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Effect of Mismatched Complementary Strands and 5′-Change in Sequence Context on the Thermodynamics and Structure of Benzo[*a*]pyrene-Modified Oligonucleotides†

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ABSTRACT: Benzo[*a*]pyrene (B[*a*]P) is a well-studied environmental carcinogen that when activated can react with DNA to form four major adducts: (+)-trans-, (−)-trans-, (+)-cis-, and (−)-cis-*anti*-B[*a*]P−dG. In this study, two oligonucleotides (5′-dCCATT-G$^{B[a]P}$−CTACC-3′ and 5′-dCCATC-G$^{B[a]P}$−CTACC-3′) were prepared, each containing the four isomeric adducts, and these were hybridized to either complementary sequences or to sequences containing an A, G, or T opposite the adducted guanine. Thermal melting curves, CD, and UV spectra of each duplex were measured and compared with the unmodified counterpart. The raw and relative thermodynamic measurements were then compared which indicated that differences occur that are both adduct and sequence dependent. These differences were next compared with the in vitro DNA polymerase incorporation data and were found to be strikingly correlated. Most significantly, for all four B[*a*]P isomers a mismatch of an A across from the adduct resulted in the least amount of relative destabilization, while the Watson−Crick complement C showed the most; in vitro studies showed that A is the preferred base incorporated across from each isomer, while C was incorporated least often. This observed correlation suggests that one factor contributing to misincorporation at an adduct site is the thermodynamic stability of the incorporated base. Structurally, the effect of sequence context and mismatched complementary strands were also compared, suggesting that all adducts tend to intercalate within the helix when they are complemented with a mismatched complementary strand. In addition, the level of this intercalation seems to be both sequence and stereoisomer dependent.

Benzo[*a*]pyrene (B[*a*]P)[1] is an important environmental carcinogen whose mutagenic properties have been the subject of extensive investigations (*1*−*10*). Cellular metabolism of B[*a*]P results in the formation of *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (racemic (±)-*anti*-B[*a*]PDE) (*11*, *12*). Reaction of this metabolite with DNA proceeds through the *trans*- and-*cis*-addition of the exocyclic amino group of guanine to the C^{10}-position of *anti*-B[*a*]PDE to form the (+)-trans, (−)-trans, (+)-cis, and (−)-cis adducts at the N^2-position of guanine (Figure 1A). The B[*a*]PDE-induced mutational data were first collected using the Ames test (*13*−*15*), but since then numerous forward mutation analyses have been carried out. In bacteria it has been shown that the G to T substitutions are the predominant base-substitution mutation, although G to A, G to C, A to T, and other base substitutions, deletions, and frameshifts were also observed (*3*, *8*−*10*, *16*). Studies of B[*a*]PDE-induced mutagenesis in eukaryotic cells have shown similar specificities (*2*−*4*, *17*). One conclusion from these studies is that

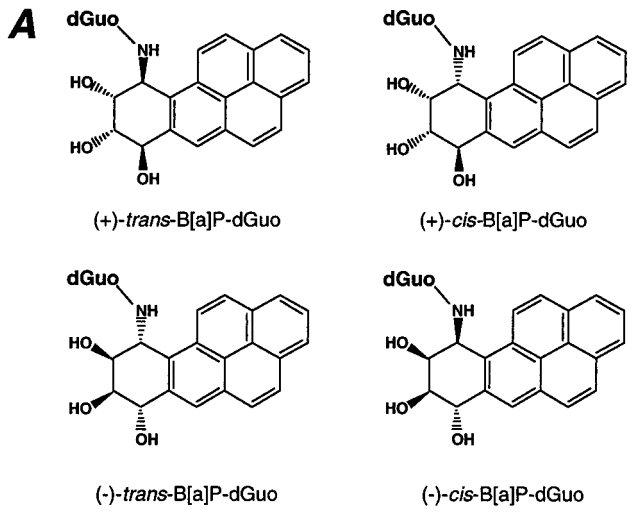

FIGURE 1: (A) Structures of the B[*a*]P adducts. (B) DNA sequences used in the melting experiments.

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[1] Abbreviations: B[*a*]P, benzo [*a*]pyrene; (±)-*anti*-BPDE, *r*7,*t*8-dihydroxy-*t*9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; oligonucleotide I, 5′-dCCATT-G$^{B[a]P}$-CTACC-3′; oligonucleotide II, 5′-dCCATC-G$^{B[a]P}$-CTACC-3′; BPI, represents modified duplexes countering oligonucleotide I; BPII, represents modified duplexes countering oligonucleotide II; M(A), represents duplexes containing a mismatch of an A across the guanine of the respective oligonucleotide; M(G), represents duplexes containing a mismatch of G across the guanine of the respective oligonucleotide; M(T), represents duplexes containing a mismatch of T across the guanine of the respective oligonucleotide.

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the mutagenic specificity at a modified guanine residue can be influenced by the base on its immediate 5′-side. For example, using site specifically modified templates containing (+)-anti-B[*a*]P−*N*-2-Gua adducts it was shown that a G to T substitution occurred exclusively in a 5′-TGC-3′ sequence, while G to T, G to A, and G to C mutations occurred in a 59:22:19 ratio in a 5′-CGG-3′ sequence (*8*, *10*).

The dependence of DNA structure on B[*a*]P isomer structure and DNA sequence has also been of particular interest. The NMR structures of four DNA duplexes, each having the sequence d(5′-CCATC-G$^{B[a]P}$-CTACC-3) but containing one of the four isomerically pure B[*a*]P adducts, have been determined (*18*−*21*). These studies have shown that in the case of the trans adducts, the B[*a*]P moiety is located in the minor groove pointing toward the 5′-end of the modified strand for the (+)-trans, and toward the 3′-end for the (−)-trans adduct. Although hydrogen bonding is still intact between the modified guanine and its complementary cytosine, in both cases there is considerable deviation from ideal hydrogen bonding angles. In the case of the cis adducts, it has been reported that the B[*a*]P moiety is intercalated within the duplex and stacked between the intact neighboring base pairs pointing toward the minor groove for the (+)-cis and the major groove for the (−)-cis adduct. In both cases the modified guanine and its cytosine partner are displaced outside the helix. It was further reported that the (+)-cis adduct can undergo a small conformational equilibrium placing the B[*a*]P moiety in the minor groove. No such conformational equilibrium was reported for the other three adducts. These structures are compared and further analyzed in a relatively recent comprehensive review (*22*).

The minor groove positioning properties of the trans adducts and the intercalation properties of the cis adducts were earlier reported by Geacintov and co-workers by conducting absorbance, fluorescence, and linear and circular dichroism measurements (*23*). In these experiments, a red-shifted absorbance maximum for the B[*a*]P absorption maxima for the two duplexes containing the cis adducts was observed when compared to their single-stranded counterpart, indicating that the pyrenyl ring was intercalated into the duplex. In contrast, a blue shift was observed for the two duplexes containing the trans adducts, indicating that the adducts are outside the helix and exposed to solvent.

In a more recent study (*24*), the NMR solution structure of a (+)-*trans*-B[*a*]P−dG was reported for a duplex containing a T 5′ to the modified G. In this sequence a certain degree of conformational equilibrium was observed between a positioning of the B[*a*]P in the minor groove and a suggested intercalation into the helix. This equilibrium was not observed in the prior studies for the (+)-trans adduct, suggesting that sequence must play a role in the three-dimensional structure in general and that having a T in the 5′ position may influence this structure.

In the present study, we have determined the effect of the four isomeric B[*a*]P adducts and sequence context on the absolute and relative stabilities and structures of complementary and mismatched DNA molecules. The two sequences studied, oligonucleotide I, d(5′-CCATT-G$^{B[a]P}$-CTACC-3′), and oligonucleotide II, d(5′-CCATC-G$^{B[a]P}$-CTACC-3′), differ from one another by the presence of either a T or C positioned 5′ to the modified guanine (Figure 1B). We find that the order of relative stabilities closely correspond to the frequency of incorporation (F_{inc}) that we and others have observed during in vitro DNA synthesis (*25*, *26*) and thus can be used as a basis to understand the mutagenesis induced by these DNA lesions.

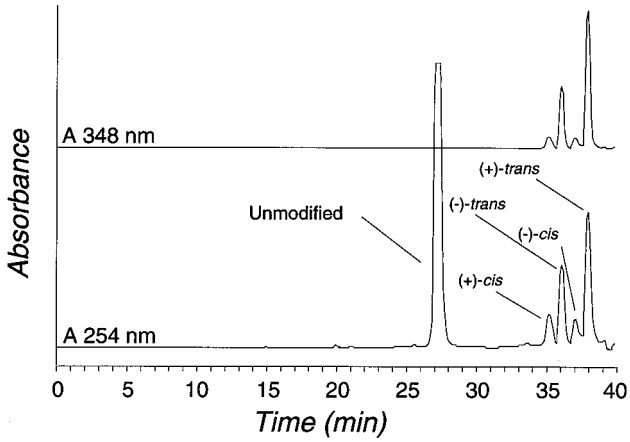

FIGURE 2: HPLC chromatogram of the reaction mixture from the modification reaction of single-stranded 11 mer with racemic benzo[*a*]pyrene. Chromatograms at wavelengths 254 and 348 nm are presented. The unmodified and all four modified oligonucleotides are easily separated and labeled as indicated. The buffer system, the column, and the gradient employed are described in Materials and Methods.

MATERIALS AND METHODS

Synthesis of anti-B[*a*]*P-Modified Oligonucleotides.* The racemic (±)-*anti*-B[*a*]PDE was purchased from the National Cancer Institute Chemical Reference Standard Repository. A 5.5 mM stock solution in 19:1 tetrahydrofuran/triethylamine was prepared and used in all covalent modification reactions. All unmodified oligonucleotides were purchased from Midland Certified Laboratories. A Varian 5000 HPLC with a polychrom 9060 diode array detector equipped with a Hamilton PRP-1 350 × 7-mm column was utilized for the purification of the unmodified oligonucleotides. The method of synthesis of the modified oligonucleotides was similar to the procedure previously described (*18*, *27*). In an Eppendorf tube, 50 to 80 ODs of HPLC-pure and desalted oligonucleotides I and II were separately dissolved in 110 μL of 10 mM potassium phosphate buffer, pH 7. Next, 150 μL of racemic (±)-*anti*-B[*a*]PDE stock solution (5.5 mM) was added to each oligonucleotide solution, and each mixture was briefly vortexed. The reaction was allowed to proceed in the dark at room temperature for 48 h with periodic brief vortexing. The reaction mixtures where then extracted 10 times with 1 mL of water-saturated ether to remove the tetrol reaction products.

Purification of the B[*a*]P-*Modified Oligonucleotides.* The (+)-trans, (−)-trans, (+)-cis, and (−)-cis-*anti*-B[*a*]P−dG isomeric covalent adducts in either oligonucleotide I or II are separable by HPLC on a C18 ODS-Hypersil column as previously described (*18*, *27*). Following ether extraction, the reaction mixtures were injected onto the ODS-Hypersil 250 × 4.6 mm column (purchased from Keystone Scientific Laboratories) at a flow rate of 1 mL/min, employing 20 mM sodium phosphate, pH 7, as buffer A, a 50:50 mixture of 20 mM sodium phosphate/methanol (v/v) as buffer B, with an elution gradient of 0−60 min, 0−90% B (Figure 2). Both reaction mixtures exhibited similar HPLC profiles. The

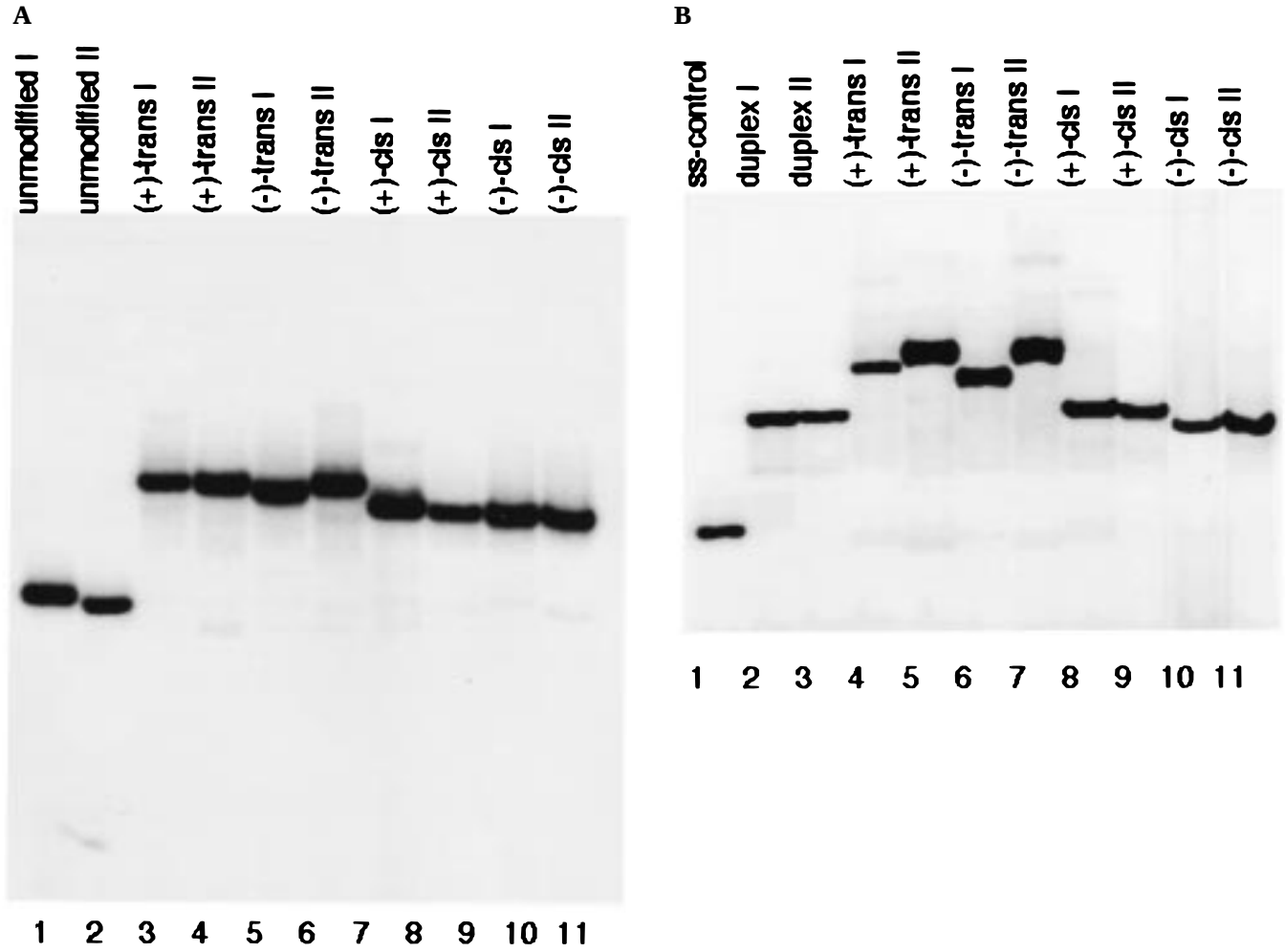

FIGURE 3: Polyacrylamide gels demonstrating purity of DNA samples. (A) Denaturing polyacrylamide gel of purified single-stranded 11-mer oligonucleotides. All HPLC- and gel-purified versions of oligonucleotide I, d(5′-CCATT-G$^{B[a]P}$-CTACC-3), and oligonucleotide II, d(5′-CCATC-G$^{B[a]P}$-CTACC-3), were ^{32}P labeled and loaded on a 20% denaturing polyacrylamide gel to establish their gel-purity. Unmodified I and II represent oligonucleotide I and II, respectively. All modified versions of the two oligonucleotides are represented by the stereochemistry of the adduct they contain. All oligonucleotides were purified to exhibit both HPLC and gel purity before being employed in all further experiments. (B) Native polyacrylamide gel of double-stranded 11-mer duplexes containing Watson−Crick complements. Duplexes were prepared and loaded on a 22% native polyacrylamide gel at 4 °C as described in Materials and Methods. Lane 1 represents an unmodified, single-stranded 11-mer control. Lanes 2 and 3, marked duplex I and II, represent the unmodified versions of the duplexes containing oligonucleotide I and II complemented against their Watson−Crick complement. All modified versions of duplex I and II, lanes 4−11, are represented by the stereochemistry of the adduct they contain.

HPLC-separated, isomerically pure, modified oligonucleotides were subject to a second HPLC purification using a shallower gradient and finally purified employing 20% denaturing gels. The purity of the oligonucleotides was then assessed by denaturing gel electrophoresis and HPLC analysis.

Nomenclature. Duplex I and Duplex II represent oligonucleotide I, d(5′-CCATTGCTACC-3′), and oligonucleotide II, d(5′-CCATCGCTACC-3′), duplexed with their Watson−Crick complements (Figure 1). A duplex with a mismatched A across from the adduct is referred to as M(A); M(T) and M(G) have analogous meanings. BPI and BPII refer to the B[*a*]P N^2-modification of the guanine in oligonucleotide I and II, respectively.

Gel Electrophoresis. All modified and unmodified versions of oligonucleotide I and II were 5′-end-labeled with ^{32}P as described (*28*), electrophoresed on 20% denaturing gel in the presence of 7 M urea, and autoradiographed employing a molecular dynamics phosphor-imager (Figure 3A). For the gel electrophoretic analyses, the double-stranded oligonucleotides where prepared by mixing a 4/1 ratio of Watson−Crick complement/5′-end-labeled versions of oligonucleotide I or II in 1 M NaCl, heated to 85 °C for 3 min, and then slowly cooled to 2 °C. The electrophoretic mobilities of the duplexes were then determined using a 22% native polyacrylamide gel at 4 °C (Figure 3B).

Measurement of Melting Curves. Absorbance vs temperature profiles (melting curves) were measured with an Aviv 14DS UV−vis spectrophotometer with a five cuvette thermoelectric controller as described previously (*29*). The concentrations of single-stranded oligonucleotides were determined by measuring 260 nm UV absorbance at 85 °C and using the calculated extinction coefficients (*30*). Equal molar ratio of oligonucleotide/complement were dissolved in 1.0 M NaCl/10 mM potassium phosphate, pH 7, buffer. Samples were next annealed and degassed by raising the temperature to 85 °C for 3 min and then cooling to −1.6 °C over a period of 25 min just prior to a melting experiment. While at 85 °C, the absorbances were measured at 260 nm for later calculation of the duplex concentration. The

extinction coefficient of duplexes was calculated by averaging the extinction coefficients of the two participating single-stranded oligonucleotides. For each experiment, duplex concentration was varied over an 80 to 100-fold range. Melting curves were determined by heating at a constant rate of 0.8 °C/min with data collection beginning at 0 °C and ending at 85−95 °C.

Determination of Thermodynamic Parameters. Thermodynamic parameters for duplex formation were obtained from melting curve data using the program MELTWIN v2.1 (*31*) as described (*32*). Data were truncated (generally using $T_M \pm 30$ °C) so that the upper and lower temperature baselines reflect the slopes in the transition region. The enthalpy and entropy of the random coil (single-stranded) to duplex equilibrium were obtained by two methods: (1) $\Delta H°$ and $\Delta S°$ from fits of individual curves were averaged, and (2) plots of reciprocal melting temperature (T_M^{-1}) versus the natural logarithm of the total strand concentration ($\ln C_T$) were fit to eq 1 (*33*):

$$T_M^{-1} = R/\Delta H° \ln C_T/4 + \Delta S°/\Delta H° \quad (1)$$

The T_M is defined as the temperature at which half of the strands are in the double helical state and half are in the random coil state. The T_M for the individual melting curves was calculated from the fitted parameters using eq 2:

$$T_M = \Delta H°/(\Delta S° + R \ln C_T/4) \quad (2)$$

where R is the gas constant [1.987 cal/(Kmol)], and the T_M is given in K. Both methods are essentially a van't Hoff analysis of the data, assuming the transition equilibrium involves only two states (i.e., duplex and random coil). Transitions are considered two-state when the $\Delta H°$ obtained from both methods agree within a difference of 15% (*34*).

CD and UV Spectra of the Duplexes. The CD spectra were determined using a Jasco J-600 spectropolarimeter, and data were collected by an IBM 386 equipped with the Jasco J-600 software. The UV spectra were determined employing a Hewlett-Packard 8452A Diode Array Spectrophotometer, and the data were collected by an ATT 386 equipped with the appropriate Hewlett-Packard software. The CD and UV samples were obtained by combining the different concentration mixtures used in the melting curves studies, employing the same buffer system (1.0 M NaCl, and 10 mM potassium phosphate, pH 7). Concentrations of the CD samples were obtained as described above. All CD and UV experiments were conducted at 20 °C. The CD data were treated by converting θ_{obs} to $\Delta\epsilon$ (M^{-1} cm^{-1}) by using eq 3 (*35*):

$$\Delta\epsilon = \theta_{obs}/(Cl \times 33000) \quad (3)$$

where θ_{obs} (in mdeg/AU) is the observed molar ellipticity, C is the concentration in molar quantities, and l is the length of the cell used in the experiment.

RESULTS

The goal of this study was to correlate the measured thermodynamic parameters of two DNA sequences containing stereochemically pure B[*a*]P adducts and each of the four bases paired at this position with the observed mutational specificities induced by these adducts. One possible method that has been used in the past is to determine the stabilities and thermodynamic parameters for the B[*a*]P-modified mismatched materials and compare these values determined for the unmodified, fully complementary samples. However, this analysis is flawed since it involves the simultaneous changing of two variables (the mispair and the adduct). We have used an alternative analysis in which the relative destabilization induced by the presence of the adduct is determined from the differences between values measured for each Watson−Crick or mismatched sequence containing or lacking the adduct. These calculated differences, or relative thermodynamic values, would account for the change in the energetics of each of the duplexes induced by only the presence of the B[*a*]P structures and thus are due only to the presence of the adduct in either the Watson−Crick or mismatched context. The smaller the magnitude of the difference, the less destabilizing is the adduct in this sequence context. It follows that these differences provide the relative stability and thermodynamic parameters that result from a mispair containing a B[*a*]P adduct and when compared as a series give the relative stability for the incorporation of each of the nucleotides opposite a B[*a*]P−dG. According to this hypothesis, the least destabilizing difference at the site of modification should lead to the most preferred base incorporated at that specific site of modification.

Determination and Analysis of the Thermodynamic Parameters. All duplexes in this study displayed monophasic melting transitions and showed concentration-dependent T_Ms, and plots of T_M^{-1} versus $\ln C_T$ were linear (correlation coefficient >0.99) over the entire 80 to 100-fold range in concentration. Thermodynamic parameters derived from the average of fits of individual melting curves and from T_M^{-1} versus $\ln C_T$ plots with respect to $\Delta G°_{37}$ and T_M are listed in Table 1. The $\Delta H°$ and $\Delta S°$ parameters of duplex formation are provided in Table S-1 as Supporting Information. For all of the duplexes, $\Delta H°$ from the two methods agree within 15%, and therefore all are considered as two-state transitions (*34*). For two-state transitions, parameters derived from the average of the curve fit parameters and from T_M^{-1} versus $\ln C_T$ plots are equally reliable (*36*), and thus the average of the results from these two methods has been used to calculate the effect of modification on the thermodynamic parameters of duplex formation that are used in subsequent determinations. The unmodified duplexes are well-predicted by the Watson−Crick, G•T, and G•A parameters (*36*, *37*), indicating that the parent duplexes in this study do not exhibit unusual behavior.

Inspection of the data presented in Table 1 indicates that all modified and unmodified duplexes containing oligonucleotide II exhibit higher melting temperatures and more stable $\Delta G°_{37}$ of formation and T_M when compared to their counterpart duplex containing oligonucleotide I. This is to be expected since oligonucleotide II has one C:G base pair substituted for a T:A pair. Also as expected, the presence of both a B[*a*]P adduct (*38*) or a mismatch leads to a duplex that is less stable than its unmodified fully complementary counterpart.

Effect of B[a]P Adducts on the Thermodynamic Parameter. Table 2 gives the differences in thermodynamics between unmodified and modified duplexes that contain either the Watson−Crick complementary base or a mismatched base on the strand opposite the B[*a*]P adduct (taken from Table

Table 1: Thermodynamic Parameters of Duplex Formation[a]

	$1/T_M$ vs C_T parameters		curve fit parameters
duplex	$-\Delta G°_{37}$ (kcal/mol)	T_M[b] (°C)	$-\Delta G°_{37}$ (kcal/mol)
Unmodified Duplexes			
I	12.3 ± 0.3	59.7	12.1 ± 0.2
I M(A)	8.2 ± 0.5	44.7	8.2 ± 0.1
I M(T)	9.1 ± 0.2	47.7	9.1 ± 0.1
I M(G)	9.3 ± 0.3	48.9	9.3 ± 0.1
II	13.5 ± 0.3	63.9	13.2 ± 0.1
II M(A)	9.5 ± 0.3	49.8	9.5 ± 0.1
II M(T)	10.5 ± 0.3	52.7	10.3 ± 0.1
II M(G)	10.2 ± 0.3	51.9	10.1 ± 0.1
Duplexes Containing (+)-Trans Adducts			
BPI	9.3 ± 1.0	51.2	9.3 ± 0.2
BPI M(A)	6.8 ± 0.4	38.5	6.9 ± 0.1
BPI M(T)	7.0 ± 0.4	39.6	7.0 ± 0.1
BPI M(G)	7.7 ± 0.6	42.5	7.6 ± 0.1
BPII	10.3 ± 0.2	55.7	10.3 ± 0.1
BPII M(A)	8.7 ± 0.1	47.4	8.7 ± 0.1
BPII M(T)	8.3 ± 0.2	46.4	8.4 ± 0.1
BPII M(G)	9.0 ± 0.3	49.3	9.0 ± 0.1
Duplexes Containing (−)-Trans Adducts			
BPI	9.6 ± 0.5	53.3	9.6 ± 0.0
BPI M(A)	7.8 ± 0.4	44.1	7.8 ± 0.0
BPI M(T)	8.2 ± 0.3	45.2	8.2 ± 0.0
BPI M(G)	7.8 ± 0.3	44.2	7.9 ± 0.0
BPII	11.0 ± 0.4	59.0	10.9 ± 0.2
BPII M(A)	8.2 ± 0.3	46.3	8.3 ± 0.1
BPII M(T)	9.0 ± 0.4	49.3	9.1 ± 0.1
BPII M(G)	8.5 ± 0.2	47.4	8.5 ± 0.0
Duplexes Containing (+)-Cis Adducts			
BPI	9.4 ± 0.6	51.2	9.5 ± 0.1
BPI M(A)	8.6 ± 0.5	48.2	8.7 ± 0.1
BPII	9.8 ± 0.5	54.9	9.4 ± 0.1
BPII M(A)	8.8 ± 0.6	49.1	8.7 ± 0.1
Duplexes Containing (−)-Cis Adducts			
BPI	7.9 ± 0.6	44.5	7.9 ± 0.0
BPI M(A)	7.6 ± 0.3	42.7	7.6 ± 0.1
BPII	9.3 ± 0.7	50.7	9.3 ± 0.1
BPII M(A)	9.3 ± 0.3	50.5	9.5 ± 0.1

[a] I and II represent oligonucleotide I, d(5′-CCATTGCTACC-3′), and oligonucleotide II, d(5′-CCATCGCTACC-3′), duplexed with their Watson−Crick complements. M(A), M(T), and M(G), respectively, represent duplexes containing a mismatch of either A, T, or G against the guanine in both sequences. BP represents N^2-modification of the guanine in both sequences with the indicated adduct. Solutions are 1.0 M NaCl, 10 mM potassium phosphate, pH 7. Errors are standard deviations from the regression analysis of the melting data. [b] Calculated for 1×10^{-4} M oligomer concentrations. Complete list of $\Delta H°$ and $\Delta S°$ information is provided in Table S-1 in the Supporting Information.

Table 2: Effect of Modification on Thermodynamic Parameters of Duplex Formation[a]

Δ(duplex)	$-\Delta\Delta G°_{37}$[b] (kcal/mol)	$-\Delta\Delta H°$[c] (kcal/mol)	$-\Delta\Delta S°$[c] (eu)	ΔT_M (°C)
Effect in Duplexes Containing (+)-Trans Adducts				
[I]−[BPI]	2.9	19.6	54.2	8.5
[I M(A)]−[BPI M(A)]	1.3	12.7	36.6	6.2
[I M(T)]−[BPI M(T)]	2.1	21.9	64.1	8.1
[I M(G)]−[BPI M(G)]	1.6	14.3	40.5	6.4
[II]−[BPII]	3.1	18.9	50.9	8.2
[II M(A)]−[BPII M(A)]	0.8	7.9	22.9	2.4
[II M(T)]−[BPII M(T)]	2.0	16.6	47.0	6.3
[II M(G)]−[BPII M(G)]	1.2	13.6	40.2	2.6
Effect in Duplexes Containing (−)-Trans Adducts				
[I]−[BPI]	2.6	21.1	59.7	6.4
[I M(A)]−[BPI M(A)]	0.4	10.8	33.6	0.6
[I M(T)]−[BPI M(T)]	0.9	10.9	32.6	2.5
[I M(G)]−[BPI M(G)]	1.4	17.1	50.4	4.7
[II]−[BPII]	2.4	18.5	52.0	4.9
[II M(A)]−[BPII M(A)]	1.2	16.3	48.4	3.5
[II M(T)]−[BPII M(T)]	1.3	13.5	39.0	3.4
[II M(G)]−[BPII M(G)]	1.7	19.1	56.2	4.5
Effect in Duplexes Containing (+)-Cis Adducts				
[I]−[BPI]	2.7	15.6	41.8	8.5
[I M(A)]−[BPI M(A)]	−0.5	6.4	21.9	−3.5
[II]−[BPII]	3.8	25.4	69.8	9.0
[II M(A)]−[BPII M(A)]	0.7	16.3	50.1	0.7
Effect in Duplexes Containing (−)-Cis Adducts				
[I]−[BPI]	4.3	24	63.7	15.2
[I M(A)]−[BPI M(A)]	0.6	6.6	19.3	2.0
[II]−[BPII]	4.1	18.9	47.9	13.2
[II M(A)]−[BPII M(A)]	0.1	4.7	14.7	−0.7

[a] I and II represent oligonucleotide I, d(5′-CCATTGCTACC-3′), and oligonucleotide II, d(5′-CCATCGCTACC-3′), duplexed with their Watson−Crick complements. M(A), M(T), and M(G), respectively, represent duplexes containing a mismatch of either A, T, or G against the guanine in both sequences. BP represents N^2-modification of the guanine in both sequences with the indicated adduct. [b] For all duplexes, averages of results obtained from the two methods reported in Table 1 have been used. [c] The $\Delta H°$ and the $\Delta S°$ parameters of duplex formation are provided in Table S-1.

1). These differences are a measure of the relative destabilizing influence of the adduct and when compared as a series give the relative stability for the incorporation of each of the nucleotides opposite a B[*a*]P−dG.

This analysis indicates that an A across from both the (+)- and (−)-trans adduct in both oligonucleotide I and II is the least destabilizing and, surprisingly, that C, the Watson−Crick complement, is the most destabilizing. The magnitude of the $\Delta\Delta G°_{37}$ varies from 0.4 kcal/mol to 1.3 kcal/mol for pairing an A, while all values for C are above 2.4 kcal/mol. The $\Delta\Delta G°_{37}$ values for T and G are all fairly close but vary sufficiently so that the order of stability for the (+)-trans is A > G > T > C, while the (−)-trans is A > T > G > C in each of the two sequences.

Because of the low yields for the modification reactions, the effect of (+)- and (−)-cis modification on the thermodynamic parameters of duplex formation for both oligonucleotides was determined only for the Watson−Crick complementary base C and for the mispair A. In all cases, the $\Delta\Delta G°_{37}$ determined indicated mispairing an A caused significantly less destabilization than was caused by a C. In fact when the modified guanine containing the (+)-cis adduct in oligonucleotide I is paired with an A, the duplex exhibits a higher stability compared with its unmodified version (Table 2).

Effect of Adduct Stereochemistry on Thermodynamic Parameters. Further analysis of Table 1 shows that that the stability as determined by the $\Delta G°_{37}$ and T_M for both oligonucleotide I and II when paired with the Watson−Crick complement (C) follows the patterns of (least stable) (−)-cis > (+)-cis > (+)-trans > (−) -trans (most stable), respectively. Interestingly, we have recently found that the relative cleavage by the UvrABC endonuclease for each of these adducts in each of these sequences follows a similar order (*26*). Zou and colleagues have also conducted UvrABC incision experiments on a construct that contains oligonucleotide II (*39*). They observed a higher level of incision efficiency for the (+) isomers compared to the (−) isomers. A similar trend of higher excision of cis isomers compared to the trans isomers was observed when the human nucleotide excision repair enzymes where employed (*40*).

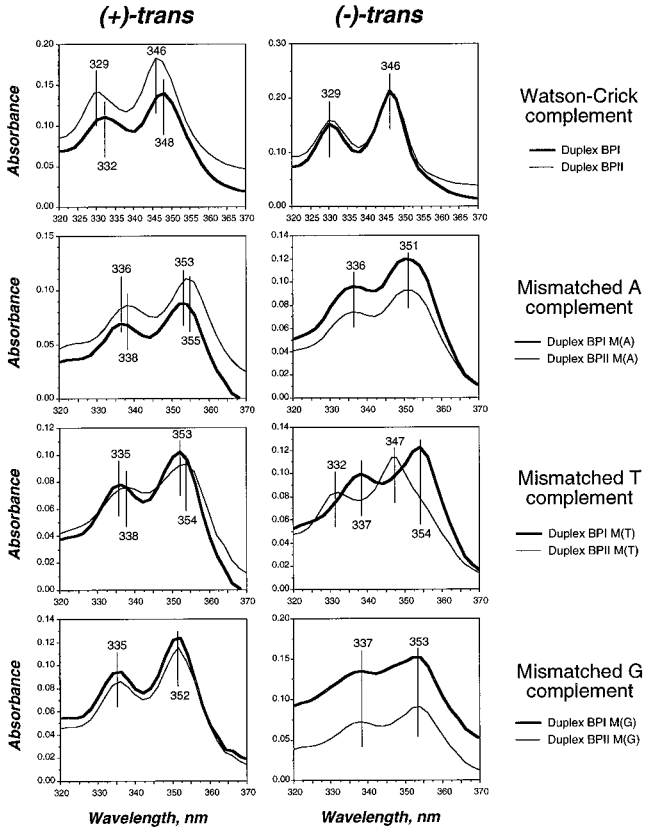

FIGURE 4: Superimposed UV spectra of the (+)-trans and (−)-trans modified duplexes. Watson−Crick, M(A), M(T), and M(G) complement markings represent the C-paired or mismatched complements positioning an A, T, or G across the guanine in both sequences, respectively. The UV spectra of all duplexes where obtained at 20 °C and the data were treated as described in Materials and Methods.

Furthermore, when a mismatch of an A is positioned across the modified guanine the cis isomers are now excised at a relatively lower level than the (+)-trans adduct. Finally, the order of adduct-dependent duplex destabilization for each mispair studied shows differences in each of the two sequences suggesting that the adducts are adopting different structures in each of these situations.

The UV and CD Spectra of the Duplexes. To characterize these structural differences, we have measured the UV and CD spectra of both oligonucleotides containing each adduct paired with each of the four bases. Although the unmodified counterparts of both duplexes BPI and BPII exhibit very similar B-DNA-like CD spectra, the CD spectra of both duplexes modified with the (+)-*trans* adduct clearly indicate that the two structures are different from one another (Figure S-1). This difference is also clearly shown in the UV spectra of these duplexes: the (+)-trans containing duplex BPI (5′-T-G*-C) exhibits two B[*a*]P-absorption maxima at 332 and 348 nm, respectively, while duplex BPII exhibits them at 329 and 346 nm, respectively (Figure 4). A shift to the red for the two B[*a*]P-absorption maxima at 329 and 346 nm has been shown to correlate with intercalation (*23*), and these structures have been confirmed by recent NMR data (*18*, *24*). The UV and CD spectra of the duplexes containing each of the three mispairs indicate that there are both sequence and mismatch-dependent changes in the structures. In general, each appears to be in a B or B-like structure (Figure S-1) with the adduct intercalated into the DNA helix

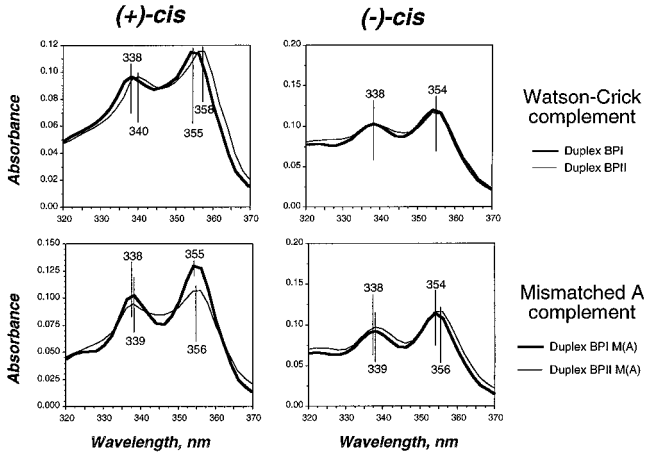

FIGURE 5: Superimposed UV spectra of both (+)-cis and (−)-cis modified duplexes. Watson−Crick and M(A) complement markings represent the C-paired or a mismatched complement positioning an A across the guanine in both sequences, respectively. The UV spectra of all duplexes where obtained at 20 °C, and the data were treated as described in Materials and Methods.

as indicated by the red shifting of the B[*a*]P-absorption maxima (Figure 4).

The UV spectra of the (−)-trans modified duplexes (BPI and BPII) containing the Watson−Crick complement also exhibit their two B[*a*]P-absorption maxima at 329 and 346 nm, indicating that the B[*a*]P moiety is outside the helix in both structures, (Figure 4) as was confirmed for BPII by NMR (*19*). The CD spectra of the (−)-trans modified duplexes containing the Watson−Crick complement are almost superimposable, and those containing the three mismatches are also very similar (Figure S-2). As was observed for the (+)-trans adducts, the mispairs across from the (−)-trans adduct result in a red shift for each of the UV B[*a*]P-absorption maxima, suggesting that the adducts are intercalated.

In the case of the (+)-cis and (−)-cis adducts, the UV spectra show red-shifted B[*a*]P-absorption maxima indicating that the adduct is intercalated in each case (Figure 5). For (+)-cis and (−)-cis BPII duplexes containing the Watson−Crick complements, the NMR structures confirm this intercalation (*20*, *21*).

DISCUSSION

In this study we have determined the thermodynamic parameters and the UV and CD spectra for two sequences containing each of the four major isomeric B[*a*]P adducts paired across from either C, A, G, or T. Ya and colleagues (*38*) have previously attempted to study the thermodynamic effect of B[*a*]P modification and mismatches across the guanine in one of these sequences (oligonucleotide II). However, in that study that used 0.1M NaCl buffer solutions, thermodynamic data for the unmodified mismatched duplexes where not obtainable and thus they reported non-two-state behavior. Therefore it was not possible to make a comparison of the effect of modification with respect to their unmodified counterparts. In our study, we have employed 1.0 M NaCl buffer solution that enabled us to obtain more stable duplexes for each sample. Based on the agreement of $\Delta H°$ parameters obtained from the average of the fits and the $1/T_M$ vs ln C_T , it appears that all of the duplexes studied

here melt with two-state transitions. Thus reliable trends in thermodynamic stabilities were obtained. Marky and collegues have also studied in detail the thermodynamics of duplex BPII containing only the trans adducts complemented only with the Watson−Crick base pair (*41*). Their results with respect to T_M and $\Delta G°$ are consistent with our reported data, where duplex BPII containing the (−)-trans adduct is more stable than its counterpart containing the (+)-trans adduct (Table 1).

Effect of Sequence and Mismatches on the Structure of Modified Duplexes. The red shift of the two B[*a*]P-absorption maxima in the UV spectrum of duplex BPI containing the (+)-trans adduct demonstrates a certain degree of adduct intercalation. In contrast, the UV spectrum of the duplex BPII does not exhibit any signs of intercalated adducts (Figure 4). This contrast is consistent with the currently published NMR solution structures of the (+)-trans adduct. Using a sequence that positioned the (+)-trans adduct in the same context as for BPI (*24*), it is suggested that the adduct was found both intercalated and on the outside of the helix, with the outside binding structure favored. Cosman et al. (*18*) showed that for BPII the (+)-trans adduct was outside the helix in the minor groove pointing toward the 5′-direction of the modified strand. Interestingly, when a mismatch is positioned across from the adduct in either sequence we find red-shifted B[*a*]P absorption maxima in all cases, suggesting substantial intercalation into the helix (Figure 4).

In case of the (−)-trans adduct, only the NMR structure of duplex BPII has been determined (*19*). This structure places the adduct in the minor groove pointing toward the 3′ end of the modified strand. Our data suggest that the structure of duplex BPI is very similar to that of duplex BPII since both exhibit their B[*a*]P absorption maxima at 329 and 346 nm, respectively (Figures 4 and S-2). As was found with the (+)-trans adduct, each of the mismatches exhibited a red shift for the B[*a*]P absorption maxima, suggesting enhanced levels of intercalation (Figure 4).

In two NMR studies (*20*, *21*), the (+) or (−)-cis adducts have been shown to be intercalated within duplex BPII, displacing the modified guanine into the minor groove. Our UV data suggest that the (+)-cis adducts in duplex BPI are also intercalated, but that this structure is not as favored as in duplex BPII (Figure 5). Positioning an A across from the modified guanine seems to have little effect on the trends of B[*a*]P intercalation in these two sequences (Figure 5).

Currently there are no published NMR studies positioning any mismatch across a BP-modified guanine, and only limited absorption studies have been conducted to try to elucidate such structures. Ya and co-workers, by performing absorption studies, have previously indicated that any mismatch across a (+)-trans or a (−)-trans modified guanine in oligonucleotide II should lead to intercalation of the adduct (*38*). Our results indicate that the structure of a B[*a*]P-modified duplex is both isomer and sequence dependent. Our study also suggests that the structure of B[*a*]P-modified duplexes that contain a mismatch across the modified guanine are dependent not only on the sequence but also on the type of mismatch.

Correlation of the Changes in the Thermodynamic Parameters with in Vitro DNA Polymerase Incorporation (F_{inc}) and in Vivo Mutational Specificities. The data presented in Table 2 show the thermodynamic differences between the adduct-containing duplexes and their unmodified counterparts. Since the modification is localized to only one base pair within all the duplexes, then the thermodynamic differences reported in Table 2 represent the effect of the modification on the stability of the whole duplex. Our hypothesis is that these differences can be correlated with the stability of incorporation of a given base across the modified guanine by a polymerase during replication.

Shibutani and co-workers (*25*) have conducted translesional synthesis studies on a DNA template containing a single stereoisomer of B[*a*]P and showed that when the adduct is situated in a 5′-C-G*-C sequence context the frequency of incorporation of dNTPs across the modified guanine follows the pattern of A > G > T > C for all four isomers. We have found that a mismatch of an A across from the modified guanine leads to the least amount of destabilization for all four adduct isomers in both sequence contexts (Table 2). More specifically, the thermodynamic trend we determined for the (+)-trans isomer in oligonucleotide II, which also positions the adduct in a 5′-C-G*-C sequence context, indicates that the likelihood of incorporation of dNTPs across the modified guanine should follow this same pattern (A > G > T > C). A similar correlation with Shibutani's data was observed for both cis isomers, suggesting a much higher likelihood of incorporation of A than the Watson−Crick complement of C. In the case of the (−)-trans adduct, our thermodynamic data suggest a pattern of A > T > G > C, which is only slightly different from the reported incorporation data of A > G > T > C by Shibutani. Interestingly, in an ongoing study we have observed a base incorporation pattern of A > G > T > C across the modified guanine of both sequences containing the (+)-trans adduct, while an A > T > G > C pattern is observed when the guanine is modified with the (−)-trans adduct (*26*). These patterns are exactly the same as the ones exhibited by our above-mentioned thermodynamic trends.

The vast majority of in vivo mutagenesis studies also show that the predominant base substitution mutation is G to T. Calculation of the mutation frequencies from these studies indicates that most progeny contain no base change at the adduct site, suggesting that a C was incorporated at the adduct site (*8*−*10*, *42*). However, in these in vivo experiments there is the additional complication of the editing processes during polymerization and mismatch repair following replication that may alter the initially incorporation ratios to ones where the normal Watson−Crick base pair predominates. For example, if these systems preferentially removed A, G, and T but allowed C to remain, possibly because in this situation a proper base pair could on occasion occur, then a template primer terminated with a C at the adduct site would be the most likely product to be extended by the polymerase or not recognized by the mismatch repair system.

Mutagenesis specificity is also affected by the sequence context in which the modified guanine is positioned. In particular, Loechler and colleagues have shown that in the sequence context of 5′-T-G*-3′ containing adducts generated by the addition of the (+)-*anti*-diol epoxide, G to T mutations are almost exclusively observed (*8*, *9*, *43*); in a 5′-C-G*-3′ sequence context greater amounts of G to C and G to A are also observed, although G to T still predominates (*9*, *10*,

43). During the process of replication, the 5′ base pair is the base pair formed after the incorporation at the modified guanine. A higher absolute stability of the final duplex formed may favor the incorporation at this 5′ position due to the higher stabilizing contribution of this base pair (Table 1). Others have observed enhanced extension for an O^6-methylguanine-modified template when the flanking base pairs were G:C vs A:T (*44*).

Finally, it is interesting to speculate as to what is the mechanism that allows purines to be incorporated preferentially at the adduct sites during in vitro DNA synthesis. In a single-stranded−double-stranded junction (primer-template) of a replication fork it appears that the (+)-trans adduct stacks over the intact adjacent 3′ base pair (with respect to the template/modified strand) and displaces the modified guanine outside the helix and within the forming groove (*45*). A similar structure placing the guanine outside the helix is observed for the cis adducts in a duplex (*20*, *21*). Therefore, the extent of adduct stacking will be strongly influenced by the adjacent newly formed 3′ base pair, the stereochemistry of the adduct, and the orientation or positioning of the next base on the template strand. This statement is supported by our data that indicate that a mismatch at the adduct site leads to increased intercalation of the adduct, suggesting that the adduct prefers a stacked position inside the duplex between the 3′ flanking base pair (with respect to the template strand) and the unpaired misincorporated base. Based on these facts, one possible mechanism to account for the observed base selection is that the purine rings are better able to stack with the intercalated adduct, resulting in a more stable structure during incorporation at this site.

In a recent study (*46*) it has been shown that the (+)-trans adduct positioned within the sequence context of 5′-C-G*-T can induce a predominant G to A, and smaller amounts of G to T transversions. Since this study was conducted in vivo, it is impossible to deduce which of the cellular processes is responsible for this phenomenon. Currently we are investigating the effect of modification of all four isomers with respect to the 5′-C-G*-T sequence context which may help determine what is contributing to these interesting mutation spectra.

In conclusion, the observed correlation between the thermodynamic data and the in vitro misincorporation data suggests that one contributing factor to misincorporation at an adduct site may be the thermodynamic stability of the incorporated base. The spectra data also suggest that the extent of intercalation of the B[*a*]P adduct within a modified duplex is dependent on the stereochemistry of the adduct, the sequence within which it lies, and base incorporated across from the adduct. How these factors relate to mutagenesis in various sequence contexts is currently under investigation.

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SUPPORTING INFORMATION AVAILABLE

The $\Delta H°$ and $\Delta S°$ parameters of duplex formation and the CD spectra of the matched and mismatched B[a]P-modified duplexes are available as Supporting Information (6 pages). Ordering information is given on any current masthead page.

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