115} following heating.

There were indications from our previous work that the model—heating causes changes in the mutational pattern via AP site formation—was not likely to be correct (Rodriguez & Loechler, 1993b). Our results herein support this by suggesting that AP sites are insufficiently mutagenic [presumably because of efficient repair [reviewed in Lindahl (1990) and Doetsch and Cunningham (1990)] given their rate of formation from (+)-*anti*-B[*a*]PDE adducts to account for the number of mutations observed at G_{115} or elsewhere in *supF*.

This conclusion means that other models for the complexity in mutational patterns for (+)-*anti*-B[*a*]PDE must be considered. Our current working hypothesis [model 1 in Rodriguez and Loechler (1993b)] is that a single adduct [probably (+)-*anti*-B[*a*]P–N^2-Gua] can adopt multiple conformations, where each can cause a different kind of mutations. Furthermore, the conformation adopted by an adduct may be influenced by various factors, including external factors, such as heating, as well as DNA sequence context. By this model, the changes seen in the mutational pattern at G_{115} reflect a heat-induced change in the conformation of an adduct at this site.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* strain ES87 and plasmid pUB3 are described in Rodriguez *et al.* (1992). Plasmid pXth1 was obtained from Dr. Bruce Demple, Harvard School of Public Health (Yi *et al.*, 1988). ES87/pXth1 cells were constructed by transformation.

Materials. All materials not explicitly mentioned were of the manufactures' highest grade purity.

Inducing AP Sites. The 3553 base pair plasmid pUB3 (Rodriguez *et al.*, 1992) was used for all experiments. AP sites were induced by the method of Lindahl and Andersson (1972) (Figure 2a). pUB3 plasmid DNA (2.9 μg/μL) was diluted with buffer (10 mM sodium citrate, 0.1 M NaCl, pH 5.0; preheated to 70 °C) to 0.12 μg/μL and heated at 70 °C for various lengths of time. To stop the reactions, samples were immediately placed on ice. Subsequently, samples were drop dialyzed at 20 °C (Marusyk & Sargeant, 1980) using a 0.025-μM filter (Millipore) against 10 mM Tris·HCl/1.0 mM EDTA (pH 8.0) for 90 min. AP sites were cleaved by treatment with 20 (or 50) units of exonuclease III (New England Biolabs) in 50 mM Tris·HCl/5 mM $CaCl_2$/10 mM 2-mercaptoethanol (pH 8.0).

AP Site Analysis. Approximately 100 and 500 ng of pUB3 containing heat-induced AP sites were analyzed concurrently, and the fraction of supercoiled plasmid in each sample was visualized following electrophoresis on a 0.8% agarose gel in the presence of 1 μg/mL ethidium bromide in TAE buffer (40 mM Tris·HCl/1 mM EDTA, pH 8.0/20 mM glacial acetic acid). Photographs of the gels were taken with Polaroid 665 positive/negative film using 366-nm light (Figure 2b). The relative intensities of the supercoiled band in each gel was determined with an Ultroscan XL enhanced laser densitometer (LKB Bromma) using GelScan XL software (Pharmacia). Five hundred nanograms of pUB3 was also analyzed because, when 100 ng of pUB3 was heated for longer periods of time (e.g., 42 and 52.5 min), the amounts of supercoiled plasmid remaining were barely visible (lanes 5 and 6, Figure 2b), making an accurate assessment by densitometry difficult at best.

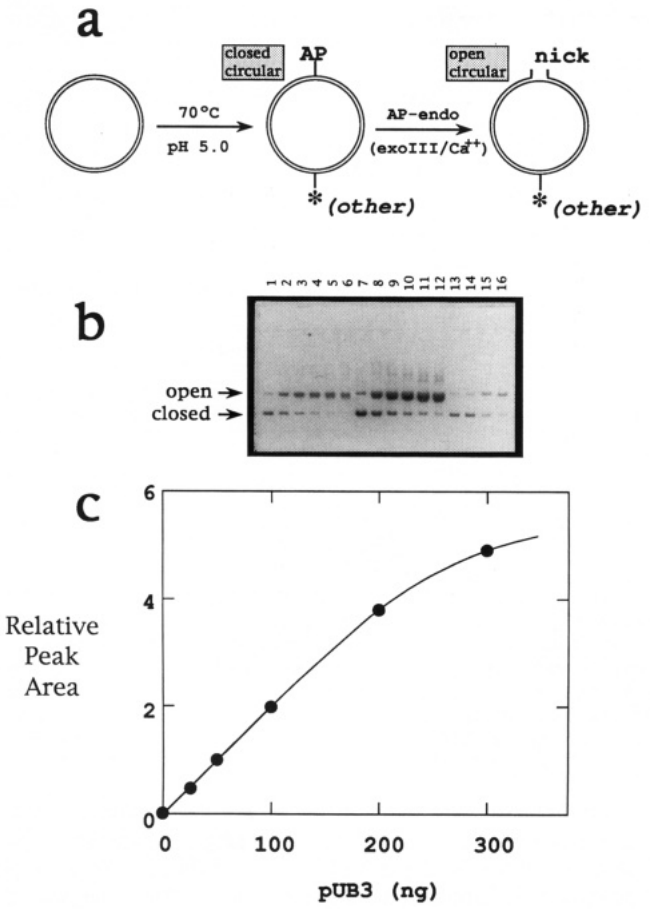

FIGURE 2: Assay for AP sites induced by the method of Lindahl and Andersson (1972) in the plasmid pUB3. (a) Supercoiled plasmid pUB3 was heated at 70 °C and pH 5.0 for varying lengths of time to induce AP sites. Treatment of this closed circular material with exonuclease III, an AP-endonuclease (Materials and Methods), caused nicking at AP sites and generated open circular pUB3. (b) Following the strategy in panel a, the material was subjected to agarose gel electrophoresis in the presence of ethidium bromide, where supercoiled pUB3 migrated more rapidly than open circular material (labeled closed and open, respectively). The gel was photographed with Polaroid positive/negative film, and the negative image is shown. Lanes 1–6 show the results for 0, 10.5, 21.0, 31.5, 42, and 52.5 min of heating, respectively, when ~100 ng was used. Lanes 7–12 show the same samples with 500 ng. The supercoiled (closed circular) band was scanned with a densitometer to determine relative peak areas. Lanes 13 and 14 show (+)-*anti*-B[*a*]PDE–pUB3 not treated and treated with exonuclease III, respectively. Lanes 15 and 16 show (+)-*anti*-B[*a*]PDE–pUB3 heated at 70 °C and pH 5.0 to induce ~1.4 AP sites, then not treated and treated with exonuclease III, respectively. Panel c shows a typical standard curve. Known amounts of supercoiled pUB3 were subjected to agarose gel electrophoresis and photographed with positive/negative film. Subsequently, the negative film was scanned with a densitometer, and panel c shows a plot of relative peak area vs concentration of supercoiled plasmid pUB3. From such standard curves, the concentration of supercoiled pUB3 (in panel b) could be estimated from their relative peak areas via interpolation.

To assess the relationship between DNA concentrations and band intensity, supercoiled pUB3 standards of known DNA concentrations were also analyzed in parallel. A standard curve was constructed by plotting peak area vs DNA concentration (Figure 2c), and the concentration of supercoiled plasmid in heated/exonuclease III treated samples was determined by interpolation. The number of AP sites/plasmid was calculated on the basis of the Poisson distribution

$$\text{AP sites/plasmid} = -\ln f$$

where f is the fraction of supercoiled pUB3 plasmid remaining after AP site induction/exonuclease III treatment, compared to the amount of supercoiled pUB3 plasmid in a control that was neither heated nor treated with exonuclease III. Lindahl and Nyberg (1972) showed that heating led to nicking at a fraction of the AP sites, although complete nicking required additional measures.

Determining the Number of AP Sites in Heated (+)-*anti*-*B*[*a*]*P–pUB3*. pUB3 plasmid was randomly adducted with (+)-*anti*-B[*a*]PDE (Rodriguez & Loechler, 1993a) and stored in 100 mM HEPES and 1 mM EDTA (pH 6.8) at –70 °C. Typically, 50–100 ng/μL of adducted and unadducted control pUB3 were heated at 80 °C for 10 min in volumes ranging from 10 to 100 μL. Samples were digested with exonuclease III and analyzed as described above.

A sample calculation follows. For experiment 1 of Table III with (+)-*anti*-B[*a*]PDE–pUB3 heated for 20 min, AP sites/plasmid = 1.05 (= $-\ln f = -\ln[0.35]$). The average is 1.08 for experiments 1–4 (Table III). To obtain a value for AP sites attributable to adducts only, the corresponding average for unadducted pUB3 is subtracted to give 0.45 (=1.08 – 0.63). There were 32.8 (+)-*anti*-B[*a*]PDE adducts/plasmid on average for experiments 1–4, while there were ~22 (+)-*anti*-B[*a*]PDE adducts/plasmid in the experiments of Rodriguez and Loechler (1993a,b). Accordingly, excess AP sites attributable to adducts are corrected to 0.30 (=0.45 × [22/32.8]).

The following procedure was performed to ensure that B[*a*]P adducts did not inhibit the actions of exonuclease III. Heated (+)-*anti*-B[*a*]PDE–pUB3 samples were first drop-dialyzed exactly as described above and then diluted 24-fold with 0.01 M sodium citrate/0.1 M NaCl (pH 5.0) to 2.1 ng/μL. Finally, AP sites were induced by heating at 70 °C for 21 min, which we estimated gave ~1.4 AP sites/plasmid. Afterward, the sample was drop-dialyzed again and digested with exonuclease III (as described).

Determining the AP-Endonuclease Activity of ES87 and ES87/pXth1 Cells. A 1-L flask containing 400 mL of Luria–Bertani (LB) medium [10% tryptone (Difco), 5% yeast extract (Difco), and 10% NaCl (J. T. Baker)] supplemented with 25 μg/mL streptomycin (Sigma) was inoculated with a 12-mL overnight culture of the strain ES87. The same was done with a strain of ES87/pXth1, except the LB medium contained 30 μg/mL chloramphenicol (Sigma), as well as streptomycin. The cells were grown by shaking vigorously at 37 °C to OD_{550} ~0.65 and then placed on ice for 10 min. The cells were decanted into 250-mL centrifuge bottles (100 mL/bottle) and again placed on ice. Half the cells (200 mL) from each strain remained on ice, while the other half (200 mL) were SOS-induced by our standard protocol (Rodriguez *et al.*, 1992), which is based upon that of Koffel-Schwartz *et al.* (1984). Afterward, both SOS-uninduced (two centrifuge bottles with 100 mL each) and SOS-induced cells (four centrifuge bottles with 200 mL each) were centrifuged at 8000 rpm for 10 min at 4 °C. SOS-uninduced cell pellets were resuspended in 200 mL of sterile, distilled water (4 °C). SOS-induced cells had been diluted four times by SOS induction; therefore, the pellets were resuspended in 100 mL of sterile distilled water (4 °C), and then two centrifuge bottles were combined so that each centrifuge bottle contained the same concentration of cells as the SOS-uninduced cells. Cells were centrifuged and pellets resuspended in 200 mL of sterile distilled water (4 °C). The cells were centrifuged again and resuspended in 8 mL of 50 mM Tris/10% glycerol/1 mM DTT (pH 7.5). Cells were lysed by passage through a French pressure cell (American Instrument Co.) at 10 000 psi. The lysates were centrifuged at 5000 rpm for 10 min (4 °C), and the supernatants were

Table II: AP Sites Induced in Unadducted pUB3 as a Function of Time of Heating at 70 °C and pH 5.0

	time of heating								
	1.05 min	2.10 min	3.15 min	4.20 min	10.5 min	21.00 min	31.50 min	42.00 min	52.50 min
AP sites induced[a]	0.016 (±0.04)	0.16 (±0.04)	0.27 (±0.04)	0.32 (±0.09)	0.55 (±0.11)	1.3 (±0.10)	2.1 (±0.23)	2.7 (±0.06)	3.5 (±0.30)
AP sites/min[b]	0.015	0.076	0.086	0.076	0.052	0.062	0.068	0.064	0.067

[a] All values are the average of three or four experiments using either 20 or 50 units of exonuclease III (Materials and Methods). [b] AP sites induced from line 2 divided by time of heating from line 1.

Table III: AP Sites Generated in Closed Circular (Supercoiled) pUB3 following Various Treatments, Including (+)-*anti*-B[*a*]PDE Adduction, Heating at 80 °C (pH 6.8), and/or Incubation with Exonuclease III

		% closed circular pUB3 (unadducted, control)				% closed circular (+)-*anti*-B[*a*]PDE–pUB3[a]			
		0 min of heating[b]		10 min of heating[b]	20 min of heating[b]	0 min of heating[b]		10 min of heating	20 min of heating
expt	units of exoIII[c]	–exoIII	+exoIII	+exoIII	+exoIII	–exoIII	+exoIII	+exoIII	+exoIII
1	20	100[d]	90	65	62	100	94	53	35
2	20	100	100	69	65	100	95	57	49
3	20	100	100	62	42	100	93	50	27
4	50	100	86	67	45	100	90	58	29
av AP sites/plasmid[e]			0.064 (±0.077)	0.42 (±0.046)	0.63 (±0.22)		0.073 (±0.023)	0.61 (±0.068)	1.08 (±0.27)
excess AP sites/plasmid[f]							0.01	0.13	0.30

[a] (+)-*anti*-B[*a*]PDE–pUB3 refers to pUB3 with 36.3, 29.9, 31.2, and 33.8 (+)-*anti*-B[*a*]PDE adducts per plasmid for experiments 1, 2, 3, and 4, respectively. [b] Time (in minutes) that unadducted pUB3 or (+)-*anti*-B[*a*]PDE–pUB3 was heated at 80 °C and pH 6.8 (Materials and Methods). [c] Units of exonuclease III (Materials and Methods). [d] Percentage of closed circular (supercoiled) pUB3 (either unadducted or adducted) after heating and treatment with exonuclease III, where comparison is to pUB3 (either unadducted or adducted) that was neither heated nor treated with exonuclease III (first column, 100%) (Materials and Methods). [e] The average number of AP sites per plasmid based upon the four experiments was determined by procedures outlined under Materials and Methods. [f] The difference between the number of AP sites in unadducted vs (+)-*anti*-B[*a*]PDE adducted pUB3. These values were normalized to 22 adducts/plasmid for comparison to (+)-*anti*-B[*a*]PDE–pUB3 in Rodriguez and Loechler (1993a,b).

kept on ice until prior to AP-endonuclease activity analysis.

AP-endonuclease enzyme assays were performed as described by Levin and Demple (1990). The substrate containing AP sites used in the enzyme assays was a generous gift from B. Demple and D. Ramotar.

Mutations Induced in pUB3 following Treatment by the Method of Lindahl. Unadducted pUB3 plasmid (16.5 μg) was ethanol precipitated and then resuspended in 25 μL of 10 mM sodium citrate/0.1 M NaCl (pH 5.0). AP sites were induced by heating the DNA at 70 °C for 0, 42, 84, and 126 min, which we estimate gave 0, 2.9, 5.8, and 8.7 AP sites, respectively, on the basis of the results in Table II. Thereafter, each sample was diluted with cold TE (pH 8.0) to a concentration of 3.3 ng/μL and placed on ice.

Competent ES87 and ES87/pXth1 cells were SOS-induced and transformed by electroporation. Cells containing pUB3 with *supF*⁻ mutations were selected on lactose minimal plates. These plasmids were isolated and sequenced. The details of these procedures are described elsewhere (Rodriguez *et al.*, 1992).

RESULTS

Quantitating AP Sites in pUB3. First, a reliable assay for AP sites was developed. AP sites were induced in supercoiled pUB3 plasmid by heating at 70 °C and pH 5.0 for various amounts of time (Figure 2a), which is the classic method of Lindahl and Andersson (1972). Thereafter, the samples were incubated with the multifunctional enzyme, exonuclease III, in the presence of Ca^{2+}, which selectively activates its AP-endonuclease activity (Rogers & Weiss, 1980). Any supercoiled pUB3 plasmids containing AP sites were nicked by exonuclease III treatment and converted to the open circular form, which was monitored in agarose gels containing ethidium bromide. These gels were photographed with positive/negative film (Figure 2b), and the negatives were analyzed densitometrically to determine the relative peak area associated with supercoiled pUB3 (labeled "closed" in Figure 2b). The concentration of supercoiled pUB3 remaining in each AP site-containing sample after exonuclease III treatment was interpolated from a standard curve (e.g., Figure 2c). The standard curve was generated when different amounts of supercoiled pUB3 standards were loaded in the same gel and analyzed similarly. Plots of peak area vs concentration of supercoiled pUB3 standards consistently gave smooth curves.

The fraction of supercoiled pUB3 remaining following exonuclease III treatment was used to estimate the average number of AP sites/plasmid on the basis of the Poisson distribution (Materials and Methods). In all cases, the data compared the decrease in peak area of supercoiled pUB3 plasmid both treated at pH 5.0/70 °C (i.e., containing AP sites) and treated with exonuclease III vs unheated/untreated pUB3 control: in two experiments 0.14 and 0.21 AP site/plasmid were observed when pUB3 was heated for 2.1 min; similarly, 0.12 and 0.16 AP sites/plasmid were observed when pUB3 was heated for 2.1 min when 2.5 times more exonuclease III was used in the same assay. This demonstrates both that the results are reproducible even at low levels of AP sites/plasmid and that the amount of exonuclease III used was sufficient to nick virtually every AP site.

Table II shows that the number of AP sites generated per unit of time is reasonably constant as estimated for the eight time points between 2.1 (0.16 AP site/plasmid) and 52.5 min (3.5 AP sites/plasmid) of heating (average, ~0.069 AP site plasmid^{-1} min^{-1}).[2] (Each data point in Table II is the average of three or four experiments, and in each case increasing the amount of exonuclease III did not affect the results.) This implies that our assay is reliable at least over this range of AP sites/plasmid.

How Many AP Sites Are Found in Heated (+)-*anti-B*[*a*]-*P–pUB3*? Plasmid pUB3 was reacted with (+)-*anti*-B[*a*]-

[2] ~0.069 AP site plasmid^{-1} min^{-1} compares reasonably well with ~0.095 AP sites plasmid^{-1} min^{-1} (following correction for relative plasmid size) determined by Lindahl and Andersson (1972), who assumed that nicks in alkali sucrose gradients were attributable to AP sites. It is possible that some of these nicks arose from other heat-induced lesions, which could account for the small difference between our values.

PDE to give between 29.9 and 36.3 adducts/plasmid (Table III) and then subsequently heated at 80 °C for 0, 10, and 20 min at pH 6.8. After the adducted samples (and unadducted controls) were heated, they were incubated with exonuclease III and analyzed by the procedures described above. When unadducted (control) pUB3 was heated for 10 and 20 min at 80 °C (pH 6.8), a small number of AP sites were induced, while only slightly more AP sites were induced in (+)-*anti*-B[*a*]PDE–pUB3 (Table III).[3] On the basis of the results in Table III, ~0.13 and ~0.30 AP sites are formed from (+)-*anti*-B[*a*]PDE adducts in (+)-*anti*-B[*a*]PDE–pUB3 following heating at 80 °C for 10 and 20 min, respectively. (The values were normalized to 22 adducts/plasmid for comparison to our mutational studies.) The reliability of these values is supported by the results in Table II.

One potential caveat to the results in Table III is that (+)-*anti*-B[*a*]PDE adducts might inhibit the action of exonuclease III. To test this possibility, ~1.4 AP sites were purposely introduced by the Lindahl method (see above) into (+)-*anti*-B[*a*]PDE–pUB3 and analyzed by treatment with exonuclease III. Virtually no nicking occurred in (+)-*anti*-B[*a*]PDE–pUB3 treated with exonuclease III when AP sites were not present [Figure 2b, lanes 13 (–exoIII) and 14 (+exoIII), respectively]. However, when AP sites were present in (+)-*anti*-B[*a*]PDE–pUB3, exonuclease III is clearly active [Figure 2b, lanes 15 (–exoIII) and 16 (+exoIII), respectively]. Thus, (+)-*anti*-B[*a*]PDE adducts did not inhibit the ability of exonuclease III to recognize AP sites.

Mutation Frequency Resulting from AP Sites Induced in pUB3 by the Method of Lindahl. Having assessed how many AP sites were formed from (+)-*anti*-B[*a*]PDE adducts in (+)-*anti*-B[*a*]PDE–pUB3, we needed to determine how mutagenic the AP sites are to estimate the relative contribution of AP sites to (+)-*anti*-B[*a*]PDE mutagenesis. To study the mutagenic potency of AP sites, pUB3 was treated at 70 °C and pH 5.0 for various times, after which treatment the DNA was transformed into ES87 cells by electroporation and the MF determined (Figure 3). The presence of 8.7 AP sites/plasmid in pUB3 increased MF from background ($\sim 1.6 \times 10^{-6}$) to $\sim 11 \times 10^{-6}$. (The data points in Figure 3 are the average of two experiments.)

Although MF did increase, this may not be due to AP site mutagenesis, because heating is known to induce other potential mutagenic lesions including uracil via cytosine deamination (Shapiro & Klein, 1966; Sowers *et al.*, 1987). In this regard, two lines of investigation were pursued.

First, we reasoned that an AP site can only be mutagenic if it is not repaired. Thus, if the level of AP-endonuclease was increased in ES87 cells, then MF should decrease if AP sites were responsible. Therefore, ES87 cells were transformed with the plasmid pXth1 (Yi *et al.*, 1988), which contains the *xth* gene that encodes the AP-endonuclease, exonuclease III. (Hereafter this cell is referred to as ES87/pXth1.) ES87/pXth1 cells were shown to have approximately 30 times more class II AP-endonuclease activity in comparison to ES87 cells (Table IV). SOS induction appeared to increase AP-endonuclease activity slightly in both strains. The results in Figure 3 show that MF in ES87/pXth1 cells (open circles)

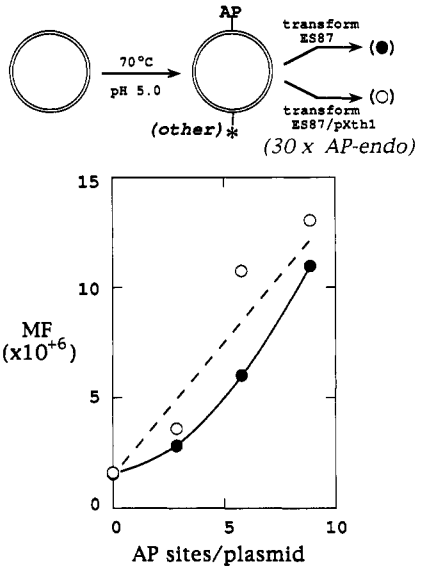

FIGURE 3: AP site mutagenesis following the heating of supercoiled plasmid pUB3 at 70 °C and pH 5.0. Supercoiled, unadducted pUB3 was heated for varying lengths of time, then transformed into *E. coli* ES87 cells, and MF was determined (Materials and Methods). MF vs the number of AP sites (as determined by the procedures outlined in Figure 2) is shown (solid circles, which is the average of two experiments). In addition, the same heated pUB3 DNA was transformed into ES87/pXth cells, which contain 30 times more AP-endonuclease activity (see text and Table IV) and MF determined (open circles, which is the average of two experiments). The solid line is the best smooth curve drawn through the results for ES87 cells, while the straight dashed line is the best straight line drawn through the entire date set.

Table IV: AP-Endonuclease Activity in ES87 and ES87/pXth1 Cells

strain	specific activity[a] (units/mg of protein) –SOS[b]	+SOS[b]
ES87	220	230
ES87/pXth1	6320	8390

[a] The specific activity of class II AP-endonucleases was determined (Materials and Methods), where units are defined according to the method of Levin and Demple (1990). [b] –SOS and +SOS imply SOS-uninduced and SOS-induced cells, respectively (Materials and Methods).

did not decrease vs ES87 cells (solid circles) at several levels of AP sites/plasmid. This result argues against AP sites being the primary cause of these mutations. The yield of progeny plasmids from transformations with unadducted pUB3 with 8.7 AP sites/plasmid was ~58% in ES87 cells and ~44% in ES87/pXth1 cells.

Second, we determined the DNA sequences of the *supF* mutants to assess if they were consistent with either of the two patterns for AP site mutagenesis (Table I). Of the 41 mutants sequenced, 36 were base substitutions and 33 were at G:C base pairs. The majority of mutations in both ES87 and ES87/pXth1 cells were GC→AT (76% and 73%, respectively; Table V).

DISCUSSION

The suggestion that AP sites might play a role in (+)-*anti*-B[*a*]PDE mutagenesis is based upon certain similarities in their mutagenic specificity (Eisenstadt *et al.*, 1982; Loeb, 1985; Mazur & Glickman, 1988; Bernelot-Moens *et al.*, 1990). This is ostensibly reasonable because a labile adduct from racemic (+/–)-*anti*-B[*a*]PDE has been described in the literature (Osborne *et al.*, 1978; King *et al.*, 1979; Sage & Haseltine, 1984; Lobanenkov *et al.*, 1986; Rill & Marsch,

[3] Heating unadducted pUB3 at 70 °C and pH 5.0 in citrate buffer induced ~0.069 AP site/min (Table II), while heating at 80 °C and pH 6.8 in HEPES buffer induced ~0.035 AP site/min (Table III). The latter is so high principally because amine buffers catalyze nicking at AP sites more readily than oxygen buffers (data not shown), presumably because amines are better nucleophiles. The difference in temperature is also significant.

Table V: Base Substitution Mutations[a] Induced in *supF* When pUB3 Was Heated at 80 °C and pH 5.0

strain	G→T[b]	G→A	G→C	A→N[c]
ES87	1[d] (5%)	16 (76%)[e]	2 (10%)	2 (10%)[f]
ES87/pXth1	3 (20%)	11 (73%)	0 (<7%)	1 (7%)[g]
total	4 (11%)	27 (75%)	2 (6%)	3 (8%)

[a] Mutations other than base substitutions were also isolated. ES87: $\Delta(78–129)$; $+C_{174}$; ΔC_{109}. ES87/pXth1: ΔG_{103}; insertion at G_{116} (no homology to any known *E. coli* gene in Genbank). [b] Assuming a mutation at a Gua in a G:C base pair in *supF*. [c] Assuming a mutation at an Ade in an A:T base pair in *supF*. [d] Number of mutants isolated of the indicated type; e.g., 16 G→A mutants were isolated from ES87 cells transformed with pUB3 containing induced AP sites. [e] One G_{116}→A also was ΔA_{128}. One G_{181}→A also had a 2 base, GG, insertion at G_{174}. [f] A_{154}→T and A_{190}→G, which was also ΔG_{109}. [g] A_{140}→G.

1990), which has been attributed to B[*a*]P–N7-Gua, and has been estimated to represent as much as ~40% of all adducts in certain cases (King *et al.*, 1979; Sage & Haseltine, 1984).

We have determined the number of AP sites formed from (+)-*anti*-B[*a*]PDE adducts (Results). In an independent line of investigation not involving (+)-*anti*-B[*a*]PDE adducts, we estimate the mutagenic potency of AP sites in our *supF* system (next section). From this information, the contribution of AP sites to (+)-*anti*-B[*a*]PDE mutagenesis can be assessed.

Mutagenicity of AP Sites in supF of pUB3 (in the Absence of (+)-anti-B[a]PDE Adducts). AP sites were introduced into unadducted ds pUB3 by heating at 70 °C and pH 5.0 (Figure 2; Table II). However, for three reasons it is unlikely that the AP sites induced by this treatment are responsible for the mutations in Figure 3 and Table V.

(*1*) GC→AT mutations predominated (Table V), which is not consistent with the published patterns of AP site mutagenesis. Assuming AP site generation at purines [as first established by Lindahl and Nyberg (1972)], GC→TA mutations should dominate (Table I) on the basis of both random (Kunkel, 1984) and site-directed mutagenesis studies (Lawrence *et al.*, 1991).

(*2*) When DNA is treated at 70 °C and pH 5.0, AP sites are induced ~1.5 times more rapidly at Gua than at Ade (Lindahl & Andersson, 1972), which correlates with the observation that AP site mutagenesis occurs ~1.5 times more frequently at Gua than at Ade by this same treatment [from Table 4 of Kunkel (1984) following correction for target size]. Of the three mutations at A:T base pairs (Table V), only one AT→TA mutation is likely to be attributable to an AP site.[4] Taken together, this predicts that only a small number of base substitution mutations at G:C base pairs in Table V can be attributed to AP site mutagenesis. Although the exact number is uncertain [~2.5 is estimated following correction for relative G:C vs A:T target size in *supF* [see Rodriguez and Loechler (1993a)]], it is certainly less than the 33 collected. Thus, the fraction of mutations at G:C vs A:T base pairs is not consistent with a major role for heat-induced AP site mutagenesis.

(*3*) MF did not decrease in ES87/pXth1 cells vs ES87 cells (Figure 3), which argues against mutagenesis by AP sites. Two caveats must be noted: (i) it is possible that some step other than the action of an AP-endonuclease is rate limiting in repair, though this seems unlikely because—following AP-endonuclease nicking at an AP site—it is doubtful that a mutation could occur by replication bypass of the AP site because of the nick beside it; (ii) the additional AP-endonuclease activity in ES87/pXth1 cells is not functional, which seems unlikely given that it is detectable in extracts from these very cells.

These three points argue that most of the mutants obtained by heating unadducted ds pUB3 are not due to AP sites, which is probably because AP sites are efficiently repaired in ds DNA in *E. coli* [reviewed in Lindahl (1990) and Doetsch and Cunningham (1990)]. All reported studies (of which we are aware) on AP site mutagenesis in *E. coli* employed ss vectors [reviewed in Loeb and Preston (1986), Kunkel (1984), and Lawrence *et al.* (1991)]. [AP sites do appear to contribute to the mutations induced when a ds mammalian shuttle plasmid is heated (Gentil *et al.*, 1984)]. The majority of mutants in Table V are more likely to be attributable to cytosine deamination to uracil, which causes GC→AT mutations (Coulondre, *et al.*, 1978) and has been studied by heating ds plasmids (Frederico *et al.*, 1990).

Although another method to introduce AP sites into pUB3 might have been pursued, the following calculations demonstrate that a value for MF/AP site can be estimated from these data that is adequate for our purposes. On the basis of the one AT→TA mutation (see above) and the four G→T mutations (Table V) and assuming pattern 1 AP site mutagenesis (Table I), which is conservative, we estimate that no more than ~23% of the mutations in Table V can be attributed to AP sites. From Figure 3 the limit on the mutation frequency per AP site (assuming a linear dose response curve) is as follows: MF/AP site ~ 0.25×10^{-6} [$=0.23 \times$ [$(11.0 - 1.6) \times 10^{-6}$ MF/8.7 AP sites]]. To build in a margin of error, we will use MF/AP site < 0.5×10^{-6}.

Do AP Sites Contribute to Mutagenesis from (+)-anti-B[a]PDE? When (+)-*anti*-B[*a*]PDE–pUB3 was treated at 80 °C for 10 min, <~0.2 AP site/plasmid formed from the 22 (+)-*anti*-B[*a*]PDE adducts/plasmid (Table III). We can safely use this same limit as an estimate for the number of AP sites/plasmid with unheated (+)-*anti*-B[*a*]PDE–pUB3 as well, because—although the actual limit is likely to be much lower on the basis of both intuition and the results in Table III—the results in Table II suggest that it is difficult to estimate reliably fewer than ~0.16 AP site/plasmid. From these estimates, the MF due to AP sites formed from (+)-*anti*-B[*a*]PDE adducts in either unheated or heated (+)-*anti*-B[*a*]PDE–pUB3 is <~0.10×10^{-6} (=[~0.2 AP site/plasmid] × [$<0.5 \times 10^{-6}$ MF/AP site]).

If the value in the previous paragraph is compared to MF = 9.0×10^{-6} for base substitution mutagenesis with 22 adducts per plasmid for unheated (+)-*anti*-B[*a*]PDE–pUB3 (Rodriguez & Loechler, 1993a), AP sites account for <~1.1% (=[<0.10]/[9.0] × 100%) of the mutations. When a similar comparison is done for heated (+)-*anti*-B[*a*]PDE–pUB3 [MF = 4.8×10^{-6} (Rodriguez & Loechler, 1993b)], AP sites account for <~2.1% (=[<0.10]/[4.8] × 100%) of the base substitution mutations. In the case of the latter [heated (+)-*anti*-B[*a*]PDE–pUB3], 115 base substitution mutants were isolated, so <2.4 of these could be due to AP sites generated from (+)-*anti*-B[*a*]PDE adducts.

AP Site from a Heat-Labile Adduct Cannot Explain the Results at G_{115}. One model for the results at G_{115} [introduction; model 2 in Rodriguez and Loechler (1993b)] is that a labile adduct (e.g., B[*a*]P–N7-Gua) is responsible for mutations prior to heating but is hydrolyzed by heating to an AP site, which is responsible for the mutational pattern after heating. Pattern 1 for AP site mutagenesis (Table

[4] Two mutations at A:T base pairs were AT→GC, which are unlikely to be attributable to AP site mutagenesis because this predicts Cyt insertion opposite an AP site, which is the least frequent insertional event (Kunkel, 1984), and one of these two was AT_{140}→GC, which is the major spontaneous base substitution hotspot (Rodriguez et al., 1992).

I)—assuming a Gua adduct—is consistent with the pattern of mutations observed at G_{115} following heating. (A test for difference gave $P \simeq 0.16$.)

Table I shows that 33 (of 115 or 29%) of the base substitution mutations were isolated at G_{115} for the heated (+)-*anti*-B[*a*]-PDE–pUB3 sample. Thus, even if all three AP site mutants in *supF* (see previous section) were assumed to be generated solely at G_{115}, the majority of mutants observed at G_{115} following heating cannot be accounted for by AP site mutagenesis, unless AP sites at G_{115} are $>\sim 10$ times more mutagenic than on average. This makes it unlikely that AP sites play a role in the changes in mutational pattern at G_{115}.

It is important to evaluate the assumptions made in these calculations. (1) A 2-fold margin of error was used in estimating how many of the mutants in Table V were due to AP sites. (2) A linear dose response curve was assumed for the relationship between MF and heat-induced AP sites. For ES87 cells, this relationship looks decidedly nonlinear (solid curve in Figure 3), and the assumption of linearity is conservative. (3) If the value of <2.4 mutants is spread over the entire *supF* gene (85 base pairs), then the number of mutations that might be attributed to AP sites formed at G_{115} would be reduced. (4) That fewer AP sites are formed in *supF* by treating pUB3 at 70 °C and pH 5.0 than on average is the only obvious potential source of an underestimation of the role of AP sites in (+)-*anti*-B[*a*]PDE mutagenesis.

For the reasons cited above, AP sites also do not contribute significantly to (+)-*anti*-B[*a*]PDE mutagenesis in general, even following heating at 80 °C for 10 min. This conclusion is bolstered by the following. Heating unadducted pUB3 for 10 min at 80 °C induced ~0.4 AP site/plasmid (Table III). In previous work we showed that this treatment increased the spontaneous MF marginally [from ~1.0 to $\sim 1.3 \times 10^{-6}$ in Rodriguez and Loechler (1993b)]. Heating (+)-*anti*-B[*a*]-PDE–pUB3 for 10 min at 80 °C induced ~0.6 AP site/plasmid (Table III), from which MF $\sim 1.5 \times 10^{-6}$ might be expected on the basis of a analogy to the results with unadducted pUB3. In fact, MF $\sim 10.5 \times 10^{-6}$ was obtained (Rodriguez & Loechler, 1993b), which implies that the adducts themselves, and not AP sites, are principally responsible for the mutations.

Although less rigorously quantitative, Schaaper *et al.* (1982) concluded it was unlikely that AP sites played a major role in mutagenesis by racemic *anti*-B[*a*]PDE in *E. coli*. Drinkwater *et al.* (1980) concluded that <1% of all mutations from racemic *anti*-B[*a*]PDE can be attributed to AP sites in a eukaryotic system.

Previously, we showed that ~5% of (+)-*anti*-B[*a*]PDE adducts are released by heating at 80 °C for 10 min (Rodriguez & Loechler, 1993b). On the basis of our unpublished data, the labile adduct is (+)-*anti*-B[*a*]P-N^2-Gua and *not* (+)-*anti*-B[*a*]P–N7-Gua, and (+)-*anti*-B[*a*]P–tetrol is released by heating, which would not be expected to generate an AP site. The latter is consistent with the findings of Moran and Ebisuzaki (1991) that labile adducts from *anti*-B[*a*]PDE do not generate AP sites *in vivo*.

Adduct Conformational Polymorphism. If the change in mutational pattern at G_{115} in *supF*, as well as other results, cannot be traced to the presence of AP sites, then what are the alternatives? A role for heat-induced chemical modification of an adduct [model 4 in Rodriguez and Loechler (1993b)] appears to be unlikely because heating does not modify the chemical structure of the (+)-*anti*-B[*a*]P-N^2-Gua adducts remaining in (+)-*anti*-B[*a*]PDE–pUB3 (Drouin and Loechler, unpublished findings). Heat-induced deamination of the cytosine opposite a Gua adduct is also unlikely to be responsible because (i) this would not account for the decrease in GC→TA mutations (Table I); (ii) this would predict an increase in GC→AT but not GC→CG mutations at G_{115}; and (iii) this does not explain why G_{115} is the only site where this occurs.

Our current working hypothesis is that, prior to heating, an adduct at G_{115} is in one conformation that causes one mutational pattern, while after heating it is in another conformation that causes a different mutational pattern. This is model 1 in Rodriguez and Loechler (1993b), which discusses in greater detail several unintuitive features of this model. If this model is correct, it may provide a unifying hypothesis for much of our base substitution mutational data. The pattern of mutation observed at G_{115} prior to heating is remarkably similar to that observed in the sequence context, 5′-T<u>G</u>-3′, where G→T mutations were virtually exclusively obtained independent of heating (Table I). By model 1, we would argue that, independent of heating, the mutagenic specificity is restricted because the Gua adduct undergoing mutation is trapped in one conformation with a Thy on its 5′-side. In contrast, the pattern of mutation observed at G_{115} after heating is remarkably similar to the pattern observed in 5′-G<u>G</u>-3′ sequences, where G→A and G→C mutations are more significant (Table I). By model 1, we would argue that the Gua adduct undergoing mutation is principally trapped in a second conformation when it has a Gua on its 5′-side.

ACKNOWLEDGMENT

We gratefully acknowledge Bruce Demple, Harvard School of Public Health, for the plasmid pXth1 and for helpful discussions, and he and Dindial Ramotar for providing the substrate used to measure the AP-endonuclease activities in *E. coli*. We also gratefully acknowledge the Cancer Research Program of the National Institute, Division of Cancer Cause and Prevention, Bethesda, MD, for providing (+)-*anti*-B[*a*]-PDE.

REFERENCES

Benasutti, M., Ezzedine, Z. D., & Loechler, E. L. (1988) *Chem. Res. Toxicol. 1*, 160–168.

Bernelot-Moens, C., Glickman, B. W., & Gordon, A. J. E. (1990) *Carcinogenesis 11*, 781–785.

Burns, P. A., Gordon, A. J. E., & Glickman, B. W. (1987) *J. Mol. Biol. 194*, 385–390.

Cavalieri, E. L., Rogan, E. G., Devanesan, P. D., Cremonesi, P., Cerny, R. L., Gross, M. L., & Bodell, W. J. (1990) *Biochemistry 29*, 4820–4827.

Cheng, S. C., Hilton, B. D., Roman, J. M., & Dipple, A. (1989) *Chem. Res. Toxicol. 2*, 334–340.

Conney, A. H. (1982) *Cancer Res. 42*, 4875–4917.

Coulondre, C., Miller, J. H., Farabaugh, P. J., & Gilbert, W. (1978) *Nature 274*, 775–780.

de los Santos, C., Cosman, M., Hingerty, B. E., Ibanez, V., Margulis, L. A., Geacintov, N. E., Broyde, S., & Patel, D. J. (1992) *Biochemistry 31*, 5245–5252.

Devanesan, P. D., RamaKrishna, N. V. S., Todorovic, R., Rogan, E. G., Cavalieri, E. L., Jeong, H., Jankowiak, R., & Small, G. J. (1992) *Chem. Res. Toxicol. 5*, 302–309.

Doetsch, P. W., & Cunningham, R. P. (1990) *Mutat. Res. 236*, 173–201.

Drinkwater, N. R., Miller, E. C., & Miller, J. A. (1980) *Biochemistry 19*, 5087–5092.

Eisenstadt, E., Warren, A. J., Porter, J., Atkins, D., & Miller, J. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1945–1949.

Frederico, L. A., Kunkel, T. A., & Shaw, B. R. (1990) *Biochemistry 29*, 2532–2537.

Gentil, A., Margot, A., & Sarasin, A. (1984) *Mutat. Res. 129*, 141–147.
Goljer, I., Withka, J. M., Kao, J. Y., & Bolton, P. H. (1992) *Biochemistry 31*, 11614–11619.
Harvey, R. G. (1991) *Polycyclic Aromatic Hydrocarbons: Chemistry and Cancer*, Cambridge University Press, Cambridge, U.K.
Horsfall, M. J., Gordon, A. J. E., Burns, P. A., Zielenska, M., van der Vliet, G. M. E., & Glickman, B. W. (1990) *Environ. Mol. Mutagen. 15*, 107–122.
King, H. W. S., Osborne, M. E., & Brookes, P. (1979) *Chem.-Biol. Interact. 24*, 345–353.
Koffel-Schwartz, N., Verdier, J. M., Bichara, M., Freund, A. M., Daune, M. P., & Fuchs, R. P. P. (1984) *J. Mol. Biol. 177*, 35–51.
Kunkle, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1494–1498.
Lawrence, C. W., Borden, A., Banerjee, S. K., & LeClerc, J. E. (1991) *Nucleic Acids Res. 18*, 2153–2157.
Levin, J. D., & Demple, B. (1990) *Nucleic Acids Res. 18*, 5069–5075.
Lindahl, T. (1990) *Mutat. Res. 238*, 305–311.
Lindahl, T., & Andersson, A. (1972) *Biochemistry 11*, 3618–3623.
Lindahl, T., & Nyberg, B. (1972) *Biochemistry 11*, 3610–3617.
Lobanenkov, V. V., Plumb, M., Goodwin, G. H., & Grover, P. L. (1986) *Carcinogenesis 7*, 1689–1695.
Loeb, L. A. (1985) *Cell 40*, 483–485.
Loeb, L. A., & Preston, B. D. (1986) *Annu. Rev. Genet. 20*, 201–230.
Loechler, E. L. (1989) *Biopolymers 28*, 909–927.
Loechler, E. L. (1991) *Methods Enzymol. 203*, 458–476.
Loechler, E. L. (1993) in *The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance* (Eaton, D. L., & Groopman, J. D., Eds.) Academic Press, Orlando, FL (in press).
Loechler, E. L., Benasutti, M., Basu, A. K., Green, C. L., & Essigmann, J. M. (1990) in *Progress in Clinical and Biological Research, Vol. 340A, Mutation and the Environment, Part A: Basic Mechanisms* (Mendelsohn & Albertini, Eds.) pp 51–60, Wiley-Liss, New York.
Mackay, W. B., Benasutti, M., Drouin, E., & Loechler, E. L. (1992) *Carcinogenesis 13*, 1415–1425.
Marnett, L. J. (1987) *Carcinogenesis 8*, 1365–1373.
Marusyk, R., & Sargeant, A. (1980) *Anal. Biochem. 105*, 403–404.
Mazur, M., & Glickman, B. W. (1988) *Somat. Cell Mol. Genet. 14*, 393–400.
Miller, J. H. (1980) in *The Operon* (Miller, J. H., & Reznikoff, W. S., Eds.) 2nd ed., pp 31–88, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Miller, J. H. (1983) *Annu. Rev. Genet. 17*, 215–238.
Moran, M. F., & Ebisuzaki, K. (1991) *Mutat. Res. 262*, 79–84.
Osborne, M. E., Harvey, R. G., & Brookes, P. (1978) *Chem.-Biol. Interact. 20*, 123–130.
Phillips, D. H. (1983) *Nature 303*, 468–472.
Phillips, D. H., Hewer, A., & Grover, P. L. (1985) *Cancer Res. 45*, 4167–4174.
Richardson, K. K., Crosby, R. M., Richardson, F. C., & Skopek, T. R. (1987a) *Mol. Gen. Genet. 209*, 536–532.
Richardson, K. K., Richardson, F. C., Crosby, R. M., Swenberg, J. A., & Skopek, T. R. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 344–348.
Rill, R. L., & Marsch, G. A. (1990) *Biochemistry 29*, 6050–6058.
Rodriguez, H., & Loechler, E. L. (1993a) *Carcinogenesis 14*, 373–383.
Rodriguez, H., & Loechler, E. L. (1993b) *Biochemistry 32*, 1759–1769.
Rodriguez, H., Bhat, U. P., Snow, E. T., & Loechler, E. L. (1992) *Mutat. Res. 270*, 219–231.
Rogers, A. G., & Weiss, B. (1980) *Methods Enzymol. 65*, 201–211.
Sage, E., & Haseltine, W. A. (1984) *J. Biol. Chem. 259*, 11098–11012.
Sayer, J. M., Chadha, A., Agarwal, H. S. K., Yeh, H. J. C., Yagi, H., & Jerina, D. M. (1991) *J. Org. Chem. 56*, 20–29.
Schaaper, R. M., Glick, B. W., & Loeb, L. A. (1982) *Cancer Res. 42*, 3480–3485.
Shapiro, R., & Klein, R. J. (1966) *Biochemistry 5*, 2358–2362.
Singer, B., & Essigmann, J. M. (1991) *Carcinogenesis 12*, 949–955.
Singer, B., & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.
Sowers, L. C., Shaw, B. R., Veigl, M. L., & Sedwick, W. D. (1987) *Mutat. Res. 177*, 201–218.
Yi, T.-M., Stearns, D., & Demple, B. (1988) *J. Bacteriol. 170*, 2898–2903.