How Stereochemistry Affects Mutagenesis by N^2-Deoxyguanosine Adducts of 7,8-Dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene: Configuration of the Adduct Bond Is More Important Than Those of the Hydroxyl Groups†

Rajiv Shukla,‡ Scott Jelinsky,‡ Tongming Liu,§ Nicholas E. Geacintov,§ and Edward L. Loechler*,‡

Department of Biology, Boston University, Boston, Massachusetts 02215, and Department of Chemistry, New York University, New York, New York 10003

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ABSTRACT: Previous work has shown that the major adduct from the (+)-*anti* diol epoxide of benzo[*a*]-pyrene (B[*a*]P), which forms at N^2-deoxyguanosine [(+)-*trans*-*anti*-B[*a*]P-N^2-dG], is capable of inducing either predominantely G → T mutations (∼95%) in a 5′-TGC-3 sequence context or predominantly G → A mutations (∼80%) in a 5′-CGT-3′ sequence context. This is likely to be attributable to the major adduct being in a different mutagenic conformation in each case. In the next phase of this work, the questions to be addressed are what conformation is associated with what mutation and why? To help define what aspect of adduct structure is important to mutagenesis, the work herein reports on the mutations induced in a single sequence context by four stereoisomers of B[*a*]P−N^2-dG: (+)-*trans*-, (+)-*cis*-, (−)-*trans*-, and (−)-*cis*-. The (+)-*trans*- and (−)-*cis*-adducts show a remarkably similar mutational pattern with G → A mutations predominating (∼80%). The (−)-*trans*- and (+)-*cis*-adducts also show a similar mutational pattern with a more even mixture of G → T, G → A, and G → C mutations. Each of these adducts has an adduct bond and three hydroxyl groups at four consecutive saturated carbons in the B[*a*]P moiety of the adduct; the stereochemistry at these four positions differs in each of the adducts. The (+)-*trans*- and (−)-*cis*-adducts are a pair sharing the *S* configuration for the adduct bond, although they are a mirror image vis-a-vis the hydroxyl groups. The (−)-*trans*- and (+)-*cis*-adducts share the opposite adduct bond stereochemistry (*R*) but differ in the stereochemistry of their hydroxyl groups. Thus, there is a correlation suggesting that *anti*-B[*a*]P−N^2-dG adduct mutagenesis is more dependent on the stereochemistry of the adduct bond than on the stereochemistry of the hydroxyl groups.

Benzo[*a*]pyrene is an example of a polycyclic aromatic hydrocarbon, which is a class of ubiquitous environmental pollutants produced by incomplete combustion that can be found in urban air, cigarette smoke, and charred foods [reviewed in Harvey (1991)]. B[*a*]P[1] is metabolically activated to the mutagenic and carcinogenic diol epoxide (+)-*anti*-B[*a*]PDE, which can react with DNA principally to give an adduct at N^2-deoxyguanosine ([+ta]-B[*a*]P−N^2-dG; Table 1; Cheng *et al*., 1989; Sayer *et al*., 1991). In fact, (+)-*anti*-B[*a*]PDE may contribute to (e.g.) human lung cancer, based upon recent findings that mutational hotspots in p53 found in lung tumor cells from smokers correspond to hotspots for (+)-*anti*-B[*a*]PDE adduction of p53 DNA (Denissenko *et al*., 1996). There are, however, caveats to this supposition since other substances might also show similar reactivity hotspots in p53 and it has been shown that B[*a*]P can potentially react with DNA following other types of metabolic activation [e.g., see Phillips *et al*. (1985), Marnett (1987), Cavalieri *et al*. (1990), and Devanesan *et al*. (1992)].

Our work has focused on random mutagenesis with (+)-*anti*-B[*a*]PDE itself, as well as adduct site-specific mutagenesis with the major adduct [+ta]-*anti*-B[*a*]P−N^2-dG in *Escherichia coli* (Benasutti *et al*., 1988; Loechler *et al*., 1990; Mackay *et al*., 1992; Rodriguez *et al*., 1992; Rodriguez & Loechler, 1993a,b, 1995; Drouin & Loechler, 1993, 1995; Loechler, 1989, 1991, 1994, 1995, 1996; Jelinsky *et al*., 1995; Hanrahan *et al*., 1997; Shukla & Loechler, 1997;

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* Author to whom correspondence should be addressed.

‡ Boston University.

§ New York University.

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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*); (−)-*anti*-B[*a*]PDE, (−)-*t*-7,*r*-8-dihydroxy-*r*-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-deoxyguanosine to C10 of (+)-*anti*-B[*a*]PDE; [+ca]-B[*a*]P−N^2-dG, the corresponding *cis*-addition adduct of N^2-deoxyguanosine and (+)-*anti*-B[*a*]PDE; [−ta]-B[*a*]P−N^2-dG, the corresponding *trans*-addition adduct of N^2-deoxyguanosine and (−)-*anti*-B[*a*]PDE; [−ca]-B[*a*]P−N^2-dG, the corresponding *cis*-addition adduct of N^2-deoxyguanosine and (−)-*anti*-B[*a*]PDE; pRE0, plasmid pTZ19R with all three *Eae*I restriction sites removed and a −1 frameshift mutation in the *lacZ*′ fragment (Jelinsky *et al*., 1995); pRT1, plasmid pRE0 with O-G added into its *Hin*cII site; O-G, an unadducted 13-mer 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 ([+ca]-, [−ta]-, and [−ca]-B[*a*]P-G144 are the corresponding oligonucleotides with [+ca]-, [−ta]-, and [−ca]-B[*a*]P−N^2-dG in the G144 sequence context); [−ta]-B[*a*]P-G141, the corresponding oligonucleotide with [−ta]-B[*a*]P−N^2-dG in the G141 sequence context; [+ta]-B[*a*]P-G144-pRT1, plasmid pRT1 with [+ta]-B[*a*]P−N^2-dG in the G144 sequence context from *supF* ([+ca]-, [-ta]-, and [−ca]-B[*a*]P-G144-pRT1 are the corresponding plasmids with [+ca]-, [−ta]-, and [−ca]-B[*a*]P−N^2-dG in the G144 sequence context from *supF*, and [−ta]-B[*a*]P-G141-pRT1 is the corresponding plasmid with [−ta]-B[*a*]P-N^2-dG in the G141 sequence context from *supF*); C-pRT1, plasmid pRT1 constructed in parallel but with no adduct; GHD, gapped heteroduplex DNA; TE, 10 mM Tris-HCl (pH 8.0)/1 mM EDTA (pH 8.0); TAE, 40 mM Tris-acetate and 1 mM EDTA (pH 8.0); MF, mutation frequency.

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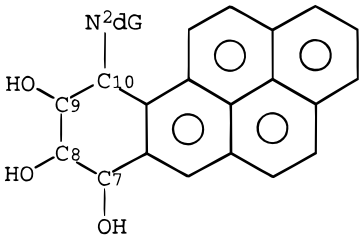
Table 1: Single Base Substitution Mutations Isolated in a 5′-CGT-3′ Sequence from B[*a*]P−N^2-G Adducts with Different Stereochemical Configurations[a] When Studied in SOS-Induced ES87 Cells[b]



G144[c] →	T	A	C	total[c]	MF[d] (%)
[+ta]-B[*a*]P-G144-pRT1[e]	10	54	2	66	0.68
[−ca]-B[*a*]P-G144-pRT1	7	75	14	96	0.11
[−ta]-B[*a*]P-G144-pRT1	35	48	58	141	0.24
[+ca]-B[*a*]P-G144-pRT1	26	16	34	76	0.58
C-pRT1[f]	1	1	0	2	0.033
C-pRT1[g]	3	4	1	8	0.013
[−ta]-B[*a*]P-G141-pRT1[h]	1	3	1	5	0.014

[a] The absolute stereochemistry at C10 (adduct bond) and C7−C9 (hydroxyl groups) for each of the adducts is as follows, where u means up and d means down: [+ta] C10 u (*S*), C9 d, C8 d, C7 u; [−ta] C10 d (*R*), C9 u, C8 u, C7 d; [−ca] C10 u (*S*), C9 u, C8 u, C7 d; [+ca] C10 d (*R*), C9 d, C8 d, C7 u. [b] C-pRT1 or B[*a*]P-G144-pRT1 was transformed into SOS-induced ES87 cells, and progeny plasmids were isolated and enriched for those having mutations in the *Tth*111I restriction site (5′-GACGCCG$_{144}$TC-3′), which includes position G144 (see text). [c] The number of each type of base substitution mutation at position G144, as well as the total, obtained for each of the indicated plasmids. Other types of mutations were also isolated and are listed here, where the number of each isolated is one unless indicated otherwise in parentheses. [+ta]-B[*a*]P-G144-pRT1: missing the 13 bp insert (4), G138 → A (7), one unreadable. [−ca]-B[*a*]P-G144-pRT1: missing the 13 bp insert (16), missing the 13 bp insert and two additional bases (67), C140 → T/C142 → T, G141 → A, C143 → T/G144 → A (2). [-ta]-B[*a*]P-G144-pRT1: missing the 13 bp insert (26), missing the 13 bp insert and two additional bases (25), A139 → G, G141 → A (2), G141 → T, C143 → T (2), C143 → G/G144 → C (2), T145 → C, C146 → G, C146 → A. [+ca]-B[*a*]P-G144-pRT1: missing the 13 bp insert (9), G138 → A (6), C141 → C, C149 → T (2), unreadable (2). C-pRT1: missing the 13 bp insert (18), G138 → A, G138 → A, G138 → T, G141 → C. C-pRT1: missing the 13 bp insert (23), missing the 13 bp insert and two additional bases (76), A139 → G, G141 → C, C142 → G, C142 → G/C143 → T, C143 → A/G144 → A. [−ta]-B[*a*]P-G141-pRT1: missing the 13 bp insert (27), missing the 13 bp insert and two additional bases (40), G141 → A, G141 → T, T145 → C, C146 → G, G138 → A, A139 → C, A139 → G, C142 → T/C143 → T, C143 → T/G144 → A (2). [d] 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). [e] Data from Shukla and Loechler (1997). [f] Data from Shukla and Loechler (1997), and the control for the results with [+ta]-B[*a*]P-G144-pRT1. [g] The control for the results with [−ca]-B[*a*]P-G144-pRT1, [−ta]-B[*a*]P-G144-pRT1, and [+ca]-B[*a*]P-G144-pRT1. [h] The control to rule out mutagenesis by the UV lesions in the strand not containing the B[*a*]P-N^2-dG adduct (see text). Note that the adduct is at position G141, while the mutations are at G144.

Kozack & Loechler, 1997). We have also done similar studies with the corresponding species for dibenz[*a, j*]-anthracene (Gill *et al*., 1993a,b; Min *et al*., 1996). (+)-*anti*-B[*a*]PDE induces a full spectrum of mutations, including base substitutions (45%), frameshifts (24%), insertions (23%), and deletions (8%) (Rodriguez & Loechler, 1993a,b). Much of our work to date has focused on base substitution mutagenesis, which primarily involves mutations at G-C base pairs, where a significant fraction of G-C → T-A, G-C → A-T, and G-C → C-G mutations were all isolated (57%, 23%, and 20%, respectively, in SOS-induced cells; Rodriguez & Loechler, 1993a,b).

We have investigated several hypotheses for this mutational complexity and concluded that it is unlikely that pathways involving minor adducts or adduct breakdown products, such as apurinic sites, are significant (Drouin & Loechler, 1993, 1995). Our work to date suggests that most of the mutations from (+)-*anti*-B[*a*]PDE can be attributed to [+ta]-*anti*-B[*a*]P−N^2-dG (see below), leading to the question: by what mechanism(s) can a single adduct induce multiple kinds of mutations? Our working hypothesis has been that a single adduct may adopt multiple conformations in DNA, each of which may induce a different mutation, and that adduct conformation can be controlled by factors such as DNA sequence context (Rodriguez & Loechler, 1993b; Loechler, 1995). In the last several years, it has become clear that B[*a*]P adducts can indeed adopt a remarkably complex diversity of conformations [reviewed in Geacintov *et al*. (1997)].

The hypothesis in the previous paragraph is simplistic, and determining exactly what conformations are most relevant to mutagenesis will be difficult (see Discussion). Whatever is the case, it is important to begin to approach the questions: which adduct conformation is associated with what mutation and why? To associate a conformation with a particular mutation, we believe that it will be helpful to have examples where [+ta]-B[*a*]P−N^2-dG induces principally one kind of mutation, which should simplify—as much as is possible—the study of the relationship between adduct conformation and mutagenesis. 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). More recently, we showed that G → A mutations predominated in a 5′-CGT-3′ sequence context (Shukla & Loechler, 1997). Our hypothesis to explain these results is that the DNA polymerase is bypassing a different adduct conformation when the G → T mutation is being induced vs when the G → A mutation is being induced and that DNA sequence context is dictating the relative proportions of these different adduct conformations.

By whatever mechanism, these findings show that there is nothing inherent about the kinds of mutations induced by [+ta]-B[*a*]P−N^2-dG, which appears to be able to induce a dramatically different mutational pattern depending on its sequence context. This conclusion was reinforced by our findings that [+ta]-B[*a*]P−N^2-dG induced yet a third pattern (a more even mixture of G → T, G → A, and G → C mutations) in a 5′-CGG-3′ sequence context (Jelinsky *et al*., 1995). It is important to note that each of these results correlated with what was obtained when (+)-*anti*-B[*a*]PDE was studied in a *supF* gene in *E. coli* cells, where G → T mutations dominated in 5′-TG-3′ sequences, G → A mutations dominated at position G144 (5′-CGT-3′), and a mixture of G → T, G → A, and G → C mutations were observed at G115 (5′-CGG-3′). These correlations reinforce the notion that [+ta]-B[*a*]P−N^2-dG is indeed the adduct responsible for the majority of the mutations induced by (+)-*anti*-B[*a*]-PDE. The role that sequence context can play in [+ta]-B[*a*]P−N^2-dG adduct mutagenesis was also evident in the work of Moriya *et al.* (1996), who showed that G → T mutations predominated in a 5′-TG-3′ sequence context, while a greater preponderance of G → A and G → C mutations were found in a 5′-GGC-3′ sequence context.

The results described in the previous paragraph permit one to conclude that a single stereochemically distinct adduct can induce different mutations in different sequence contexts. As a corollary, herein we have investigated how mutagenesis is affected by varying the stereochemistry of this type of N^2-dG adduct in a single sequence context. We have assessed the relative significance of the stereochemistry of the adduct bond at C10 vs the hydroxyl groups at C7, C8, and C9 in B[*a*]P−N^2-dG by studying four stereochemically distinct adducts, namely, [+ta]-, [+ca]-, [−ta]- and [−ca]-B[*a*]P−N^2-dG (structures in Table 1), in the G144 (5′-C<u>G</u>T-3′) sequence context. This information should prove useful in deciding what aspects of B[*a*]P−N^2-dG structure are important to adduct conformation relevant to mutagenesis.

EXPERIMENTAL PROCEDURES

The 7*R*,8*S*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-B[*a*]PDE] (lot 92-356-91-19), and the 7*S*,8*R*-dihydroxy-9*R*,10*S*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene [(−)-*anti*-B[*a*]PDE] (lot 93-449-49-19) were each purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository. All 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, Shukla & Loechler, 1997). Strains and plasmids were as described previously (Rodriguez & Loechler, 1992; Shukla & Loechler, 1997).

Synthesis and Purification of Oligonucleotides. The oligonucleotide 5′-GACG$_{141}$CCG$_{144}$TCATCC-3′ (O-G) was purchased from Midland Certified Reagent Co. (Midland, TX), and purified by reverse-phase HPLC prior to adduction (Benasutti *et al*., 1988). A total of five oligonucleotides were synthesized and are designated [+ta]-B[*a*]-G144, [+ca]-B[*a*]-G144, [−ta]-B[*a*]-G144 and [−ca]-B[*a*]-G144, with each having the indicated adduct in a 5′-C<u>G</u>T-3′ sequence context corresponding to G144 in the *supF* gene; and [−ta]-B[*a*]P-G141, which has the indicated adduct in a sequence context corresponding to G141 in *supF*. Each of these was synthesized from O-G using a general approach described previously (Cosman *et al*., 1990; Mao *et al*., 1995), as outlined in Jelinsky et al. (1995) and Shukla and Loechler (1997), although the pH of the reaction mixture was lowered from ∼11 to 7.0 for [+ca]-B[*a*]-G144 and [−ca]-B[*a*]-G144. We have found that, at the lower pH value, the yields of *cis*-adducts are more comparable to those of *trans*-adducts (M. Cosman, J. Chen, T.-M. Liu, and N. E. Geacintov, unpublished results). Reverse-phase HPLC purification and subsequent analysis to establish both the stereochemistry and the position of the adduct were effectively identical to those described in Jelinsky *et al.* (1995) and Shukla and Loechler (1997) following published procedures (Cheng *et al*., 1989; Cosman *et al*., 1990; Geacintov *et al*., 1991; Mao *et al*., 1995).

Following HPLC purification, each oligonucleotide was ^{32}P-radiolabeled at the 5′ end and purified successively by both denaturing and native (i.e., nondenaturing) polyacrylamide (20%) gel electrophoresis as described previously (Jelinsky *et al*., 1995; Shukla & Loechler, 1997). 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 to study mutagenesis by B[*a*]P−N^2-Gua adducts in the G144 sequence context was effectively identical to our previous study of mutagenesis by [+ta]-B[*a*]P−N^2-dG alone in the G144 sequence context (Shukla & Loechler, 1997). A total of six plasmids were constructed. The plasmids designated [+ta]-B[*a*]P-G144-pRT1, [+ca]-B[*a*]P-G144-pRT1, [−ta]-B[*a*]P-G144-pRT1, and [−ca]-B[*a*]P-G144-pRT1 have a B[*a*]P−N^2-dG adduct of the indicated stereochemistry in the G144 sequence context. The plasmids designated C-pRT1 and [−ta]-B[*a*]P-G141-pRT1 represent controls; they have no adduct and a [−ta] adduct in the G141 sequence context, respectively. Each of these plasmids was transformed into SOS-induced ES87 cells and progeny plasmids were isolated as described previously (Jelinsky *et al*., 1995; Shukla & Loechler, 1997). Our strategy was developed such that the sequence context of G144 (but not G141) is embedded in a unique *Tth*111I restriction site (5′-GACN$_3$<u>G</u>TC-3′; G144 underlined), which provided a means to isolate progeny plasmids with mutations at G144, because they are *Tth*111I-insensitive, as described previously (Shukla & Loechler, 1997). [We note that mutations at G141 should not be detectable based on the reported recognition sequence for *Tth*111I; however, we have consistently observed a few G141 mutations, suggesting that *Tth*111I specficity extends beyond what is reported.] MF at G144 in the *Tth*111I site was calculated essentially identically to the way that we have done previously for the same adduct in a *Pst*I site (Mackay *et al*., 1992), in an *Eae*I site (Jelinsky *et al*., 1995), and in a *Tth*111I site (Shukla & Loechler, 1997).

RESULTS

Synthesis and Purification of Oligonucleotides Containing Different Stereoisomers of B[a]P-N^2-dG in the G144 Sequence Context (5′-C<u>G</u>T-3′). In the work reported herein, mutagenesis by the stereoisomers [+ca]-, [−ta]-, and [-ca]-B[*a*]P−N^2-dG were studied in the 5′-C<u>G</u>T-3′ sequence context, corresponding to G144 in *supF* of the plasmid pUB3 (Rodriguez & Loechler, 1993a,b). Herein we also include data from our previous work on [+ta]-B[*a*]P−N^2-dG in the same sequence context (Shukla & Loechler, 1997). For purposes of comparison, [+ta]-B[*a*]P−N^2-dG is presented in the same figures with [+ca]-B[*a*]P−N^2-dG, even though the mutagenesis studies on [+ta]-B[*a*]P−N^2-dG were done in earlier experiments with material constructed previously. In the interest of brevity, it is sometimes easier to describe this work as if [+ta]-B[*a*]P−N^2-dG was done simultaneously.

Oligonucleotides containing B[*a*]P−N^2-dG adducts with four different stereoisomeric configurations ([+ta]-, [+ca]-, [−ta]-, and [−ca]-; Table 1) were synthesized by reacting (+)-*anti*-B[*a*]PDE or (−)-*anti*-B[*a*]PDE with the unadducted oligonucleotide O-G (5′-GACG$_{141}$CCG$_{144}$TCATCC-3′) as described previously (Experimental Procedures; Jelinsky *et al*., 1995; Shukla & Loechler, 1997). In each case, reaction products were separated by reverse-phase HPLC and several peaks were isolated for further analysis (data not shown). The nature of the B[*a*]P−N^2-dG adduct in each oligonucleotide was determined by both CD spectroscopy and digestion to the mononucleoside level followed by cochromatography with known adduct standards (data not shown), essentially using procedures reported previously (Cheng *et al*., 1989; Cosman *et al*., 1990; Geacintov *et al*., 1991). Modified

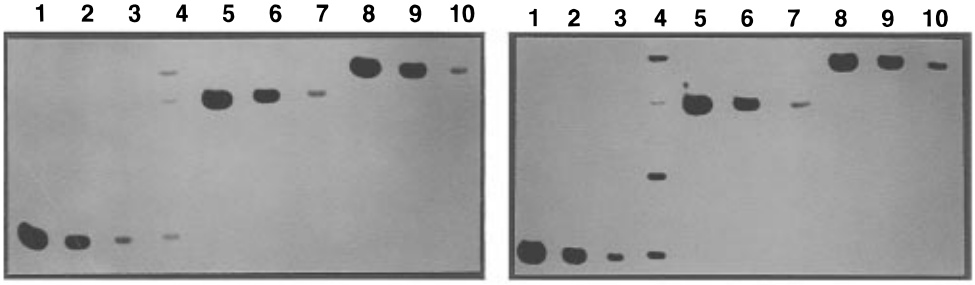


FIGURE 1: Analysis of unadducted O-G and [+ta]-, [+ca]-, [−ta]-, and [−ca]-B[*a*]P-G144, which have [+ta]-B[*a*]P−N^2-dG in the G144 sequence context. Following the reaction of (+)-*anti*-B[*a*]PDE or (−)-*anti*-B[*a*]PDE with O-G (5′-$GACG_{141}CCG_{144}$TCATCC-3′), the products were separated by reverse-phase HPLC according to Mao *et al*. (1995). Peaks containing a single B[*a*]P−N^2-dG adduct at G144 were isolated (Experimental Procedures), and further purified (along with O-G) by both denaturing and native polyacrylamide gel electrophoresis. The images are following native polyacrylamide gel electrophoresis and phosphorimaging. (Left panel) lanes 1−3, O-G; lanes 5−7, [+ca]-B[*a*]P-G144; lanes 8−10, [+ta]-B[*a*]P-G144. Lane 4 shows a mixture of the three oligonucleotides. (Right panel) Lanes 1−3, O-G; lanes 5−7, [−ca]-B[*a*]P-G144; lanes 8−10, [−ta]-B[*a*]P-G144. Lane 4 shows a mixture of the three oligonucleotides, plus [−ta]-B[*a*]P-G141. For each oligonucleotide containing an individual adduct, the relative amounts loaded were 1.0, 0.1, and 0.01 (left to right) in successive lanes.

Maxam−Gilbert DNA sequencing with dimethylsulfate (DMS) (data not shown) was used to assess which oligonucleotides had an adduct at position G144 using a procedure described previously (Mao *et al*., 1995). An oligonucleotide with a [−ta] adduct at position G141 was also isolated as a control (see below).

Following HPLC purification, each adducted oligonucleotide, and the unadducted control (O-G), were purified by both native and denaturing polyacrylamide gel electrophoresis (data not shown) as described in Jelinsky *et al.* (1995). After these three steps of purification, the oligonucleotides were characterized. Lanes 1−3 of Figure 1 (left panel) show O-G at serial 10-fold increasing concentrations when analyzed by native polyacrylamide gel electrophoresis. Lane 3 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 1, which is 100-fold less intense. A similar conclusion is reached for both [+ca]-B[*a*]P-G144 (lanes 5−7) and [+ta]-B[*a*]P-G144 (lanes 8−10). Lane 4 (Figure 1, left panel) shows that each of these oligonucleotides resolves from the others by this technique. Figure 1 (right panel) shows similar results for O-G (lanes 1−3), [−ca]-B[*a*]P-G144 (lanes 5−7), and [−ta]-B[*a*]P-G144 (lanes 8−10), as well as a mixture of these three oligonucleotides plus [−ta]-B[*a*]P-G141 (lane 4). Each of these oligonucleotides contains no contaminant at the <1% level. A careful quantitative analysis of all of the data in Figure 1 gave the following results. O-G in the left panel had two contaminants at the 0.2% and 0.3% level that each migrated slightly more slowly, while O-G in the right panel had one contaminant at the 0.2% level that migrated slightly more slowly. [+ca]-, [+ta]-, [−ca]-, and [−ta]-B[*a*]P-G144 had no contamination at the limit of detection (<0.1% level). Finally, [−ta]-B[*a*]P-G141 was also purified; it contained a contaminant at the ∼3% level. [−ta]-B[*a*]P-G141 and its contaminant could be separated sufficiently to establish that both had an adduct at G141, so the contaminant is probably [−ca]-B[*a*]P-G141. [−ta]-B[*a*]P-G141, which is used as a control, is sufficiently pure for the purposes of our experiments (see below).

*Construction of Plasmids Containing B[a]*P-N^2*-dG Adducts.* The basic strategy to construct plasmids with each of these B[*a*]P−N^2-dG adducts in the G144 sequence context was identical to the procedure in Shukla and Loechler (1997; see Figure 1). The products are designated C-pRT1, [+ta]-B[*a*]P-G144-pRT1, [+ca]-B[*a*]P-G144-pRT1, [−ta]-B[*a*]P-G144-pRT1, and [−ca]-B[*a*]P-G144-pRT1 for the plasmids containing no adduct, [+ta]-, [+ca]-, [−ta]-, and [−ca]-B[*a*]P−N^2-dG, respectively, at G144. [−ta]-B[*a*]P-G141-pRT1 has [−ta]-B[*a*]P−N^2-dG at G141. Each plasmid product has a single adduct in a unique *Tth*111I site located in the polylinker region of a pTZ19r-like plasmid, which has a ColE1 origin of replication. The plasmids were double-stranded and had a large number of UV lesions (∼20) incorporated into the strand of the plasmid that did not contain the B[*a*]P−N^2-dG adduct in order ultimately to minimize the yield of progeny plasmids from the strand not containing B[*a*]P−N^2-dG; i.e., to minimize the problem of "strand bias" [Koffel-Schwartz *et al*., 1987; discussed in Loechler (1996)].

The individual adduct-containing plasmids were characterized to show that the B[*a*]P−N^2-dG-containing plasmids did indeed contain a lesion. Each plasmid 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. Figure 2, left panel, shows that C-pRT1 (lane 2), [+ca]-B[*a*]P-G144-pRT1 (lane 3), and [+ta]-B[*a*]P-G144-pRT1 (lane 4) each gives a single major band when analyzed by denaturing polyacrylamide gel electrophoresis. Figure 2, right panel, shows that C-pRT1 (lane 2), [−ta]-B[*a*]P-G144-pRT1 (lane 3), and [−ca]-B[*a*]P-G144-pRT1 (lane 4) each gives a single major band when analyzed by denaturing polyacrylamide gel electrophoresis. In each case the major band derived from an adduct-containing plasmid migrates more slowly than the one derived from C-pRT1 (lane 1), because the adduct retards migration. Each of the adduct-containing plasmids showed minor bands, which appeared to migrate 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, 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; Shukla & Loechler, 1997).[2]

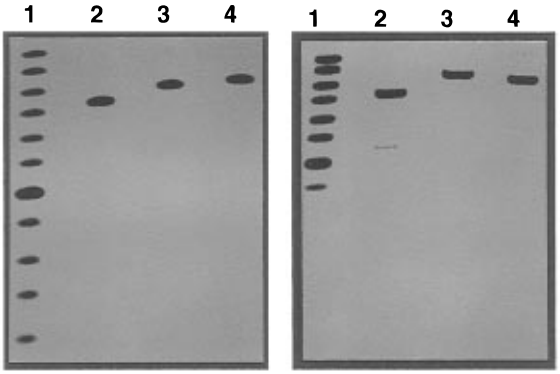


FIGURE 2: Characterization of C-pRT1 and [+ta]-, [+ca]-, [−ta]-, and [−ca]-B[*a*]P-G144-pRT1, which have B[*a*]P−N^2-dG adducts of the indicated stereochemistry in the G144 sequence context of the plasmid pTR1, following the liberation of an adduct-containing fragment. Each plasmid was digested with *Pst*I and *Bam*HI, which should liberate the adduct in a 27-nucleotide fragment of sequence 5′-GGTCGACGCCG$_{144}$TCATCCGACTCTAGAG, where the underlining shows the sequence of the original oligonucleotide (i.e., O-G) incorporated into the plasmid. The images are following denaturing polyacrylamide gel electrophoresis and phosphorimaging. (Left panel) C-pRT1 (lane 2), [+ca]-B[*a*]P-G144-pRT1 (lane 3), and [+ta]-B[*a*]P-G144-pRT1 (lane 4). (Right panel) C-pRT1 (lane 2), [−ta]-B[*a*]P-G144-pRT1 (lane 3), and [−ca]-B[*a*]P-G144-pRT1 (lane 4). For size comparisons, lane 1 in both panels shows a ladder with bands at every two nucleotides between 12 and 32 in length, although the bands from 12 to 16 are too light to be seen in the right panel.

A second 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). Each B[*a*]P-N2-dG-containing plasmid was shown to be more insensitive to cleavage by *Tth*111I than the corresponding non-adduct-containing, control plasmid C-pRT1 (data not shown) in experiments essentially identical to those performed for [+ta]-B[*a*]P-G144-pRT1 in our recent study (Shukla & Loechler, 1997).

Adduct-Derived Mutagenesis. C-pRT1, [+ta]-B[*a*]P-G144-pRT1, [+ca]-B[*a*]P-G144-pRT1, [−ta]-B[*a*]P-G144-pRT1, and [−ca]-B[*a*]P-G144-pRT1 were each transformed into SOS-induced ES87 cells. [We have used ES87 in all of our recent adduct site-specific studies because we wished to compare our results to our random adduction experiments with (+)-*anti*-B[*a*]PDE, in which we used ES87 cells (Rodriguez & Loechler, 1993a,b).] 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 based on a procedure that we have used in all of our adduct site-specific studies (Mackay *et al*., 1992; Jelinsky *et al*., 1995; Shukla & Loechler, 1997). The types of mutations isolated at G144 for each of the plasmids are reported in Table 1. Mutations at positions other than G144 were also observed (Table 1; footnote *c*) but either are present at too low a frequency to be reliably attributed to an adduct or are unlikely to be due to an adduct and were ignored. Apparent MF is also reported in Table 1 for each of the experiments; these values are hard to interpret for reasons discussed in Jelinsky *et al.* (1995).

[2] An explanation for why we have chosen not to purify closed circular, adduct-containing constructs can be found in footnote 4 in Shukla and Loechler (1997).

We considered the possibility that the mutations obtained with [+ta]-B[*a*]P-G144-pRT1, [+ca]-B[*a*]P-G144-pRT1, [−ta]-B[*a*]P-G144-pRT1, and [−ca]-B[*a*]P-G144-pRT1 (Table 1) might be attributed to the UV lesions incorporated into the non-B[*a*]P-containing DNA strand (for the purposes of eliminating "strand bias"; see above). For this to be true, the B[*a*]P adducts would have to enhance mutagenesis by the UV lesions, since MF for the B[*a*]P-containing plasmids was greater than MF for the non-B[*a*]P-containing, control plasmid (C-pRT1; Table 1), which also contained UV lesions. In essence, the notion is that the presence of the B[*a*]P moiety might be eliminating strand bias against mutagenesis due to the UV lesions. If such a mechanism were operative, then it seems sensible to expect that the presence of a B[*a*]P moiety in the vicinity of G144 would also enhance this putative pathway of UV lesion mutagenesis. To evaluate this possibility, we constructed a plasmid with [−ta]-B[*a*]P−N^2-dG at position G141 and determined MF at G144 (Table 1). The fact that [−ta]-B[*a*]P-G141-pRT1 gave approximately the same MF as C-pRT1 argues against this mechanism.

DISCUSSION

It is likely that the mutations observed in the progeny plasmids were due to adducts at position G144 in the constructs for the following reasons. [A more thorough discussion of these arguments can be found at the beginning of the Discussion section in Shukla and Loechler (1997).] (1) The purified oligonucleotides containing B[*a*]P−N^2-Gua adducts are extremely pure (Figure 1); each adducted oligonucleotide had <0.1% contamination. (2) MF at G144 from the adduct-containing plasmids was higher than MF for the control with no adduct (C-pRT1) by >10-fold in each case, except [−ca]-B[*a*]P−N^2-dG (Table 1). (3) It is extremely unlikely that the mutations at G144 are from the UV lesions in the strand that does not contain the B[*a*]P−N^2-G for reasons discussed in the last paragraph of Results.

Effect of Sequence Context and Adduct Stereochemistry on Mutagenesis. We have previously shown that a single stereochemically distinct adduct (i.e., [+ta]-B[*a*]P−N^2-dG) is able to induce a different pattern of mutations in different sequence contexts. For example, it induces G → A mutations in one sequence context [5′-C$\underline{G}$T-3′; Table 1, data from Shukla and Loechler (1997)], or G → T mutations in another (5′-T$\underline{G}$C-3′; Mackay *et al*., 1992). This is the most dramatic change in base substitution mutagenic specificity reported to date, and it shows that there is nothing inherent to the kind of mutations that a particular adduct can induce. In another sequence context (5′-C$\underline{G}$G-3′), we also showed that [+ta]-B[*a*]P−N^2-dG can induce a more even mixture of G → T, G → A, and G → C mutations (Jelinsky *et al*., 1995). The work of Moriya *et al*. (1996) also shows that sequence context can affect B[*a*]P−N^2-dG adduct mutagenesis.

In a corollary to the work summarized in the previous paragraph, herein we have shown that different stereochemically distinct adducts can induce different mutations in the same sequence context. [+ta]-B[*a*]P−N^2-dG and [−ca]-B[*a*]P−N^2-dG gave a preponderance of G → A mutations (∼80%), while [+ca]-B[*a*]P−N^2-dG and [−ta]-B[*a*]P−N^2-dG gave a more even mixture of G → T, G → A, and G → C mutations (Table 1).

These data bear on the question: what aspect of stereochemistry is important for defining the mutagenic pattern of

an adduct? In a broad sense, there are two stereochemical regions of B[*a*]P−N^2-dG adducts that might be relevant to mutagenesis: the chirality of the adduct bond at the C10 position of the B[*a*]P moiety vs the chirality of the hydroxyl groups at C7, C8, and C9 of the B[*a*]P moiety (Table 1). [+ta]- and [−ca]-B[*a*]P−N^2-dG both share the *S* absolute configuration for the adduct bond at C10, although they are mirror images with respect to stereochemistry of the hydroxyl groups. These two adducts gave a similar mutagenic pattern (principally G → A mutations), suggesting that mutagenesis is influenced more by the stereochemistry of the adduct bond than the absolute configuration of the three hydroxyl groups. If the stereochemistry of the hydroxyl groups were to dominate, then one would expect that (e.g.) the pattern of mutagenesis from [+ta]- and [+ca]-B[*a*]P−N^2-dG would be more similar, which they are not. A similar conclusion can be reached on the basis of the similarity of the mutagenic patterns associated with the 10*R*, [+ca]- and [−ta]-B[*a*]P−N2-dG adducts (Table 1). Nevertheless, a careful examination of the data set in Table 1 reveals that there are more subtle differences between the 10*S* and the 10*R* adduct pairs, indicating that the absolute configurations of the three hydroxyl groups do play a secondary role in determining the patterns of mutations. The most striking differences are that the 10*S* [−ca] adduct appears to give rise to a proportionally higher fraction of G → C mutations than the 10*S* [+ta] adduct, while the 10*R* [−ta] adduct gives a proportionally higher fraction of G → A mutations than the 10*R* [+ca] adduct.

Correlations between the structural characteristics of stereoisomeric pairs of 10*S* and 10*R* B[*a*]P−N^2-dG adducts have also been noted in other studies. The NMR solution structures of the two 10*R* and the two 10*S* *anti*-B[*a*]P−N^2-dG lesions in a 5′-CGC-3′ sequence context in double-stranded oligonucleotides were determined by Cosman et al. (1992, 1993, 1996) and by de los Santos et al. (1992). These results were analyzed in detail by Geacintov et al. (1997), who showed that the 10*S* [+ta]- and [−ca]-B[*a*]P−N^2-dG lesions on the one hand, and the 10*R* [−ta]- and [+ca]-B[*a*]P−N^2-dG lesions on the other, are conformationally related to one another with similar relative orientations of the covalently linked B[*a*]P and dG residues. In addition, Christner et al. (1994) and Chary et al. (1995a,b) have reported that the pattern of inhibition of DNA polymerases *in vitro* depends predominantly on the *R* and *S* absolute configurations of N^6-dA residues covalently linked to the C10 position of B[*a*]P. Evidence that stereochemistry affects B[*a*]P−N^2-dG mutagenesis is also evident in the work of Moriya *et al.* (1996).

Relationship between Adduct Conformation and Adduct Mutation. Our view is that differences in the configurations of these related stereoisomeric adducts affects the population of conformations that each adopts in DNA, which in turn affects the mutational outcome. While this idea may be sensible in a general way, it is simplistic, and formulating it in greater mechanistic detail, and its investigation, will be difficult for at least five interrelated reasons. First, the investigation of the conformation of bulky adducts currently is conducted principally on double-stranded oligonucleotides, and it is not clear whether this information will be relevant to mutagenesis, which is occurring at a single/double-stranded DNA junction in the presence of a DNA polymerase [discussed in Loechler (1991)]. Second, this is complicated further by the fact that mutagenesis occurs in a cell, and there may be other factors that influence qualitative and/or quantitative aspects of mutagenesis accordingly, such as DNA repair, leading vs lagging strand DNA synthesis, DNA topology, binding of other proteins to DNA, etc. Third, the conformation most directly relevant to mutagenesis may be difficult to investigate since it is actually the transition state associated with the rate-determining step in the mutagenic bypass of the adduct by the relevant DNA polymerase [discussed in Loechler (1996)]. Fourth, a description of the mutagenic process would also include information about other intermediates in the pathway; e.g., it may prove useful to identify the ground state that is most closely associated with or is the direct precursor to the mutagenic transition state. However, this ground state may very well be a minor conformation, which will complicate its identification [discussed in Loechler (1996)]. Fifth, the fact that a single adduct may be able to induce different mutations depending on what conformation it adopts may complicate mutagenesis for another reason. On the one hand, interconversion between relevant mutagenic conformations may be relatively rapid, even in the presence of a DNA polymerase, in which case the initial conformation of the adduct in double-stranded DNA encountered by the DNA polymerase would be largely irrelevant to the mutagenic outcome. Alternatively, interconversion between relevant mutagenic conformations might be relatively slow (compared to the rate of polymerization), in which case the initial conformation of the adduct encountered by the DNA polymerase could in principle dictate the mutagenic pathway. The latter would mean that the population of adduct conformations in double-stranded DNA could have a profound influence on the pathway of mutagenesis.

Regarding the fifth point in the previous paragraph, we have discussed the possibility that *in some cases* adduct conformation in double-stranded DNA may indeed be preserved in some important way at the single/double-stranded DNA junction in the presence of a DNA polymerase—i.e., that interconversion between adduct conformations may be slow—and in that sense the conformation of an adduct in double-stranded DNA may be relevant to the pathway of mutagenesis [e.g., Loechler (1995) and references therein]. This is based on the fact that some of our data are difficult to explain by any other mechanism (Rodriguez & Loechler, 1993b, 1995; Drouin & Loechler, 1993, 1995). We hasten to add that there are other examples in our data where there is no need to invoke such a possibility.[3]

The notion that adduct conformational interconversion is relatively slow compared to the rate of DNA polymerization is counterintuitive in that the commonly held view is that DNA is very flexible and that conformational changes in DNA occur relatively rapidly. However, this issue currently is difficult to evaluate definitively, since there is little data on the rates of interconversion between different conformations, especially in the case of dramatically different conformations with bulky adducts. For example, one can imagine situations where different mutagenic conformations might involve an adduct switching grooves and flipping over.

[3] We note that one of the senior authors (N.E.G.) is uncomfortable with this interpretation of the data, which is principally attributable to the other senior author (E.L.L.), although neither senior author has been able to think of a reasonable alternative explanation.

Such a complex change of conformation would surely require multiple steps and be relatively slow as a result. Even the view that the rate of conformational interconversion should increase once a DNA polymerase encounters an adduct might not necessaily be true, given that DNA polymerases appear to surround DNA near their active site and hold it relatively rigidly.

It is important to add that the simplistic hypothesis that adduct mutational complexity is due to adduct conformational complexity--may be true independent of whether it is true that adducts can get trapped in conformations in double-stranded DNA. In any event, as models are developed to try to understand how B[*a*]P−N^2-dG adducts induce mutations, the information described herein may prove useful since it suggests that attention should be more focused on the stereochemistry of the adduct bond.

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