The Major, N^2-dG Adduct of (+)-*anti*-B[a]PDE Shows a Dramatically Different Mutagenic Specificity (Predominantly, G → A) in a 5′-CGT-3′ Sequence Context†

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ABSTRACT: Mutations induced by the (+)-*anti* diol epoxide of benzo[a]pyrene [(+)-*anti*-B[a]PDE] were described previously in the *supF* gene of the *Escherichia coli* plasmid pUB3 [Rodriguez *et al.* (1993) *Biochemistry, 32,* 1759]. (+)-*anti*-B[a]PDE induced a complex pattern of mutations, including insertions, deletions, frameshifts, as well as base substitution mutations, which for G:C base pairs alone included a significant fraction of G:C → T:A, A:T and C:G mutations. A variety of results suggest that most of these mutations arise from the major adduct ([+ta]-B[a]P-N^2-dG), raising the question how can a single adduct induce different kinds of mutations? Our working hypothesis in this regard is that (1) an adduct can adopt multiple conformations; (2) different conformations cause different mutations; and (3) adduct conformation is controlled by various factors, such as DNA sequence context. To investigate what conformation is associated with what mutation, it is essential to find examples where [+ta]-B[a]P-N^2-dG induces principally one kind of mutation as a prelude to the study in that same context of the conformation-(s) potentially relevant to mutagenesis. Earlier work indicated that (+)-*anti*-B[a]PDE gave a preponderance of G → A mutations in a 5′-CGT-3 sequence context, and herein it is shown that these mutations are likely to be attributable to the major adduct, since in this same sequence context [+ta]-B[a]P-N^2-dG studied site specifically also induces principally G → A mutations (∼82%). Previously, [+ta]-B[a]P-N^2-dG was shown to induce principally G → T mutations (∼97%) in a 5′-TGC-3′ sequence context. Thus, by simply altering its surrounding sequence context this adduct can give a preponderance of either G → A or G → T mutations. This is the most dramatic change in base substitution mutagenic specificity for an adduct described to date and illustrates that the qualitative pattern of mutagenesis by a bulky adduct can be remarkably diverse.

We have been studying mutagenesis by (+)-*anti*-B[a]PDE and its major adduct ([+ta]-*anti*-B[a]P-N^2-dG) (Figure 1) in *Escherichia coli* (Benasutti *et al*., 1988; Loechler, 1989, 1991, 1994, 1995; Loechler *et al*., 1990; Mackay *et al*., 1992; Rodriguez *et al*., 1992; Rodriguez & Loechler, 1993a,b, 1995; Drouin & Loechler, 1993, 1995; Jelinsky *et al*., 1995; Hanrahan *et al*, 1997; Kozack & Loechler, 1997), as well as the corresponding species for dibenz[a,j]anthracene (Gill *et al*., 1993a,b; Min *et al*., 1996). (+)-*anti*-B[a]PDE is a metabolite of benzo[a]pyrene (B[a]P),[1] which is an example of a polycyclic aromatic hydrocarbon and is a ubiquitous environmental contaminant [reviewed in Harvey (1991)].

It is generally believed that the mutagenic potential of (+)-*anti*-B[a]PDE contributes to the carcinogenic potential of B[a]P [reviewed in Harvey (1991)]. Furthermore, there is some evidence that (+)-*anti*-B[a]PDE may be an important contributor to (e.g.) human lung cancer causation based upon recent findings that mutational hotspots in p53 found in lung tumor cells of smokers correspond to hotspots for (+)-*anti*-B[a]PDE adduction of p53 DNA (Denissenko *et al*., 1996). However, this conclusion needs further substantiation since other substances might also show similar reactivity hotspots in p53, and B[a]P can potentially react with DNA following other types of metabolic activation [e.g., Phillips *et al.* (1985), Marnett (1987), Cavalieri *et al.* (1990), and Devanesan *et al.* (1992)].

(+)-*anti*-B[a]PDE induces all classes of mutations in *E. coli*, including base substitutions (45%), frameshifts (24%), insertions (23%), and deletions (8%) (Rodriguez & Loechler, 1993a). Much of our work to date has been focused on base substitution mutagenesis, which primarily involves mutations at G:C base pairs, where a significant fraction of G:C → T:A, AT, and C:G mutations were all isolated (57%, 23%, and 20%, respectively in SOS-induced cells; Rodriguez & Loechler, 1993a,b).

This kind of mutational complexity is typical of bulky mutagens/carcinogens, although the underlying mechanism for it remains unclear. Upon the basis of a variety of results,

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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*); [+ta]-B[a]P-N^2-dG, the major adduct, which is formed via *trans* addition at N^2-dG to C10 of (+)-*anti*-B[a]PDE; pRT0, pTZ19R with all three *Eae*I restriction sites removed and a −1 frame shift mutation in the *lacZ*′ fragment (Jelinsky *et al*., 1995); pRT1, pRT0 with O-G added into its *Hin*cII site; O-G, an unadducted decamer with the G144 DNA sequence context (5′-GACGCCG_{144}TCATCC-3′); [+ta]-B[a]P-G144, O-G modified to contain [+ta]-B[a]P-N^2-dG in the G144 sequence context; [+ta]-B[a]P-G144-pRT1, pRT1 with [+ta]-B[a]P-N^2-dG in the G144 sequence context from *supF*; C-pRT1, pRT1 constructed in parallel with B[a]P-tG144-pRE1, but with no adduct; GHD, gapped heteroduplex DNA; TE, 10mM Tris-HCl (pH 8.0)/1mM EDTA (pH 8.0); TAE, 40 mM Tris-acetate, 1 mM EDTA (pH 8.0).

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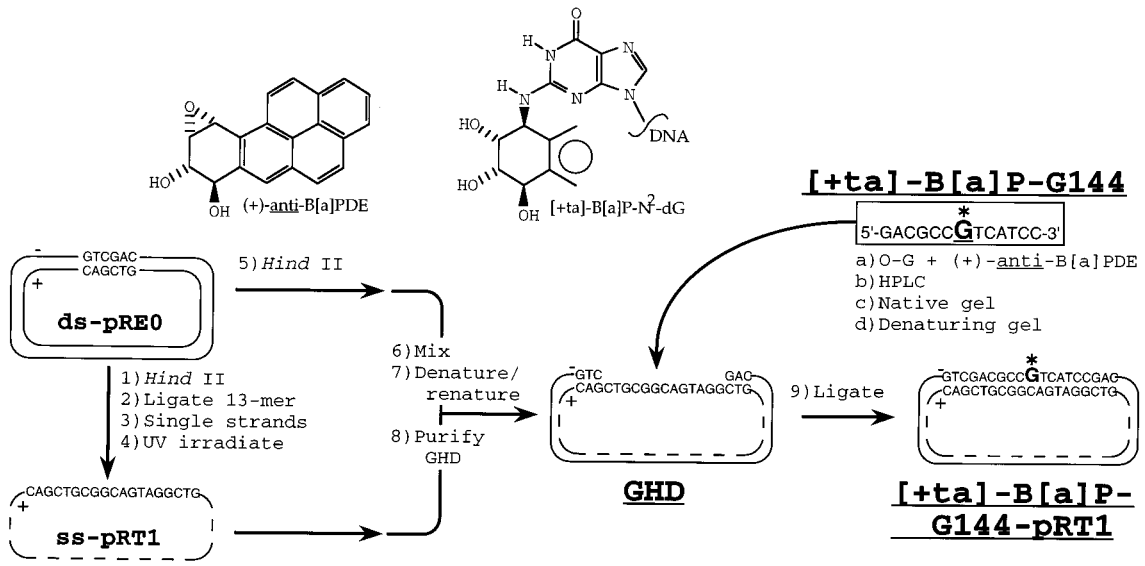

FIGURE 1: Structures of (+)-*anti*-B[a]PDE and its major adduct [+ta]-B[a]P-N^2-dG and the strategy to situate this adduct in a sequence context corresponding to G144. Steps a − d: [+ta]-B[a]P-G144 was formed by reaction of O-G with (+)-*anti*-B[a]PDE and purified by HPLC, as well as native and denaturing gel electrophoresis. Steps 1 and 2: construction of pRT1 by the introduction of a duplex oligonucleotide (5′-GACGCCGTCATCC-3′/5′-GGATGACGGCGTC-3′) into the unique *Hind*II site in pRE0. Steps 3 and 4: ss-pRT1 was isolated and UV irradiated. Steps 5−8: ds-pRE0 was digested with *Hind*II and mixed with UV irradiated ss-pRT1 and denatured/renatured to give gapped heteroduplex DNA (GHD), which was isolated and purified. Step 9: [+ta]-B[a]P-G144 was covalently incorporated into the GHD via ligation to give [+ta]-B[a]P-G144-pRT1.

we have proposed that most of the mutations from (+)-*anti*-B[a]PDE arise from the major adduct, [+ta]-B[a]P-N^2-dG (Cheng *et al*., 1989; Sayer *et al*., 1991), via adopting multiple conformations with different mutagenic consequences, where factors such as DNA sequence context influence adduct conformation (Rodriguez & Loechler, 1993b; Loechler, 1995). Preliminary results (S. A. Jelinsky, & E. L. Loechler, unpublished results) suggest that this hypothesis is correct. In the last several years, it has become clear that B[a]P adducts can indeed adopt a variety of conformations, and a review describing this rich conformational complexity has recently appeared (Geacintov *et al*., 1997).

If this hypothesis is true, then the next questions become what conformation is associated with what mutation and, finally, why. To associate a conformation with a particular mutation, it is necessary to find examples where [+ta]-B[a]P-N^2-dG induces principally one kind of mutation, so that adduct conformation can be studied. Previously, we established that [+ta]-B[a]P-N^2-dG induces principally G → T mutations in a 5′-TGC-3′ sequence context (Mackay *et al*., 1992). An NMR study in this same sequence context revealed that the major conformation had the guanine of the adduct base paired with its complementary cytosine and the pyrene moiety in the minor groove pointed toward the base on the 5′ side of the adduct (Fountain & Krugh, 1995). However, there was a minor adduct conformation, whose structure could not be determined.

In our studies with (+)-*anti*-B[a]PDE in the mutational target *supF* (Rodriguez & Loechler, 1993b), a preponderance of G → A mutations were observed at position G144 (5′-CGT-3′) in SOS-induced cells. Herein, we describe the construction of a plasmid using site-specific means that contains [+ta]-*anti*-B[a]P-N^2-dG in a sequence context that is identical to the G144 context in *supF* for three bp on the 5′ side and five bp on the 3′ side of the adduct. Using this plasmid, we show that [+ta]-*anti*-B[a]P-N^2-dG induces predominantly G → A mutations.

MATERIALS AND METHODS

The 7*R*,8*S*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene [(+)-*anti*-B[a]PDE] was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Lot 92-356-91-19). All (+)-*anti*-B[a]P-containing material was handled as described previously (Benasutti *et al*., 1988), including working under yellow lights with it. All other materials were as described previously (Jelinsky *et al*., 1995), except *Tth*111I, which was obtained from New England Biolabs. Strains were described in detail elsewhere (Rodriguez & Loechler, 1992), but we note that ES87 cells are wild-type for all known DNA repair functions and have the genotype *ara*, *thi*, Δ*pro-lac*, *strA*/F′ (*pro*$^+$, *lacI*^{Q}, *lacI*^{am26}, *lacZ*Δ*M15*). All plasmids have a ColE1 (ds-DNA) and f1 (ss-DNA) origin of replication derived from pTZ19R, and have been described previously (Jelinsky *et al*., 1995) with the exception of pRT1 (see below).

Synthesis and Purification of [+ta]-B[a]P-G144. The oligonucleotide (5′-GACG$_{141}$CCG$_{144}$TCATCC-3′) (O-G) was purchased from Midland Certified Reagent Co. (Midland, TX) and purified by HPLC prior to adduction (Benasutti *et al*., 1988). The (+)-*anti*-B[a]PDE-modified oligonucleotide [+ta]-B[a]P-G144 was synthesized from O-G using the general approach described previously (Cosman et al., 1990; Mao et al., 1995), as outlined in Jelinsky *et al.* (1995). Purification and subsequent analysis to establish both the stereochemistry and position of the adduct were virtually identical to those described in Jelinsky *et al.* (1995) following published procedures (Cheng *et al*., 1989; Cosman et al., 1990; Geacintov et al., 1991; Mao *et al*., 1995).

HPLC-purified oligonucleotides were 5′-end, ^{32}P-radiolabeled and purified successively by both denaturing and native (i.e., nondenaturing) polyacrylamide (25%) gel electrophoresis (Figure 1; steps c and d) as described previously (Jelinsky *et al*., 1995). Purity was established as >99% (see Results) following gel electrophoresis and visualization using a

Molecular Dynamics Phosphoimager Model SF with the software ImageQuant (version 3.3).

Plasmid Constructions, Transformations, and Mutagenesis Studies. The strategy in Figure 1 was developed to study mutagenesis by [+ta]-B[a]P-N^2-Gua in the G144 sequence context. For this strategy to work, the plasmid must contain only a single *Tth*111I site (5′-GACN_3GTC-3′; G144 underlined) with the adduct in it. The starting plasmid pRE0 (Jelinsky *et al*., 1995) has no *Tth*111I site and had 4 bp removed from the unique *Sph* I in its polylinker, thereby, introducing a −1 frame shift mutation into the *lacZ*′ fragment making it out of frame. A second plasmid, pRT1, was constructed from pRE0 by inserting a duplex decamer (5′-GACGCCGTCATCC-3′/5′-GGATGACGGCGTC-3′), which contained the G144 sequence context embedded in a *Tth*111I site, into pRE0 (Figure 1; steps 1, and 2) using the method of Basu *et al.* (1987); the addition of 13 bp restores the reading frame in *lacZ*′.[2]

ss-pRT1 was irradiated with a saturating dose of UV lesions (4800 J/m^2) to give ∼20 lesions per strand (Figure 1; steps 3 and 4; Mackay *et al*., 1992).[3] Thereafter, ss-pRT1 was denatured/renatured (i.e., heteroduplexed) with pRE0, which had been cleaved with *Hinc*II to give blunt-end, ds-linear DNA (Figure 1, steps 5−7). The product of interest, an open circular molecule with a 13 nucleotide gap (gapped heteroduplex DNA [GHD]), was purified by agarose gel electrophoresis (Figure 1, step 8). O-G and [+ta]-B[a]P-G144 were each ligated individually into this gapped duplex to give products designated: C-pRT1 and [+ta]-B[a]P-G144-pRT1, respectively (Figure 1, step 9).

Following ligation, C-pRT1 and [+ta]-B[a]P-G144-pRT1 were each purified by gel exclusion chromatography (Jelinsky *et al*., 1995). We note that more extensive purification of these vectors was not performed for several reasons.[4]

[+ta]-B[a]P-G144-pRT1 and C-pRT1 were each transformed into ES87 cells using a procedure that is identical to the one that we have used in all of our mutagenesis studies (Rodriguez et al., 1992; Jelinsky *et al*., 1995). ES87 cells were used that were SOS induced by the procedure of Koffel-Schwartz *et al.* (1984) (UV irradiation at 254 nm; total dose, 12.6 J/m^2, which gives a cell survival of ∼50%).

[+ta]-B[a]P-N^2-dG was incorporated into a *Tth*111I restriction endonuclease recognition site in [+ta]-B[a]P-G144-pRT1, as opposed to an *Eae*I site in B[a]P-G115-pRE1 (Jelinsky *et al*., 1995). Accordingly, characterizations and mutant enrichments involving [+ta]-B[a]P-G144-pRT1 were performed with *Tth*111I instead of *Eae*I. We note that digestions involving *Tth*111I were conducted at 65 °C. Mutations in the vicinity of the adduct would result in the elimination of the unique *Tth*111I restriction site, which includes position G144, in pRT1. This serves as the basis for the mutant enrichment procedure, which was essentially identical to the one used for the same adduct in a *Pst*I site (Mackay *et al*., 1992) or in an *Eae*I site (Jelinsky *et al*., 1995). Mutant enrichment required only three rounds of enrichment because *Tth*111I cleaved its recognition sequence more effectively than *Eae*I, which required at least four rounds of mutant enrichment. MF at any particular site in the *Tth*111I site of C-pRT1 and [+ta]-B[a]P-G144-pRT1 was calculated essentially as described previously (Mackay *et al*., 1992; Jelinsky *et al*., 1995).[2]

RESULTS

Synthesis and Purification of an Oligonucleotide Containing [+ta]-B[a]P-N^2-dG in the G144 Sequence Context. An oligonucleotide with [+ta]-B[a]P-N^2-dG in the G144 sequence context ([+ta]-B[a]P-G144) was synthesized by reacting (+)-*anti*-B[a]PDE with the corresponding unadducted oligonucleotide O-G (5′-GACG_{141}CCG_{144}TCATCC-3′) as described previously (Jelinsky *et al*., 1995). Products were separated by reverse phase HPLC, and several peaks were isolated for further analysis (data not shown). Oligonucleotides containing [+ta]-B[a]P-N^2-dG adducts were identified by both CD spectroscopy and digestion to the mononucleoside level followed by cochromatography with a known adduct standard (data not shown) as done previously (Cheng *et al*., 1989; Cosman et al., 1990; Geacintov et al., 1991). Importantly, two major peaks were observed, and each contained [+ta]-B[a]P-N^2-dG. The location of the adduct in each case was determined by modified Maxam-Gilbert DNA sequencing with DMS (data not shown) as done previously (Mao et al., 1995). One species had an adduct in the position corresponding to G144, while the other had an adduct in the position corresponding to G141; no work was performed with the latter.

Following HPLC purification, [+ta]-B[a]P-G144 and an unadducted control (O-G) were purified by both native and denaturing polyacrylamide gel electrophoresis (data not shown) according to our published methods (Jelinsky *et al*., 1995). Our experience is that all three steps are necessary to achieve material that is >99% pure for reasons discussed in Jelinsky *et al.* (1995). After these three steps of

[2] Cells containing pRE0 give a white colony when plated on X-gal, a substrate for β-galactosidase, because pRE0 has a −1 frame shift in its *lacZ*′ fragment. In contrast, cells containing pRT1 have the reading frame restored and give a blue colony. This phenotypic difference between starting plasmid, pRE0, and the final product, pRT1, allows us to readily distinguish the two. In addition, by analyzing the ratio between blue and white colonies we are able to determine the mutation frequency as described previously (Mackay *et al*., 1992; Jelinsky *et al*., 1995).

[3] Ultimately, ss-pRT1 becomes the strand that does not contain [+ta]-B[a]P-N^2-dG (dashed line in Figure 1) in [+ta]-B[a]P-G144-pRT1. The UV lesions significantly decreases the yield of progeny plasmids derived from this strand (Mackay *et al*., 1992).

[4] We have never purified B[a]P-containing, closed circular plasmids for four reasons. First, in general, we wished to minimize the chance of damaging the adduct in [+ta]-B[a]P-G144-pRT1 by minimizing both the extent to which this material was manipulated, and the time between the completion of construction and the mutagenesis experiments, which was never more than seven days. Second, closed circular [+ta]-B[a]P-G144-pRT1 was not purified because this would require exposure to UV light in the presence of ethidium bromide, which can damage B[a]P-containing material both photochemically [e.g., see Margulis *et al*. (1991) and Li *et al*. (1995)] and potentially photodynamically. Third, we were concerned that the presence of ethidium bromide might affect adduct conformation, and thereby adduct mutagenesis, although we have since shown that this is unlikely. Finally, we have tried to match the conditions in our random mutagenesis studies with (+)-*anti*-B[a]PDE (Rodriguez & Loechler, 1993a,b) vs our adduct site-specific studies with [+ta]-B[a]P-N^2-dG (Mackay *et al*., 1992; Jelinsky *et al*., 1995; herein) as closely as possible. To this end we have been sure that (1) both types of studies were done with ds-DNA; (2) both studies utilized similar plasmids with identical origins of replication (ColE1); (3) in each case the adduct was in the proper strand vis-a-vis leading vs lagging strand DNA synthesis; (4) in each case the adduct was in the proper strand vis-a-vis the transcribed vs nontranscribed DNA strand; (5) the same cells (i.e., ES87) were used; (6) the level of SOS induction was the same (see below); and (7) other conditions (e.g., salts, etc.) were similar. With this in mind, in our random mutagenesis studies with (+)-*anti*-B[a]PDE, ∼30% of the plasmid DNA was unavoidably open circular. [+ta]-B[a]P-G144-pRT1 contains ∼44% open circular material (Figure 3), which is reasonably similar.

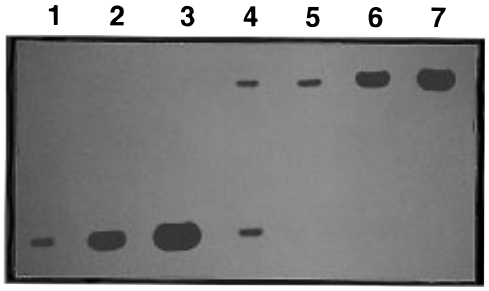

FIGURE 2: Analysis of unadducted O-G and [+ta]-B[a]P-G144, which has [+ta]-B[a]P-N^2-dG in the G144 sequence context. Following the reaction of (+)-*anti*-B[a]PDE with O-G (5′-GACGCCG$_{144}$TCATCC-3′), the products were separated by HPLC according to Mao *et al*., (1995). A peak containing a single [+ta]-B[a]P-N^2-dG at G144 ([+ta]-B[a]P-G144) was isolated (Materials and Methods) and further purified (along with O-G) by both denaturing and native polyacrylamide gel electrophoresis. Native polyacrylamide gel electrophoresis and phosphorimaging gave the results in lanes 1−3 for O-G (relative amounts loaded = 0.01, 0.1 and 1.0, respectively) and lanes 5−7 for [+ta]-B[a]P-G144 (relative amounts loaded = 0.01, 0.1, and 1.0, respectively). Lane 4 shows a mixture of O-G and [+ta]-B[a]P-G144.

purification, the products were analyzed by both denaturing and native polyacrylamide gel; the latter is shown (Figure 2), because it gives better resolution. Lanes 5−7 show [+ta]-B[a]P-G144 in serial 10-fold increasing concentrations; lane 7 has a single band with no contaminating bands at the level of <1%, as judged by comparison to the intensity of the band in lane 5, which is 100-fold less intense. Lanes 1−3 show similar results for O-G, and there is also no contaminants at the level of <1%.

Construction of C-pRT1 and [+ta]-B[a]P-G144-pRT1. The basic strategy for our studies (Figure 1) follows that described in Jelinsky et al. (1995). One key feature is that an adduct at position G144 is embedded in a *Tth*111I restriction site (5′-GACN$_3$GTC-3′; G144 position underlined), which must ultimately be a unique site in our construct. A plasmid pRE0 was constructed (Jelinsky *et al*., 1995) by introducing a −1 frame shift mutation into the polylinker region, such that the *lacZ*′ fragment is out-of-frame. A second plasmid, pRT1, was constructed by inserting a duplex 13mer, which contained the G144 sequence context embedded in a *Tth*111I site, into pRE0 (Materials and Methods); the addition of 13 bp restores the reading frame in *lacZ*′.[2]

ss-pRT1, which had been UV irradiated (Materials and Methods) in order to minimize the generation of progeny plasmids from the strand not containing the B[a]P-adduct (see below), was denatured/renatured (i.e., heteroduplexed) with pRE0, which was cleaved with *Hinc*II to give blunt-end, ds-linear DNA.[3] The product of interest, an open circular molecule with a 13 nucleotide gap (gapped heteroduplex DNA [GHD]) was purified by agarose gel electrophoresis. O-G and [+ta]-B[a]P-G144 were each ligated individually into this gapped duplex to give products designated C-pRT1 and [+ta]-B[a]P-G144-pRT1, respectively. The efficiency of ligation was estimated to be ∼68% and ∼56%, respectively (data not shown).

The products C-pRT1 and [+ta]-B[a]P-G144-pRT1 were characterized. One means to establish that a particular restriction site contains an adduct has been to show that the adduct blocks cleavage by the corresponding restriction endonuclease (*Tth*111I in this case). [+ta]-B[a]P-G144-pRT1 is insensitive to cleavage by *Tth*111I (Figure 3, lane 4). [Some closed circular [+ta]-B[a]P-G144-pRT1 is converted to open circular DNA due to the fact that *Tth*111I digestions are done at 65 °C.) Closed circular C-pRT1, which contains no adduct, is mostly cleaved to linear material by *Tth*111I (Figure 3; lane 2).

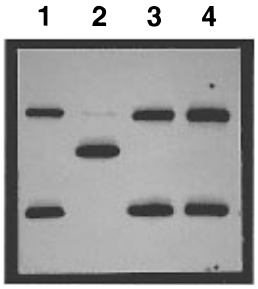

FIGURE 3: Characterization of C-pRT1 and [+ta]-B[a]P-G144-pRT1 by *Tth*111I digestions. C-pRT1 was either not treated (lane 1) or treated (lane 2) with *Tth*111I. B[a]P-tG144-pRE1 was either not treated (lane 3) or treated (lane 4) with *Tth*111I. Following agarose gel electrophoresis, the gel was dried and analyzed by phosphorimaging. Bands at three positions are observed, and correspond to open circular, linear, and closed circular plasmid (top to bottom).

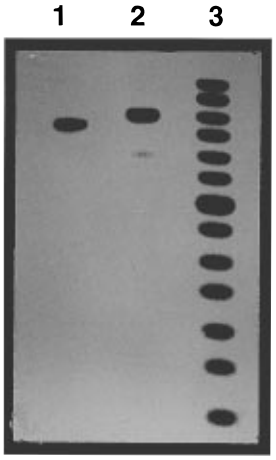

FIGURE 4: Characterization of C-pRT1 and [+ta]-B[a]P-G144-pRT1 following the liberation of an adduct-containing, 27-nucleotide fragment. C-pRT1 (lane 1) and B[a]P-tG144-pRE1 (lane 2) were each digested with *Pst*I and *Bam*HI, which should liberate a 27-nucleotide fragment of sequence: 5′-GGTCGACGCCG$_{144}$-TCATCCGACTCTAGAG, where the underlining shows the sequence of the original oligonucleotide (i.e., O-G and [+ta]-B[a]P-G144) incorporated into the GHD. The samples were separated by denaturing polyacrylamide gel electrophoresis, and analyzed by phosphorimaging. For size comparisons, lane 3 shows a ladder with bands at every two nucleotides between 8 and 32 nucleotides in length.

A second method was used to show that [+ta]-B[a]P-G144-pRT1 did indeed contain a lesion. [+ta]-B[a]P-G144-pRT1 was digested with *Pst*I and *Bam*HI, which should liberate an adduct-containing, 27 nucleotide fragment (sequence: 5′-GGTCGACGCCGTCATCCGACTCTAGAG-3′, where the underlining shows the sequence of O-G) following denaturation. When analyzed by denaturing polyacrylamide gel electrophoresis, [+ta]-B[a]P-G144-pRT1 gives one major band (Figure 4; lane 2), which migrates more slowly than the corresponding major band from C-pRT1 (lane 1), because the presence of the adducts retards migration. [+ta]-B[a]P-G144-pRT1 gave minor bands, which appeared at positions ∼23 and ∼17 and are likely to be attributable to ligation of a small fraction (∼5%) of the adducted oligonucleotide into the gapped duplex on the 3′ but not the 5′ side, and on the 5′ but not the 3′ side,

Table 1. Base Substitution Mutations in the *Tth*111I Site in Progeny Plasmids Derived from [+ta]-B[a]P-G144-pRT1, Which Contains [+ta]-B[a]P-N^2-Gua at Position G144, and the Corresponding Nonadduct-Containing, Control Plasmid (C-pRT1) Following Transformation into SOS-Induced ES87 Cells[a]

G144 →	T	A	C	total[b]	MF (in %)[a]
[+ta]-B[a]P-G144-pRT1	10	54	2	66	0.68
C-pRT1	1	1	0	2	0.033

[a] C-pRT1 or [+ta]-B[a]P-G144-pRT1 was transformed into SOS-induced ES87 cells, progeny plasmids isolated and enriched for those having mutations in the *Tth*111I restriction site (5′-GACGCCG$_{144}$TC-3′), which includes position G144 (see text). [b] Total number of mutants isolated with mutations at G144. Other mutations were also isolated. For [+ta]-B[a]P-G144-pRT1, four were missing the 13 base pair insert, seven were G138 → A, and one was unreadable. For C-pRT1, eighteen were missing the 13 base pair insert, and there was one each of the following: G138 → A, G138 → T, and G141 → C. [c] MF is given in percent (e.g., 0.68 = 0.68%, or 0.68 × 10^{-2}) at the indicated site and was calculated as described in Jelinsky *et al*., (1995).

respectively. These kinds of incomplete ligation products have been observed in the past using similar construction strategies (Benasutti *et al*., 1988; Gill *et al*., 1993b; Jelinsky *et al*., 1995).

Mutants Derived from [+ta]-B[a]P-G144-pRT1. [+ta]-B[a]P-G144-pRT1 and C-pRT1 were transformed into SOS-induced ES87 cells. [ES87 was used because we wished to compare these results to our previous random adduction experiments with (+)-*anti*-B[a]PDE, in which we used ES87 cells (Rodriguez & Loechler, 1993a,b).] On the basis of transformations, the ratio of progeny yield from C-pRT1: [+ta]-B[a]P-G144-pRT1 was 1.0:0.74. [These values are difficult to interpret for reasons discussed in Jelinsky *et al.* (1995).]

Mutations at position G144 eliminate the unique *Tth*111I site in pRT1 rendering progeny plasmids resistant to cleavage by *Tth*111I, which was used as the basis of the enrichment for mutations in the G144 region (Jelinsky *et al*., 1995). After three rounds of mutant enrichment by *Tth*111I digestions, plasmid DNA was isolated from individual colonies and the positions of the mutations determined by DNA sequencing.

[+ta]-B[a]P-G144-pRT1 principally induced G → A base substitutions (82%) with MF ∼0.68% for the G144 position (Table 1). C-pRT1 gave a much lower MF (∼0.033%). [Values for MF reported in Table 1 are internally consistent, but cannot be interpreted as the inherent MF for [+ta]-B[a]P-N^2-Gua in the G144 sequence context for the same reasons as discussed earlier Jelinsky *et al*., (1995).] Stray mutants were also observed (Table 1; footnote 2), but are unlikely to be attributable to the adduct and were ignored.

DISCUSSION

Mutations at G144 Are Likely To Be Due to [+ta]-B[a]P-N^2-Gua. There are several reasons why the mutations observed in the progeny plasmids derived from transformations into SOS induced ES87 cells are likely to be due to the [+ta]-B[a]P-N^2-dG adduct situated at position G144 in [+ta]-B[a]P-G144-pRT1, rather than a putative contaminant.

(1) The oligonucleotide with [+ta]-B[a]P-N^2-Gua at G144 ([+ta]-B[a]P-G144) contained no detectable contaminants at the <1% level (Figure 2), and in fact careful quantitative analysis reveals that it must be <0.1%. This level of contamination is lower than the observed MF (0.68%; Table 1) from [+ta]-B[a]P-G144-pRT1. Thus, for a putative contaminant to be responsible for the mutations, one of two possibilities would have to be true. First, the putative contaminant could have copurified with [+ta]-B[a]P-G144. This seems unlikely given that three different purification steps were used, and this procedure readily separates isomers, including oligonucleotides with *cis* vs *trans* adducts, and with adducts at different guanines in the same oligonucleotide (data not shown; Jelinsky *et al*., 1995). Second, this putative contaminant could be at a lower level and still be responsible for mutations if it were preferentially ligated into the plasmid, which is hypothetically possible given that [+ta]-B[a]P-G144 was present in an ∼20-fold molar excess compared to GHD. This implies that the putative contaminant could represent at most ∼2% (=∼20 × 0.1%) of the ligation material, which means that it would have to cause a mutation ∼34% (=[0.68%/2%]100%) of the time it was bypassed. Of course, if this putative adduct were repaired, this value would have to be increased accordingly. In most cases, notably for bulky adducts [discussed in Loechler (1996)], adduct mutagenesis is not this high, especially when DNA repair is possible. Preliminary experiments suggest that this putative contaminant could not have been an oligonucleotide containing the other characterized guanine adduct, which forms via *cis* addition of (+)-*anti*-B[a]PDE to N^2-dG, because the latter does not copurify with [+ta]-B[a]P-G144, and gives a different mutational pattern, where G → A mutations do not dominate. *In toto* it seems unlikely that mutations can be attributed to a trace contaminant of purified oligonucleotide [+ta]-B[a]P-G144.

(2) MF at G144 from [+ta]-B[a]P-G144-pRT1 is higher than MF from the control with no adduct (C-pRT1) by >10-fold (Table 1). Thus, it is unlikely that [+ta]-B[a]P-G144-pRT1 can be attributed to the presence of a contaminating plasmid that contains the G144 → A mutation.

(3) One alternative possibility is that the mutations at G144 with [+ta]-B[a]P-G144-pRT1 are from the UV lesions in the strand that does not contain [+ta]-B[a]P-N^2-Gua. This would require [+ta]-B[a]P-N^2-Gua at G144 in the non-UV-lesion-containing strand in [+ta]-B[a]P-G144-pRT1 to enhance mutagenesis by the UV lesions compared to C-pRT1, which contains no adduct in the non-UV-lesion-containing strand. Such a mechanism could not be rigorously excluded in one of our previous studies (Jelinsky *et al*., 1995). To investigate this possibility, we have incorporated [-ta]-B[a]P-N^2-dG at position G141 in a pRT1 vector and in preliminary experiments have found that MF at G144 is virtually the same as with C-pRT1 (data not shown). This makes the possibility under consideration in point 3 extremely unlikely.

(4) Qualitatively, [+ta]-B[a]P-N^2-Gua (Table 1) gave predominantly G144 → A mutations (Table 1), which was also obseved for (+)-*anti*-B[a]PDE itself: 8/9 mutants at G144 were G → A with (+)-*anti*-B[a]PDE (+SOS/+heat; Rodriguez & Loechler, 1993b). Thus, the putative contaminant would have to have fortuitously given the same mutations at G144 as were obtained with (+)-*anti*-B[a]PDE.

In summary, although none of the arguments discussed above are rigorously definitive, their collective weight makes it likely that the mutations at G144 are indeed due to [+ta]-B[a]P-N^2-dG and not to a putative contaminant.

Implications of the G144 → A Mutations from [+ta]-B[a]P-N^2-dG. Perhaps the most remarkable finding in this study is that a single adduct, [+ta]-B[a]P-N^2-dG, is fully capable of inducing a preponderance of either G → A mutations as shown herein using a 5′-CGT-3′ sequence context, or G → T mutations as reported in one of our earlier

studies using a 5′-TGC-3′ sequence context (Mackay *et al*., 1992). This is the most dramatic change in base substitution mutagenic specificity of which we are aware. Although several other studies have shown that adduct mutagenic specificity can depend on DNA sequence context [examples can be found in Loechler (1996), including one of our earlier studies (Jelinsky *et al*., 1995) where we showed that [+ta]-B[a]P-N^2-dG can also induce a mixture of G → T, A, and C mutations in a 5′-CGG-3′ sequence], the mutational variation has typically been less extreme. Thus, changing DNA sequence context can dramatically affect the pattern of bulky adduct-induced base substitution mutagenesis, which has already been established for frameshift mutagenesis based on studies of the major adduct of 2-acetylaminofluorene (Burnouf *et al*., 1989; Lambert *et al*., 1992; Napolitano *et al*., 1994).

The fact that we have established a sequence context in which G → A mutations predominate suggests that studies of the conformation(s) of [+ta]-B[a]P-N^2-dG in this same context may help us understand what conformation is responsible for G → A mutations. This will be complicated for at least two reasons. First, mutations from [+ta]-B[a]P-N^2-dG may not be caused by the dominant conformation, which we have discussed previously [e.g., Loechler (1996)]. Second, most structural studies are done with ds-oligonucleotides, and this information may not be entirely relevant to mutagenesis, which is occurring at a ss/ds-junction in the presence of a DNA polymerase, as we have discussed previously [e.g., Loechler (1991)]. Nevertheless, there is precedent for the notion that information gained from the study of ds-oligonucleotides may be relevant to mutagenesis [e.g., Garcia *et al.* (1993) and Milhe *et al.* (1996)]. In addition, we have discussed the possibility that in some cases, adduct conformation in ds-DNA may indeed be preserved in some important way at the ss/ds-junction, and in that sense, adduct conformation in ds-DNA may be relevant to and predictive of what is important to mutagenesis [e.g., Loechler (1995) and references therein]. Finally, there may be examples where the dominant conformation is the mutagenic conformation.

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