Mutagenesis by the (+)-*anti*-Diol Epoxide of Benzo[*a*]pyrene: What Controls Mutagenic Specificity? †

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ABSTRACT: Mutagenesis by (+)-*anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide [(+)-*anti*-B[a]PDE], an important mutagenic/carcinogenic metabolite of benzo[*a*]pyrene (B[a]P), is being studied in order to understand the factors that influence mutagenesis both quantitatively and qualitatively. A new mutational system, which permits the selection of *supF*⁻ mutations in an *Escherichia coli* plasmid, pUB3, was used. The work described herein is an extension of previous work, which involved plasmid adduction and then immediate transformation (Rodriguez & Loechler, 1993), and began with the observation that mutation frequency (MF) decreased ~2-fold when the (+)-*anti*-B[a]PDE-adducted plasmid pUB3 is either (1) frozen and then thawed prior to transformation or (2) heated at 80 °C for 10 min prior to transformation. Several results suggest that this decrease is *not* due to the loss of labile adducts. To begin to understand this phenomenon, the mutagenic spectra are compared for (+)-*anti*-B[a]PDE in *supF* for the unheated (187 mutants), the freeze/thawed (134 mutants), and the heated (254 mutants) samples. In general, freeze/thawing and heating cause a decrease in all classes of mutations. Considering substitution mutations at G·C base pairs, which predominate, the mutagenic specificity for the combined data sets is GC → TA (57%), GC → AT (23%), and GC → CG (20%). This raises the question, how does (+)-*anti*-B[a]PDE generate this complex mutagenic specificity, which contrasts with the situation for, e.g., simple methylating agents? One factor is that mutagenic specificity at a particular guanine residue can be influenced by the base on its immediate 5′-side, most notably where mutations are virtually exclusively restricted to GC → TA in 5′-TG-3′ sequence contexts. One unexpected finding may provide additional insight. G_{115} in *supF*, which is the major hot spot for base-pairing mutagenesis, is the only site where the qualitative pattern of mutagenesis is significantly affected by heating the (+)-*anti*-B[a]PDE-adducted plasmid prior to transformation. Without heating, G_{115} → T mutations predominate, but following heating there is a statistically significant increase in the fraction of G_{115} → A and G_{115} → C mutations. The most likely model to explain this and other results is (1) a particular DNA adduct can adopt multiple conformations, (2) the conformation adopted by an adduct can be influenced by various factors, including DNA sequence context, as well as heating and freeze/thawing, and (3) each of these conformations can cause a different pattern of mutation. Three alternative models, which are less likely but cannot be rigorously excluded, are also considered, including models involving roles for labile adducts and/or AP sites.

The pattern of mutations induced by a particular mutagenic pathway appears to be expressed in the pattern of mutations induced when protooncogenes (or tumor suppressor genes) are activated, as revealed in studies of oncogenes derived from tumor cells (Barbacid, 1987; Balmain & Brown, 1988; Hollstein et al., 1991; Vogelstein & Kinsler, 1992). In mutagenesis studies this has been termed "mutational specificity" (Miller, 1980, 1983), and more recently, in reference to oncogene activation, this has been termed a "fingerprint" (Vogelstein & Kinsler, 1992).

The issue of mutagenic patterns can be divided into quantitative aspects (e.g., what influence "hot spots" vs "cold spots") and qualitative aspects (e.g, what influence GC → TA vs GC → AT vs GC → CG mutations, or "mutagenic specificity"). DNA sequence context is one factor that is generally recognized to influence both quantitative aspects [examples include Miller (1980, 1983), Burns et al. (1987), Richardson et al. (1987a,b), LeClerc et al. (1988), Singer, et al. (1989), Horsfall et al. (1990), Lawrence et al. (1990), and Dosanjh et al. (1991)] and qualitative aspects [reviewed in Loechler (1993)] of mutagenesis. More attention has been focused upon the former than the later. It seems reasonable to imagine that the carcinogenic potency of a mutagen/carcinogen must reflect the ability of that agent to follow quantitative and qualitative rules of mutagenesis that effectively activate a constellation of oncogenes and tumor suppressor genes.

To deepen our understanding of what influences mutagenesis quantitatively and qualitatively, we are studying mutagenesis by (+)-*anti*-B[a]P-7,8-dihydrodiol-9,10-epoxide [(+)-*anti*-B[a]PDE][1] (Rodriguez & Loechler, 1993) and its adducts (Mackay et al., 1992). (+)-*anti*-B[a]PDE is generally regarded to be the most important mutagenic/carcinogenic metabolite of benzo[*a*]pyrene (B[a]P), which belongs to the polycyclic aromatic hydrocarbon family of environmental carcinogens [reviewed in Conney (1982), Phillips (1983), Singer and Grunberger (1983), and Harvey (1991)]. [B[a]P

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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 guanine at the N^2-position (Figure 1); (+)-*anti*-B[a]P-N7-Gua, an adduct, which is not well characterized, that we refer to as the "labile adduct"; (+)-*anti*-B[a]PDE-pUB3, pUB3 adducted with ~22 adducts of (+)-*anti*-B[a]-PDE/plasmid (Rodriguez & Loechler, 1993); MF, mutation frequency; AP sites, apurinic/apyrimidinic sites; cpm, counts per minute; ds, double stranded, ss, single stranded; Gua, guanine; Ade, adenine; Thy, thymine.

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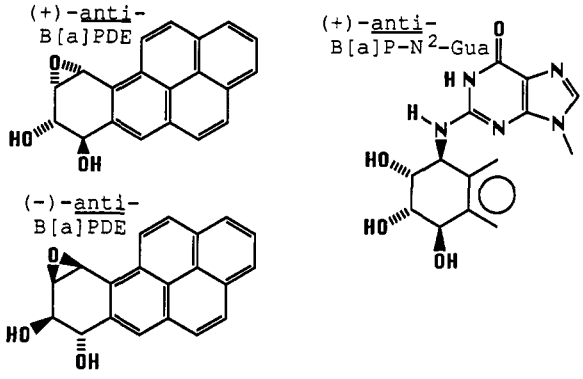

FIGURE 1: Structures.

may also be activated by other pathways (Phillips et al., 1985; Marnett, 1987; Cavalieri et al., 1990; Devanesan et al., 1992).] (+)-*anti*-B[a]PDE and its enantiomer, (–)-*anti*-B[a]PDE, primarily bind covalently in nucleic acids to form an adduct at N^2-Gua (Jeffrey et al., 1976; Koreeda et al., 1976; Osborn et al., 1976; Straub et al., 1977; for DNA, Cheng et al., 1989; Sayer et al., 1991), but may also bind to a lesser extent to deoxyadenosine to give an N^6–Ade adduct (Meehan et al., 1977; Jeffrey et al., 1979). There is some evidence for the formation of N7-Gua adducts (Osborne et al., 1978; King et al., 1979; Sage & Haseltine, 1984; Lobanenkov et al., 1986; Rill & Marsch, 1990) and for adduction at deoxycytosine residues (Straub et al., 1977; Meehan et al., 1977; Sage & Haseltine, 1984; Rill & Marsch), 1990).

The mutagenic specificity of racemic (±)-*anti*-B[a]PDE has been studied in bacteria (Eisenstadt et al., 1982; Levin & Ames, 1986; Bernelot-Moens et al., 1990), in rodent and human cells using shuttle vector systems (Yang et al., 1987; Roilides et al., 1988), in CHO cells (Carothers & Grunberger, 1990; Mazur & Glickman, 1988), and in human cells using endogenous target genes (Chen et al., 1990, 1991; Yang et al., 1991; Keohavong & Thilly, 1992).

All of the mutational studies described above used racemic (±)-*anti*-B[a]PDE. This prompted us to study mutagenesis by (+)-*anti*-B[a]PDE (Rodriguez & Loechler, 1993) in isolation from (–)-*anti*-B[a]PDE, which is a minor metabolite of B[a]P, using a new system that we recently developed (Rodriguez et al., 1992). (+)-*anti*-B[a]PDE was reacted with the plasmid pUB3 in vitro (the product is designated (+)-*anti*-B[a]PDE-pUB3) and then immediately transformed into *Escherichia coli* (ES87) cells, which were either SOS-uninduced or SOS-induced using a simple procedure (Koffel-Schwartz et al., 1984). There are several features of our system that make it attractive for studying mutagenesis, including that a high fraction (i.e., 176 of 255 or 69%) of all possible base-pairing mutations have been detected phenotypically in *supF* [Kraemer & Seidman, 1989; Rodriguez and Loechler (1993) and references therein].

We were also interested in comparing (+)-*anti*-B[a]PDE mutagenesis to our site-directed studies on (+)-*anti*-B[a]-P-N^2-Gua, which induces principally G → T mutations in a 5′-T<u>G</u>*C-3′ sequence context (Mackay, et al., 1992).

In the course of our work, we were concerned about the loss of labile adducts (Osborne et al., 1978; King et al., 1979; Sage & Haseltine, 1984; Lobanenkov et al., 1986; Rill & Marsch, 1990) during plasmid manipulation, which prompted us to take several precautions, including transforming the adducted plasmid immediately after adduction (Rodriguez & Loechler, 1993). As addressed herein, this concern initially seemed warranted when we discovered that mutation frequency (MF) decreased ~2-fold when pUB3 adducted with (+)-*anti*-B[a]-PDE was heated at 80 °C for 10 min prior to transformation.[2] However, various results, including the fact that merely freeze/thawing the adducted plasmid also caused an ~2-fold decrease in MF, suggested that something other than the loss of labile adducts was responsible. To begin the process of understanding the source of this ~2-fold decrease in mutagenesis, we have determined the mutational spectra of (+)-*anti*-B[a]-PDE in *supF* of pUB3 following either heating or freeze/thawing, and we compare them to our previous data, which involved neither heating nor freeze/thawing (Rodriguez & Loechler, 1993).

MATERIALS AND METHODS

Strains, Plasmids, Mutant Isolation, Reagents, and Media. *E. coli* strain ES87 and plasmid pUB3, as well as transformation, mutant selection, DNA sequencing, MF calculation, and reagents and media, were as described previously (Rodriguez et al., 1992; Rodriguez & Loechler, 1993).

Adductions with (+)-anti-BP[a]PDE. ^{3}H-Labeled (+)-*anti*-B[a]PDE (Chemsyn Science Laboratories and the National Cancer Institutes Carcinogen Reference Standard Repository; catalogue number R702H; lot CSL-88-155-13) was handled with considerable caution as described previously (Benasutti et al., 1988a). Adductions of pUB3 with (+)-*anti*-B[a]PDE were as described previously (Rodriguez & Loechler, 1993). Approximately 5 μg of pUB3 was reacted with ~90 ng (0.28 nmol) of ^{3}H-(+)-*anti*-B[a]PDE (984 mCi/mmol) at room temperature, which gave ~22 adducts/plasmid (Rodriguez & Loechler, 1993). The total volume of the reaction was 120 μL. After 1 h of reaction, the DNA was ethanol precipitated (Rodriguez & Loechler, 1993) and the pellet was resuspended in 100 μl of HEPES buffer (100 mM HEPES and 1 mM EDTA, pH 6.8; 0 °C). Beginning with the ethanol precipitation, these steps took approximately 60 min.

Heating and Freeze/Thawing. (+)-*anti*-B[a]PDE-pUB3 was transformed immediately into ES87 cells (typically completed within ~60 min) to generate the data that were neither heated nor freeze/thawed (Rodriguez & Loechler, 1993). Alternatively, (+)-*anti*-B[a]PDE-pUB3 was heated for 10 min at 80 °C and then chilled in ice (0 °C) prior to transformation.[2] On other occasions, the heated, adducted DNA was frozen (at –70 °C) and subsequently thawed prior to transformation by electroporation. Freezing was always accomplished by placing the sample in an Eppendorf tube at –70 °C in a freezer, while thawing involved merely hand-holding followed by incubation on ice.

Statistics. χ^2 analysis was used to test for statistical significance. In certain cases, the data had to be normalized for the number of sites where a particular mutation could be detected; e.g., G → T mutations could be detected at 16 and 7 sites in 5′-G<u>G</u>-3′ sequences and 5′-T<u>G</u>-3′ sequences, respectively (Rodriguez & Loechler, 1993).

Analysis of Labile Adducts. The following procedure is essentially identical to that used at times in our study of the labile N7-Gua adduct of aflatoxin B_1 (Benasutti et al., 1988b).

pBU3 was adducted with ^{3}H-(+)-*anti*-B[a]PDE. Approximately 1.5 μg of adducted DNA was transferred into a screw-cap microfuge tube and the volume was adjusted to 200 μL with HEPES buffer (as above). After the sample was layered with 175 μL of Nujol mineral oil (Perkin-Elmer), the capped

[2] Heating at 80 °C for 10 min did not cause significant denaturation of pUB3, which required higher temperatures (e.g., 100 °C) as assessed by evaluating hyperchromicity at 260 nm (data not shown).

Table I: Effect of Heating and Freeze/Thawing upon the Mutation Frequency and Yield of pUB3 Adducted with (+)-*anti*-B[a]PDE, Following Transformation into SOS-Induced ES87 Cells

		–heat[a]			+heat[a]			
expt		F/T[b] 0	F/T 1	F/T 2	F/T 0	F/T 1	F/T 2	F/T 3
1	MF[c] (×10⁶)[c]	25	11.4	10.3		12.9	9.1	9.5
	yield[d]	0.046	0.064	0.063		0.079	0.112	0.077
2	MF (×10⁶)	18.7		9.4	8.9			
	yield	0.082		0.066	0.072			
3	MF (×10⁶)	17.8		8.3				
	yield	0.092		0.141				

[a] pUB3 that was adducted with (+)-*anti*-B[a]PDE was either not heated (–heat) or heated (+heat) at 80 °C for 10 min prior to transformation. [b] F/T strands for freeze/thawing, where 0, 1, 2, and 3 indicates the number of rounds of freeze/thawing that were performed (Materials and Methods). [c] Mutational frequency ($\times 10^{+6}$) as determined by procedures outlined in Materials and Methods. [d] Yield is defined as the ratio of progeny plasmids obtained from transformation of (+)-*anti*-B[a]PDE-pUB3 divided by the yield of progeny plasmids from unadducted pUB3. In general, heating caused a small decrease (~11%) in progeny plasmid yield for unadducted pUB3.

microfuge tube was either freeze/thawed or put at 0 or 80 °C. After various times of incubation the sample was chilled on ice, and the water layer was removed using a Finnpipette. The original microfuge tube was then rinsed with an additional 100 μL of HEPES buffer, which was combined with the original 200 μL. To this, ice-cold ethanol (750 μL; 0.15 M sodium acetate; pH 5.2) was added. After 10 min on ice, the DNA was centrifuged (30 min at 4 °C). The supernatant, which contains hydrolyzed adducts, was transferred into a scintillation vial, to which Aquasol-2 scintillation cocktail (New England Nuclear) was added, and the sample was counted (cpm_s). The pellet, which contains DNA and nonhydrolyzed adducts, was resuspended in 300 μL of HEPES buffer and rinsed with 650 μL of cold ethanol; both were combined and counted (cpm_p). We showed that [cpm_s + cpm_p] was equal (± 10%) to the total cpm added. The fraction of tritium released was merely Fr = [$cpm_s/(cpm_s + cpm_p)$].

Tritium release was not due to ^{3}H-exchange. A sample (300 μL) heated for 22 h at 80 °C was transferred into a new microfuge tube and 700 μL of distilled water was added. The solvent was evaporated to 50 μL using a Speed-Vac concentrator. To this, 250 μL of HEPES buffer was added and the procedure was repeated (as above). The level of tritium in the supernatant was no more than 17% lower than from a sample run in parallel that was not subjected to evaporation. ^{3}H-Water would have been removed during evaporation.

RESULTS

Effect of Heating and Freeze/Thawing on (+)-anti-B[a]-PDE Mutagenesis. Our mutational assay involves the adduction of the plasmid pUB3 with (+)-*anti*-B[a]PDE in vitro (designated (+)-*anti*-B[a]PDE–pUB3) and its subsequent transformation into *E. coli* ES87 cells (Rodriguez et al., 1993). In experiments utilizing mutational target genes in plasmids, the adducted DNA is frequently frozen and later thawed prior to transformation (or transfection). In this regard, adductions with (+)-*anti*-B[a]PDE could be problematic given that labile adducts are formed (Osborne et al., 1978; King et al., 1979; Sage & Haseltine, 1984; Lobanenkov et al., 1986; Rill & Marsch, 1990). However, we suspected that this issue would not be significant because heat-labile adducts would not be expected to hydrolyze significantly following simply freeze/thawing. Nevertheless, to preclude this concern, we transformed (+)-*anti*-B[a]PDE-pUB3 immediately after adduction (Rodriguez & Loechler, 1993). Concurrently, we began an investigation of these concerns.

In contrast to expectations, a single round of freeze/thawing of (+)-*anti*-B[a]PDE-pUB3 caused an ~2-fold decrease in mutational frequency (Table I, experiment 1). Given this, it seemed possible that additional cycles of freeze/thawing might cause a larger decrease. However, experiments 1, 2, and 3 (Table I) show that two rounds of freeze/thawing did not decrease MF significantly further. In addition, (+)-*anti*-B[a]-PDE-pUB3 was purposely heated at 80 °C for 10 min prior to transformation;[2] again, MF decreased by no more than ~2-fold (Table I, experiment 2). There was no apparent synergism between heating and freeze/thawing; MF decreased by ~2-fold when both were performed (Table I, experiment 1). Repeated freeze/thawing of the heated sample also appeared to have no further effect upon MF (Table I, experiment 1). It did not appear that storage of the frozen (+)-*anti*-B[a]PDE-pUB3 sample for several months (vs several days) significantly affected MF (data not shown).

The fraction of survivors for (+)-*anti*-B[a]PDE-pUB3 versus unadducted pUB3 was ~0.073 for the samples that were neither heated nor freeze/thawed (Table I). The average value for all of the heated and/or freeze/thawed samples was ~0.084. Thus, the ~2-fold decrease in MF following heating or freeze/thawing appeared to be more attributable to a decrease in mutant yield than to enhanced survival.

The series of unexpected findings described above did not fit a pattern that would suggest that the ~2-fold decrease in MF following either freeze/thawing or heating had anything to do with the loss of labile adducts. Nevertheless, it seemed reasonable to imagine that the pursuit of these observations might provide some insight into the relationship between DNA structure, (+)-*anti*-B[a]PDE adducts, and mutagenesis. We wished to determine (1) whether freeze/thawing or heating released a significant fraction of (+)-*anti*-B[a]PDE adducts and (2) what kinds of mutations were lost following either freeze/thawing or mild heating.

Effect of Freeze/Thawing and Heating on Adducts of (+)-anti-B[a]PDE. A labile adduct was identified from the reaction of DNA with racemic (±)-*anti*-B[a]PDE and was judged to be B[a]P-N7-Gua on the basis of its lability and the enhanced exchange of its C8 proton (Osborne et al., 1978; King et al., 1979). This is not definitive so we use the term "labile adduct" and not B[a]P-N7-Gua. It accounts for ~20–40% of adducts from (±)-*anti*-B[a]PDE (King et al., 1979; Sage & Haseltine, 1984). Alkali-labile sites are thought to arise from this adduct with both (+)-*anti*-B[a]PDE and (–)-*anti*-B[a]PDE (Rill & Marsch, 1990). It is generally assumed that hydrolysis of labile adducts yields AP sites, but this has been questioned (Moran & Ebisuzaki, 1991).

Hydrolysis with $t_{1/2} \simeq 3$ h at 37 °C was reported for the labile adduct of *anti*-B[a]PDE (King et al., 1979). The corresponding N7-Gua adduct of aflatoxin B_1 is considerably more stable under similar conditions ($t_{1/2} \simeq$ 48–100 h at 37 °C; Groopman et al., 1980). On the basis of our extensive work (Benasutti et al., 1988b), we knew that >90% of AFB_1-

N7-Gua adducts were hydrolyzed by heating at 80 °C for 10 min at neutral pH. Thus, it seemed sensible to expect that the labile adduct from (+)-*anti*-B[a]PDE would also hydrolyze when treated similarly. On the basis of this analogy, it had been our original intention to use mild heating to liberate the labile adduct in an effort to study the effect of its loss on mutagenesis by (+)-*anti*-B[a]PDE. The notion was that labile adducts (e.g., B[a]P-N7-Gua) would be converted to AP sites, which might be less mutagenic because they are efficiently repaired in double-stranded (ds) DNA in *E. coli* (see Discussion). Our observation that heating decreased MF ~2-fold initially suggested to us that this was sensible; however, the ~2-fold decrease following freeze/thawing suggested otherwise.

To evaluate the loss of labile adducts, pUB3 was adducted with ^{3}H-(+)-*anti*-B[a]PDE and the loss of tritium, which is indicative of adduct hydrolysis, from DNA was studied (Materials and Methods). After three successive rounds of freeze/thawing, ~0% was released in two experiments, while in a third experiment, ~3% appeared to be released. Thus, virtually no tritium (~1% on average) was lost following three successive rounds of freeze/thawing. In two experiments, 10 min of heating at 80 °C showed that 4.4% and 5.3% of the tritium were released.

Heating at 80 °C for prolonged periods of time can release a large fraction (>50%) of adducts with a half-time in the range of several hours. (Based upon this, it appears that the labile adduct of (+)-*anti*-B[a]PDE is much more stable at 80 °C than expected based upon analogy to AFB_1-N7-Gua.) We have shown that the major adduct, (+)-*anti*-B[a]P-N^2-Gua, in a ss oligonucleotide is stable when heated at 80 °C for 10 min at neutral pH and in 0.1 N NaOH (Benasutti et al., 1988a). In fact, this adduct is stable in ss oligonucleotides when heated at 90 °C for 30 min in 1 M piperidine (Mao et al., 1992). Taken together, these results appear to indicate that (+)-*anti*-B[a]P-N^2-Gua is more stable in ss oligonucleotides than in ds-DNA. Furthermore, the simplest interpretation of these results is that (+)-*anti*-B[a]P-N^2-Gua is the "labile adduct" in ds-DNA.

Obviously, the situation is complex, and a more detailed analysis of this phenomenology is under investigation. Nevertheless, for the purposes of the work herein, we can conclude that 99% adducts remained after freeze/thawing and 95% adducts remained after heating at 80 °C for 10 min. Thus, an insignificant fraction of (+)-*anti*-B[a]PDE adducts is lost from pUB3 following either freeze/thawing or heating.

Spontaneous Mutagenesis. Table II gives information about spontaneous mutagenesis for SOS-induced cells transformed with nonadducted pUB3 that was either not heated[3] (Table II; 71 mutants, MF ~ 1.0×10^{-6}) or heated at 80 °C for 10 min (Table II; 154 mutants, MF ~1.3×10^{-6}). In previous work we showed that the spontaneous mutational spectrum in our system was mainly due to insertion elements and large deletions in both non-SOS- and SOS-induced cells (Rodriguez et al., 1992), which is often the case in *E. coli* (Galas & Chandler, 1989; Kleckner, 1981; Schaaper et al., 1986; Shapiro, 1988; Albertini et al., 1982; Schaaper & Dunn, 1991). This is also true for heated/unadducted pUB3 when transformed into SOS-induced cells (Table II). The identifiable insertion elements obtained for heated/unadducted pUB3 are catalogued in Table III and are similar to those obtained without heat (Rodriguez et al., 1992). Although heating of nonadducted pUB3 does not appear to significantly affect the frequency of base substitutions, frameshift mutations, insertions, or deletions (Table II), there appear to be some subtle changes. (The term "frameshift" is not appropriate when considering a tRNA gene, such as *supF*, but it is convenient.) Base substitutions with heating are shown in Figure 2.

Effects of Heating (+)-anti-B[a]PDE-pUB3 on Mutagenesis. Table II also categorizes the types of mutants induced in *supF* by (+)-*anti*-B[a]PDE with and without heating in SOS-induced cells. Base substitutions, insertions, and large deletions, and to a lesser extent frameshift mutations, all contributed to the ~2-fold decrease in mutagenesis noted above.

The decrease in insertions upon heating (+)-*anti*-B[a]PDE-pUB3 was mainly due to an ~4-fold decrease in insertion element mutagenesis, which was dominated by an ~8-fold decrease in identifiable insertion elements. The nature of the insertion elements derived from heating (+)-*anti*-B[a]PDE-pUB3 (Table IV) was similar to those obtained previously (Table III; Rodriguez et al., 1992; Rodriguez & Loechler, 1993). Although the heating of unadducted pUB3 did not affect deletion mutagenesis, heating (+)-*anti*-B[a]PDE-pUB3 caused an ~4-fold decrease in the deletion from bp 68 to 119, whose end points lie within a 10-base-pair direct repeat (Table II).

Frameshift mutagenesis is frequently dominated by the deletion or insertion of a single base pair in a run of consecutive base pairs [Lambert et al. (1992) and references therein], as we have observed in our previous study for unheated (+)-*anti*-B[a]PDE-pUB3 (Rodriguez & Loechler, 1993). Heating (+)-*anti*-B[a]PDE-pUB3 had little effect on frameshift mutagenesis at G_{99}–G_{100} or G_{172}–G_{176} (Table V). However, heating (+)-*anti*-B[a]PDE-pUB3 caused the frameshift mutational frequency to drop ~2-fold for sequences with three consecutive G·Cs (i.e., at G_{108}–G_{110} and G_{122}–G_{124}). Frameshift insertion mutagenesis was only observed at G_{172}–G_{176} (Table V) and heating had no effect on it.

Base substitution mutation are shown in Figure 3. We estimate that ~7 mutants in our collection of 110 G·C base-pairing mutations in the presence of heating (+)-*anti*-B[a]-PDE-pUB3 can be attributed to spontaneous events. Considerable attention will be focused upon mutations at G_{115}, where 19% and 29% of the base-pairing mutations with unheated and heated (+)-*anti*-B[a]PDE-pUB3, respectively, occurred. We estimate that at G_{115}, ~0 of 15 mutants without heating and ~1 of 33 mutants following heating can be attributed to spontaneous events. G_{115} is unusual in that heating caused a change in mutagenic specificity, where GC → TA mutations (13/15) dominated prior to heating, while the fraction of GC → TA mutations (15/33) decreased and GC → AT (7/33) and GC → CG (11/33) mutations increased following heating (Figure 3, Table VI). This change is statistically significant ($P \simeq 0.005$).

Although G·C → A·T transitions and G·C → C·G transversions in general did not change following heating of (+)-*anti*-B[a]PDE-pUB3 (Table II), when G_{115}, which is unusual as noted above, is excluded from the data set, an ~2-fold decrease in MF for G·C → A·T and G·C → C·G occurs. It appears that GC → TA mutations preferentially decreased upon heating (~3-fold), but when all data at G_{115} were excluded, this preference is only marginally statistically significant ($P \simeq 0.09$). Mutations at A·T base pairs also appeared to decrease upon heating of (+)-*anti*-B[a]PDE-pUB3 (0.54×10^{-6} vs 0.21×10^{-6}).

[3] No distinction was made between mutants collected from unadducted pUB3 that was stored at 4 °C for extended periods of time and this same DNA following freeze/thawing.

Table II: Mutations Detected in the *sup*F Gene of pUB3, both with and without Heating Unadducted pUB3 and (+)-*anti*-B[a]PDE-pUB3, as well as Freeze/Thawing of the Latter, Following Transformation into SOS-Induced ES87 Cells

	spontaneous mutations		B[a]PDE-induced mutations		
type of mutation	−heat	+heat	−heat	+heat	+freeze/thaw
base substitutions					
G·C → A·T	4[a] (0.06)[b]	15 (0.12)	12 (1.3)	27 (1.1)	12 (0.99)
G·C → T·A	2 (0.03)	15 (0.12)	53 (5.7)	55 (2.3)	20 (1.5)
G·C → C·G	5 (0.07)	3 (0.02)	13 (1.4)	28 (1.2)	4 (0.30)
A·T → G·C	6 (0.09)	4 (0.03)	3 (0.32)	1 (0.04)	0 (<0.08)
A·T → T·A	1 (0.01)	0 (<0.01)	1 (0.11)	4 (0.16)	1 (0.08)
A·T → C·G	2 (0.03)	1 (0.01)	1 (0.11)	0 (<0.04)	4 (0.30)
Total	20 (0.29)	38 (0.32)	83 (9.0)	115 (4.8)	42 (3.2)
frameshift mutations					
+G·C	0 (<0.01)	0 (<0.01)	9 (0.97)	25 (1.0)	3 (0.24)
ΔG·C	1 (0.01)	5 (0.04)	31 (3.3)	63 (2.6)	24 (1.8)
ΔA·T	0 (<0.01)	0 (<0.01)	3 (0.32)	3 (0.12)	5 (0.38)
other frameshifts	0 (<0.01)	1[c] (0.01)	0 (<0.11)	3[d] (0.12)	0 (<0.08)
total	1 (0.01)	6 (0.05)	43 (4.6)	94 (3.9)	32 (2.4)
insertions					
insertion elements	35 (0.51)	78 (0.65)	27 (2.9)	9 (0.37)	40 (3.0)
no sequence[e]	1 (0.01)	1 (0.01)	17 (1.8)	22 (0.91)	9 (0.68)
other insertions	5[f] (0.07)	11[g] (0.09)	1[h] (0.11)	0 (<0.04)	1[i] (0.08)
total	41 (0.59)	90 (0.75)	45 (4.9)	31 (1.3)	50 (3.8)
deletions					
bp 68–119	8 (0.12)	17 (0.14)	16 (1.7)	11 (0.45)	10 (0.76)
other deletions	1[j,k] (0.01)	3[k,l] (0.03)	0 (<0.11)	1[k,m] (0.04)	2[n] (0.15)
total	9 (0.13)	20 (0.17)	16 (1.7)	12 (0.50)	12 (0.91)
others, total	0 (<0.01)	0 (<0.01)	0 (<0.11)	2[o] (0.08)	1[p] (0.08)
total mutants	71	154	187	254	137
mutational frequency[q] (×10^{+6})	1.03	1.28	20.2	10.5	10.4

[a] Number of mutants isolated of the indicated type. [b] Mutational frequency (×10^{+6}) as determined by procedures outlined in Rodriguez et al. (1992). [c] One mutant had two G·Cs deleted in G_{172}–G_{176}. [d] One insertion was identified with two G·C base pairs added at G_{172}–G_{176}, and a second insertion was identified with three G·C base pairs added at G_{172}–G_{176}. One mutant had two G·Cs deleted in G_{172}–G_{176}. [e] In many instances, plasmids with molecular weights greater than pUB3 itself (i.e., >~3.5 kb) were isolated that could not be sequenced. We presume that these result from insertion elements that disrupt the binding of the DNA sequencing primer. [f] One mutant, which gave a plasmid of 6.0 kb, was isolated with an insertion after bp137 that showed homology to *rpsM*; there was no obvious homology between the inserted sequence and *supF*. One insertion was identified that duplicated the sequence 5′-TTCCCC-3′, which is located between G_{170} and G_{175}. Three large insertions were identified with no significant homology to any *E. coli* gene reported in GenBank. [g] Four mutants which gave plasmids of 4.5 kb and one mutant which gave a plasmid of 7.2 kb, were isolated with an insertion after bp137 that showed homology to *rpsM*; there was no obvious homology between the inserted sequence and *supF*. In addition, six large insertions were identified with no significant homology to any *E. coli* gene reported in GenBank. [h] One large insertion was identified with no significant homology to any *E. coli* gene reported in GenBank. [i] One large insertion was identified with no significant homology to any *E. coli* gene reported in GenBank. [j] One large deletion from bp 150 to 171 was detected. [k] In one transformation, 2, 10, and 16 deletions from bp 150 to 171 were detected for the unadducted, +SOS, unheated sample; unadducted, +SOS, heated sample; and (+)-*anti*-B[a]PDE adducted, +SOS, heated sample, respectively. This cluster of large deletions from a single transformation was of dubious origin and was not included in the data set. [l] Three large deletions, one from bp 65 to 110 in *supF*, another from bp 128 to 139 in *supF*, and a third from bp 28 in *supF* to bp 924 in pUB3, were isolated. [m] One large deletion from bp 116 in *supF* to bp 472 in pUB3 was isolated. [n] One large deletion from bp 65 to 110 was isolated. One large deletion from bp 148 to 168 was isolated. [o] Two double mutants were isolated: one consisting of $G_{116}G_{117}$ → AT and the second identified with $G_{168}G_{169}$ → TT. [p] One double mutant consisting of a G_{111} deletion and a T_{107} → C mutation was detected. Alternatively, this can be interpreted as a T_{107} deletion and a G_{111} → C mutation. [q] Three significant figures are reported for the mutation frequency, although fewer are warranted. The indicated mutants were isolated in nine experiments. The mutation frequency (Materials and Methods) is the average of the most reliable experiments.

Effects of Freeze/Thawing (+)-anti-B[a]PDE-pUB3 on Mutagenesis. Table II also categorizes the types of mutants induced in *supF* by (+)-*anti*-B[a]PDE-pUB3 following freeze/thawing in SOS-induced cells. Base substitutions, frameshift mutations, and large deletions, and to a lesser extent insertions, all contributed to the ~2-fold decrease in mutagenesis noted above. There are many similarities between the effects of heating and freeze/thawing on mutagenesis; the differences are highlighted below.

Unlike heating, which caused an ~4-fold decrease, freeze/thawing had no effect upon the MF for identifiable insertion elements. The nature of the insertion elements (Table IV) is similar to those obtained previously as noted above. Table II shows that upon freeze/thawing (+)-*anti*-B[a]PDE-pUB3, an ~2-fold decrease in frameshift MF was observed, which is slightly greater than the decrease with heating. One significant difference was an ~4-fold decrease in frameshift insertion mutagenesis at G_{172}–G_{176} (Table V), which showed no such decrease following heating. MF for base-pairing mutagenesis at G·C base pairs decreased ~3-fold. The pattern of GC → TA vs GC → AT vs GC → CG mutations following freeze/thawing is approximately the same as following heating (a test for difference gave $P \simeq 0.18$) when data for G_{115} were excluded. If there is a difference, it appears that GC → CG mutations decrease more upon freeze/thawing (~6-fold) than upon heating (~2-fold). Base substitution mutations following freeze/thawing are given in Figure 2.

DISCUSSION

Heating, Freeze/Thawing, and the Decrease in Mutagenesis. The work described herein began both with a concern about the potential inadvertent loss of labile adducts during DNA manipulation and with the thought that this lability might be used to dissect a role for different adducts in (+)-*anti*-B[a]PDE mutagenesis.

If the labile adduct were mutagenic and heating (or freeze/thawing) converted it to an AP site, which was not mutagenic due to efficient DNA repair, then this could provide a rationale for the decrease in mutagenesis observed following heating. A second possibility is that the decrease could be attributable to the breakdown of the labile adduct to give (+)-*anti*-B[a]P

Table III: Insertion Element Mutants from Experiments with Unadducted pUB3 Heated at 80 °C for 10 min Prior to Transformation into SOS-Induced ES87 Cells

site[a]	no. (size)[b]	site[a]	no. (size)[b]
	IS1[c]		
47	2 (2 @ 4.3)	125	1 (4.3)
57	1 (4.3)	130	1 (4.3)
60	1 (4.3)	136	1 (4.3)
66	6 (6 @ 4.3)	142	1 (4.3)
91	16 (15 @ 4.3, 1 @ 6.9)	165	5 (4 @ 4.3, 1 @ 8.0)
	IS5		
137	8 (7 @ 4.6, 1 @ 7.2)		
	IS10		
38	1 (4.6)	96	2 (2 @ 4.6)
55	2 (2 @ 4.6)	116	14 (13 @ 4.6, 1 @ 8.9)
91	1 (4.6)		
	γ-δ		
73	1 (8.9)	134	1 (4.5)
87	2 (2 @ 8.9)	140	1 (8.9)
89	2 (2 @ 8.9)	142	1 (8.9)
98	1 (8.9)	149	1 (4.5)
106	1 (7.2)	151	2 (2 @ 8.9)
127	1 (8.1)	162	1 (8.9)

[a] The number of the last base in *supF* prior to the site of the insertion. [b] The number of occurrences at the indicated site; the numbers in parentheses indicates the approximate size of the insertion (in kilobases) based upon a comparison to standards in an agarose gel. [c] The likely source of the inserted sequences based upon sequence homology (Pearson & Lipman, 1988).

tetraol, a process which would simply leave unadducted Gua in DNA. This is a minor pathway for the hydrolysis of AFB_1-N7-Gua (Groopman et al., 1980; Benasutti et al., 1988b), but it could predominate for the labile adduct of (+)-*anti*-B[a]PDE, which could rationalize the results of Moran and Ebisuzaki (1991).

The following raise doubts about a role for the loss of labile adducts in the ~2-fold decrease in MF following heating and freeze/thawing described in Results: (1) Following freeze/thawing or heating at 80 °C for 10 min, most (i.e., 99% or 95%, respectively) of the adducts, remained (Results). (2) Freeze/thawing appeared to decrease MF by approximately as much as heating (Table I). If labile adducts were significantly involved in the formation of a particular class of mutations, then intuitively one expects a lower MF in the heated than the freeze/thawed data. This was only observed for insertion element mutagenesis (Table II). (3) In most cases there was a great deal of similarity between the quantitative kinds of mutational changes brought about by heating vs freeze/thawing (Table II). (4) It is difficult to see how this thinking could explain the change in mutagenesis at G_{115} following heating (Table VI), unless this is the only site where the heating of a labile adduct gives (e.g.) an AP site that is appreciably mutagenic.

To retain a role for hydrolysis of labile adducts and/or AP sites in the decrease in MF requires one to propose that a small fraction of labile adducts are particularly prone to hydrolysis, that they are liberated to a similar extent by either freeze/thawing or heating, and that they are much more mutagenic than their stable counterparts. This is unlikely, but a role for labile adducts (and/or AP sites) remains a formal possibility, is considered below and will be addressed in future experiments.

An alternative explanation for the ~2-fold decrease in mutagenesis following freeze/thawing or heating is that there may be one structural conformational for an adduct from which no mutagenesis occurs, while there may be other structural conformations from which mutagenesis does occur. [Bulky adducts are most frequently replicated without mutation (Burnouf et al., 1989; Reid et al., 1990; Mackay et al., 1992; Naser-Bradley and Essigmann, personal communication).] If true, then heating (or freeze/thawing) might cause some of the mutagenic conformations to relax to their nonmutagenic counterparts. Differences in mutagenesis following heating vs freeze/thawing might reflect subtle differences in the amount (or pathway) of this putative relaxation process.

Role of Sequence Context on (+)-anti-B[a]PDE Base-Pairing Mutagenesis. When combined, the mutagenic specificity of the unheated, heated, and freeze/thawed data sets (Table II) for substitution mutations at G·C base pairs, which predominate, is GC → TA (57%), GC → AT (23%), and GC → CG (20%). This raises the question, why does (+)-*anti*-B[a]PDE show such a complex mutagenic specificity, which contrasts with the situation with (e.g.) simple methylating agents (Miller, 1980, 1983; Burns et al., 1987; Richardson et al., 1987a,b; Horsfall et al., 1990)?

As shown in Table VI, GC → TA, GC → AT, and GC → CG mutations were all obtained to a significant extent in 5′-G<u>G</u>-3′, 5′-A<u>G</u>-3′, and 5′-C<u>G</u>-3′ sequence contexts. In contrast, GC → TA mutations are virtually exclusively induced in 5′-T<u>G</u>-3′ sequences independent of heating. [This is statistically significant ($P \simeq 0.003$).] G·C → T·A mutations have been detected in both 5′-T<u>G</u>G-3′ (at G_{99}, G_{102}, and G_{118}) and 5′-T<u>G</u>C-3′ (at G_{127} and G_{141}) sequence contexts, which suggests that the base on the 3′-side of the mutated Gua does not affect the outcome. Although no mutations were detected in the sole 5′-T<u>G</u>A-3′ sequence context in *supF* (G_{146}), no mutations have ever been detected by us or others at this site (Kraemer & Seidman, 1989; Rodriguez & Loechler, 1993), suggesting that it may be phenotypically silent. *supF* has no 5′-T<u>G</u>T-3′ sequences.

The influence of the base on the 5′-side of the Gua undergoing mutation can be seen by several other comparisons. Considering Figure 3, only 2/23 mutations were not G·C → T·A for 5′-T<u>G</u>G-3′ sequences, while 15/42 mutations were not G·C → T·A mutations for 5′-G<u>G</u>G-3′ sequences. Finally, G_{118} is a 5′-T<u>G</u>GCC-3′ sequence, where only G·C → T·A mutations were obtained, while G_{115} is a 5′-C<u>G</u>GCC-3′ sequence, where all base-pairing mutations were obtained (Figure 3). In each of these comparisons, the mutagenic specificity is altered by merely changing the 5′-flanking base to a Thy, in spite of the sequence similarity on the 3′-side of the Gua undergoing mutation.

Mutations induced by (+)-*anti*-dibenz[*a*,*j*]anthracene diol epoxide [(+)-*anti*-BD[*a*,*j*]ADE] in pUB3 (Gill et al., 1992) have also been determined in the pUB3/ES87 system, and although G·C → A·T mutations predominated (51%), 17/19 mutations in 5′-T<u>G</u>-3′ sequence contexts were G·C → T·A. This result suggests that the influence of a 5′-Thy on Gua mutagenesis may not be compound-specific.

G_{115} is unusual in that its mutational pattern appears to change following heating (Figure 3, Table VI). This contrasts with the second hottest spot for base pairing mutation, G_{123}, which is a 5′-G<u>G</u>-3′ sequence context, where heating causes at most a marginal change in mutational pattern (Figure 3, where $P \simeq 0.14$ was estimated for the probability of a change). Following heating, all 5′-G<u>G</u>-3′ sequences considered together show at most a marginal change in mutations [Table VI; although the fraction of GC → CG mutations in 5′-G<u>G</u>-3′ sequences appears to increase upon heating, this is marginally statistically significant ($P \simeq 0.12$).] 5′-T<u>G</u>-3′

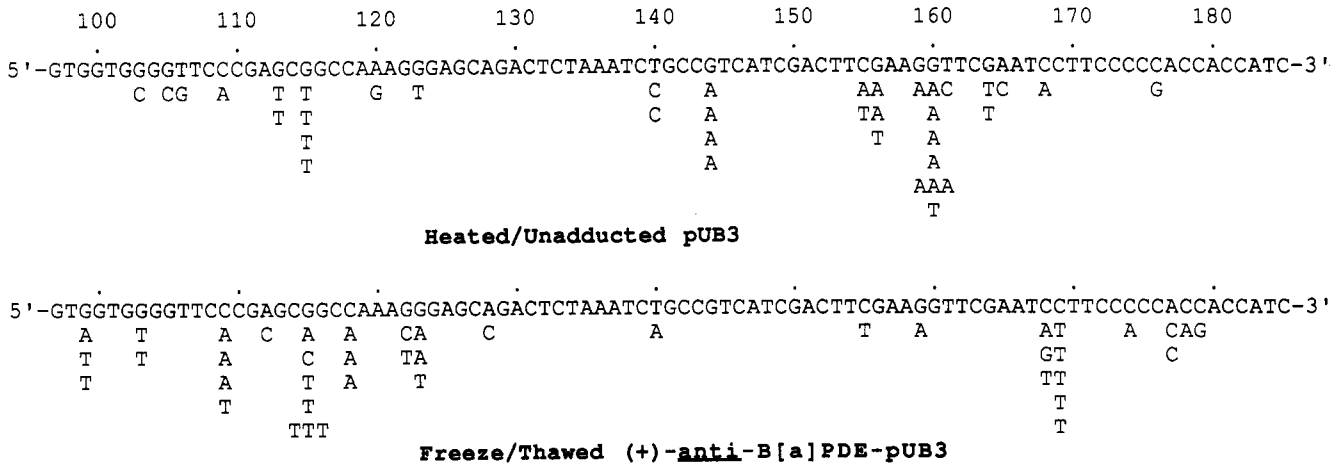

FIGURE 2: (Top) Spontaneous mutational spectrum for base-pairing mutations detected in the *supF* gene of unadducted/heated (80 °C for 10 min) pUB3 following transformation into SOS-induced ES87 cells. (Bottom) (+)-*anti*-B[a]PDE-induced mutationl spectrum for base-pairing mutations detected in the *supF* gene of pUB3 that was freeze/thawed prior to transformation into SOS-induced ES87 cells. The *supF* sequence as it appears in tRNA is given using the numbering system common to the plasmid pZ189 (Kraemer & Seidman, 1989), where the promoter is between bp24 and bp58, the pre-tRNA is between bp59 and bp98, and the tRNA gene is between bp99 and bp183.

Table IV: Insertion Element Mutants from Experiments with (+)-*anti*-B[a]PDE-pUB3 Heated at 80 °C for 10 min or Freeze/Thawed Prior to Transformation into SOS-Induced ES87 Cells

	number (size)	
site[a]	+heat[b]	+freeze/thaw[b]
	IS1[c]	
49	1 (4.3)	
58		1 (4.3)
60		1 (4.3)
66		3 (3 @ 4.3)
91	2 (2 @ 4.3)	2 (4.3, 8.0)
165	2 (2 @ 4.3)	1 (4.3)
171		6 (6 @ 4.3)
172		1 (4.3)
	IS5	
137		1 (8.9)
	IS10	
38		17 (15 @ 4.6, 2 @ 8.9)
94		4 (3 @ 4.6, 1 @ 8.9)
116	1 (4.6)	2 @ 4.6
	γ-δ	
89	1 (8.9)	2 (4.5, 7.5)
134	2 (2 @ 7.5)	

[a] The number of the last base in *supF* prior to the site of the insertion. [b] The number of occurrences at the indicated site; the numbers in parentheses indicates the approximate size of the insertion (in kilobases) based upon a comparison to standards in an agarose gel. [c] The likely source of the inserted sequences based upon sequence homology (Pearson & Lipman, 1988).

sequences show virtually no change as discussed above. The mutagenic specificity at G_{133}, which is an unusual site (Rodriguez & Loechler, 1993), also does not change upon heating (Figure 3).

This raises the question, what is the cause of the heat-induced changes in mutations at G_{115} and does it relate to the differences in mutagenesis seen in (e.g.) 5′-TG-3′ vs 5′-GG-3′ sequence contexts? Several models are considered.

Model 1: *Multiple Conformations for a Single Adduct.* We have used site-directed methods to study mutagenesis by the major adduct, (+)-*anti*-B[a]P-N²-Gua, in a 5′-TG*C-3′ sequence context, and G → T mutations predominated (Mackay et al., 1992). This provides strong circumstantial evidence that GC → TA mutations in 5′-TG-3′ sequence contexts with (+)-*anti*-B[a]PDE (Table VI) can be attributed to the (+)-*anti*-B[a]P-N²-Gua adduct. However, do these results imply that (+)-*anti*-B[a]P-N²-Gua only induces G → T mutations? The results at G_{115} may be revealing.

According to model 1, the adduct responsible for mutations at G_{115} is in one conformation prior to heating [possibly a conformation similar to one in 5′-TG-3′ sequences on the basis of a similar mutational pattern (Table VI)] and is in another conformation following heating (possibly a conformation similar to 5′-GG-3′ sequences; Table VI). Our view is that—by model 1—the initial conformation of the adduct at G_{115} is under kinetic control, while heating leads to the preferred thermodynamic conformation. We note that two conformations are proposed for the sake of simplicity; however, in fact there may be multiple conformations, and heating merely causes redistribution.

Model 1 can be a unifying hypothesis because it might rationalize most of the data. Namely, a single adduct can adopt multiple conformations, each of which can cause a different kind of mutation(s). Furthermore, adduct conformation may be influenced by various factors, including DNA sequence context as well as external factors, such as heating. The major adduct, (+)-*anti*-B[a]P-N²-Gua, is the most likely candidate. This raises several questions.

Is There Evidence That the Adducts of Bulky Mutagens/Carcinogens Can Adopt Different Conformations in Different Sequence Contexts? One conformation (to date) for (+)-*anti*-BP-N²-Gua in a duplex oligonucleotide has been reported and the pyrene moiety is in the minor groove and pointed toward the 5′-base (Cosman et al., 1992). However, it is suspected that B[a]PDE adducts undergo conformational rearrangements following adduction [discussed in Loechler (1991)], and other conformations are possible, as revealed in experimental studies (Eriksson et al., 1988; Roche et al., 1991; de los Santos et al., 1992) and theoretical studies (Aggarval et al., 1983; Anderson et al., 1987; Rao et al., 1989; Loechler, 1989).

Significantly, Geacintov and colleagues studied (+)-*anti*-B[a]PDE adducts in duplex DNA by linear dichroism (Roche et al., 1991) and showed that the spectrum for (+)-*anti*-B[a]-PDE adducts varied significantly as the DNA sequence context varied. Although the conformational basis for this difference is not known definitively, it suggests that adduct conformation can be influenced by sequence context. Fuchs and colleagues have evidence that C8-Gua adducts of 2-acetylaminofluorene adopt different conformations in different sequence contexts,

Table V: Frameshift[a] Mutagenesis Induced by (+)-*anti*-B[a]PDE in *supF* of pUB3 either without Heating, with Heating, or with Freeze/Thawing Prior to Transformation into SOS-Induced ES87 Cells

frameshift mutation	*supF*[b] sequence	−heat	+heat	+freeze/thaw[c]
		Spontaneous Mutations		
ΔG at G_{113}[b]	TCCCGAGCGGCCA	0[d] (<0.01)[e]	1 (0.01)	
ΔG at G_{108}–G_{110}	CGCTCGGGAACCC	0 (<0.01)	1 (0.01)	
ΔG at G_{172}–G_{176}	TGGTGGGGGAAGG	1 (0.01)	3 (0.02)	
total		1 (0.01)	5 (0.04)	
		(+)-*anti*-B[a]PDE Mutations		
ΔG at G_{99}–G_{100}	ACCTGTGGTGGGG	2 (0.22)	6 (0.25)	3 (0.24)
ΔG at G_{102}–G_{105}	GTGGTGGGGTTCC	1 (0.11)	6 (0.25)	2 (0.15)
ΔG at G_{108}–G_{110}	CGCTCGGGAACCC	13 (1.4)	15 (0.62)	3 (0.24)
ΔG at G_{115}–G_{116}	CCGAGCGGCCAAA	0 (<0.11)	1 (0.04)	0 (<0.08)
ΔG at G_{122}–G_{124}	CCAAAGGGAGCAG	3 (0.32)	2 (0.08)	1 (0.08)
ΔG at G_{144}	TCTGCCGTCATCG	0 (<0.11)	1 (0.04)	0 (<0.08)
ΔG at G_{172}–G_{176}	TGGTGGGGGAAGG	12 (1.3)	32 (1.3)	15 (1.1)
+G at G_{172}–G_{176}	TGGTGGGGGAAGG	9 (0.97)	25 (1.0)	3 (0.24)
ΔA at A_{106}–A_{107}	CTCGGGAACCCCA	1 (0.11)	3 (0.12)	0 (<0.08)
ΔA at A_{112}	TTCCCGAGCGGCC	1 (0.11)	0 (<0.04)	0 (<0.08)
ΔA at A_{135}–A_{137}	TGGGGGAAGGATT	0 (<0.11)	0 (<0.04)	1 (0.08)
ΔA at A_{170}–A_{171}	TGGGGGAAGGATT	1 (0.11)	0 (<0.04)	4 (0.30)
other frameshifts		0 (<0.11)	3[f] (0.12)	0 (<0.08)
total		43 (4.6)	94 (3.9)	32 (2.4)

[a] The term frameshift is not appropriate when considering a tRNA gene, such as *supF*, but it is convenient. [b] All sequences are given in the 5′ → 3′ direction and are designated according to the deletion of the middle Gua or Ade (underlined) in a run of base pairs. [c] No distinction was made between unadducted pUB3 that was stored at 4 °C for extended periods of time and pUB3 that was also freeze/thawed. [d] Number of mutants detected. [e] Mutation frequency ($\times 10^{+6}$) for the individual mutations. [f] At G_{172}–G_{176}, one insertion of two G·C base pairs, one insertion of three G·C base pairs, and one deletion of two G·Cs base pairs were isolated.

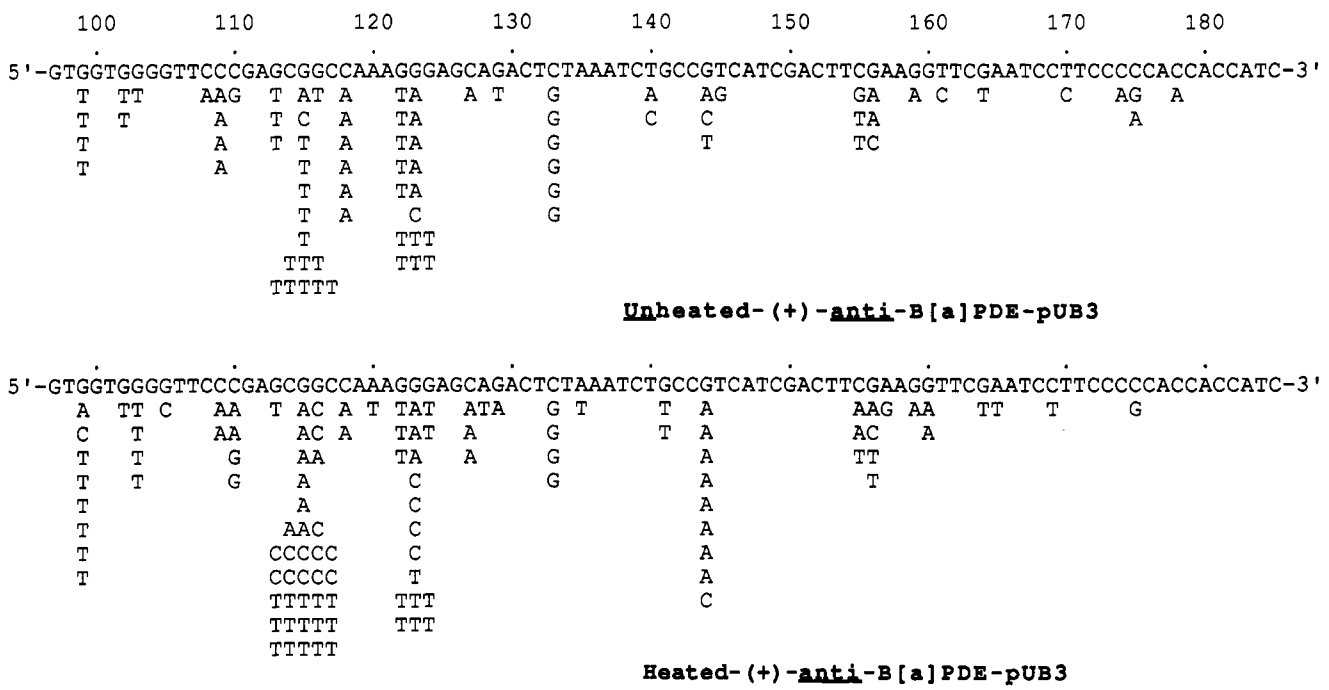

FIGURE 3: (Top) (+)-*anti*-B[a]PDE-induced mutational spectrum for base-pairing mutations detected in the *supF* gene of pUB3 following neither heating nor freeze/thawing prior to transformation into SOS-induced ES87 cells; these results are from Rodriguez and Loechler (1993). (Bottom) (+)-*anti*-B[a]PDE-induced mutational spectrum for base-pairing mutations detected in the *supF* gene of pUB3 following heating (80 °C for 10 min) prior to transformation into SOS-induced ES87 cells. The *supF* sequence as it appears in tRNA is given using the numbering system common to pZ189 (Kraemer & Seidman, 1989), where the promoter is between bp24 and bp58, the pre-tRNA is between bp59 and bp98, and the tRNA gene is between bp99 and bp183.

which is a phenomenon they call "adduct structural polymorphism" (Veaute & Fuchs, 1991; Belguise-Valladier & Fuchs, 1991). In collaborative work with Dr. B. Singer, we have experimental evidence that O⁶MeGua, *the simplest adduct*, adopts at least two different conformations with different biological consequences (data not shown).

Is There Evidence That a Particular Bulky Adduct Can Induce Different Kinds of Mutations Depending upon Its Sequence Context? A site-directed study with *O*⁶-benzylguanine in rat cells (Mitra et al., 1989) demonstrated that this adduct caused principally G → A mutations in one sequence context (5′-CGG-3′), while it caused G → T, A, and C mutations in another sequence context (5′-GGA-3′). Other examples in the literature hint at this same conclusion (reviewed in Loechler, 1993).

Do Different Conformations of Bulky Adducts Not Readily Interconvert? Unheated (+)-*anti*-B[a]PDE-pUB3 was adducted at room temperature for 1 h and manipulated for approximately 1 h at 0 °C prior to transformation. Thus, the results in G_{115} can only be explained by model 1 if the putative multiple conformations do *not* readily interconvert even upon formation of a replication fork. Although intuitively unrea-

Table VI: Number of Mutants when Analyzed According to the Base on the 5′-Side of a Mutated Guanine in the *supF* Gene of pUB3, either with or without Heating (+)-*anti*-B[a]PDE-pUB3 Prior to Transformation into SOS-Induced ES87 Cells

sequence context		G→T	G→A	G→C	total (MF)[a]
5′-T<u>G</u>-3′ (all)	-heat	13[b]	0	0	13 (1.4)
	+heat	14	1	1	16 (0.66)
5′-A<u>G</u>-3′ (all)	-heat	9	1	6	16 (1.7)
	+heat	4	3	4	11 (0.45)
5′-G<u>G</u>-3′ (all)	-heat	16	5	2	23 (2.5)
	+heat	15	6	8	29 (1.2)
5′-C<u>G</u>-3′	-heat	2	5	4	11 (1.2)
(all, except G_{115})[c]	+heat	7	10	4	21 (0.87)
5′-C<u>G</u>$_{115}$-3′[c]	-heat	13	1	1	15 (1.6)
	+heat	15	7	11	33 (1.4)
total	-heat	53	12	13	78 (8.4)
	+heat	55	27	28	110 (4.5)

[a] The number in parentheses is MF ($\times 10^{+6}$). [b] Number of mutants isolated of the indicated type; e.g., 16 G → T mutations were isolated when the Gua undergoing mutation had a Gua on its 5′-side. [c] As discussed in the text, G_{115} is a special site and, thus, is considered separately from the other 5′-C<u>G</u>-3′ sequence contexts.

sonable, it is not inconceivable given the consequences of (e.g.) moving the B[a]P moiety of (+)-*anti*-B[a]P-N^2-Gua from the minor to the major groove, which would accompany an anti to syn rotation about the glycosidic bond. Other conformational differences are also possible.

Does Model 1 Imply That the Labile Adduct Is Not a Premutagenic Lesion? There are examples of certain adducts that are significantly less mutagenic than structural analogues. For example, the major mutagenic adduct of methylating agents, O^6-methylguanine, is a minor adduct [reviewed in Singer and Essigmann (1991)]. Alternatively, it is possible that the labile adduct is (+)-*anti*-B[a]P-N^2-Gua in an unusual conformation in ds DNA.

Model 2: A Role for AP Sites in anti-B[a]PDE Mutagenesis. AP sites are known to be mutagenic (Kunkel, 1984; Loeb & Preston, 1986; Lawrence et al., 1990), and might be generated from (+)-*anti*-B[a]PDE adducts. A significant role for AP sites in B[a]PDE mutagenesis in mammalian cells seems unlikely given that Drinkwater et al., (1980) concluded that <1% of mutations from (±)-*anti*-B[a]PDE were due to AP sites. In addition, our results for *E. coli* (Drouin and Loechler, submitted for publication) show that <~1.5% and <~3% of (+)-*anti*-B[a]PDE base-pairing mutations for unheated and heated (+)-*anti*-B[a]PDE-pUB3, respectively, can be attributed to mutagenesis by AP sites. Finally, a role for AP sites seems unlikely given that freeze/thawing appears to induce many of the same changes as heating, both quantitatively (Table I) and qualitatively (Table II).

It is possible that a heat-labile adduct (e.g., B[a]P-N7-Gua) at G_{115} causes one kind of mutation and is converted by heating to an AP site that causes a different kind of mutation. Lawrence et al. (1990) have described two patterns for AP-site mutagenesis: assuming AP-site generation at Gua adducts, G → T, G → A and G → C, mutations occur at 81%, 4%, and 15%, respectively, for pattern 1 and at 55%, 25%, and 20%, respectively, for pattern 2. The second pattern is not inconsistent with the results at G_{115} following heating (a test for difference gave $P \simeq 0.16$). This model is under investigation but appears unlikely (Drouin and Loechler, submitted for publication).

Model 3: Different Adducts Preferentially Formed in Different Sequence Contexts. N^2-Gua and N7-Gua (i.e., the proposed "labile adduct") adducts generated from (+)-*anti*-B[a]PDE may be preferentially formed in slightly different sequence contexts (Boles & Hogan, 1986; Rill & Marsch, 1990). If each adduct was responsible for a different kind of mutation (e.g., G → T vs G → A), then this might explain how sequence context could influence mutagenic specificity. The multiple adduct model is likely to be important in UV-light mutagenesis (Banerjee et al., 1988, 1990; Lawrence et al., 1990; LeClerc et al., 1991) and has been considered by us for bulky adducts (Loechler, 1989). While not rigorously excluded for (+)-*anti*-B[a]PDE, the multiple adduct model cannot easily explain the results at G_{115}, although it may be important elsewhere. Both *trans*- and *cis*-(+)-*anti*-B[a]-P-N^2-Gua adducts form (Cheng et al., 1989; Sayer et al., 1991), but a significant role for the latter in mutagenesis seems unlikely given that it represents only ~1% of total adducts.

Model 4: Heat-Induced Chemical Changes in an Adduct at G_{115}. As noted above, (+)-*anti*-B[a]P-N^2-Gua is extremely heat-stable in ss oligonucleotides (Benasutti et al., 1988a; Mao et al., 1992). In a duplex plasmid the adduct bond is most labile, based upon our preliminary work, where principally (+)-*anti*-B[a]P-tetraol is released by heating (data not shown). In contrast, the glycosidic bond appears to be more stable, both because no base adducts are released upon heating and because AP sites are not readily formed in (+)-*anti*-B[a]P-pUB3 following heating (Drouin and Loechler, submitted for publication).

Chemical lability of the glycosidic bond is formally model 2. Lability of the adduct bond should generate Gua in DNA, which is not expected to lead to mutagenesis. This leaves unusual and unexpected chemistry. While possible, given the years of study of (+)-*anti*-B[a]PDE adducts, some indication of this should have emerged.

CONCLUSIONS

Our working hypothesis, which most easily rationalizes most of the data presented herein, involves the notion that an adduct can adopt multiple conformations, each of which can cause a different kind of mutation(s). Furthermore, the conformation adopted by an adduct can be influenced by various factors, including DNA sequence context as well as heating and freeze/thawing. However, other models cannot be rigorously excluded, and experiments are in progress to distinguish among them. It should be noted that if mutagenesis is affected by environmental factors, such as heating and freeze/thawing, then the notion of a unique "mutational spectrum" for a particular chemical may prove to be elusive.

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