Tullius, T. D. (1987) *Trends Biochem. Sci. 12*, 297-300.
Tullius, T. D., Dombroski, B. A., Churchill, M. E. A., & Kam, L. (1987) *Methods Enzymol. 155*, 537-558.
Van Dyke, M. W., Hertzberg, R. P., & Dervan, P. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5470-5474.
Ward, B., Rehfuss, R., Goodisman, J., & Dabrowiak, J. C. (1988) *Nucleic Acids Res. 16*, 1359-1369.
Wolffe, A. P., & Brown, D. D. (1987) *Cell 51*, 733-740.
Youngquist, R. S., & Dervan, P. B. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2565-2569.
Zimmer, C. (1975) *Prog. Nucleic Acid Res. Mol. Biol. 1*, 399-411.
Zimmer, C., & Wähnert, U. (1986) *Prog. Biophys. Mol. Biol. 47*, 31-112.
Zimmer, C., Kakiuchi, N., & Guschlbauer, W. (1984) *Nucleic Acids Res. 10*, 1721-1732.

Sequence Preferences of Covalent DNA Binding by *anti*-(+)- and *anti*-(−)-Benzo[*a*]pyrene Diol Epoxides†

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ABSTRACT: The sequence preferences of formation of piperidine-labile adducts of guanine by individual (+)- and (−)-isomers of *trans*-7,8-dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [anti-(+)- and anti-(−)-BPDE] were examined by techniques analogous to chemical DNA sequencing. Data were obtained on over 1200 bases with *anti*-(−)-BPDE and 1000 bases with *anti*-(+)-BPDE. Guanines on average yielded more labile adducts than other bases, and the reactivities of guanines with both *anti*-(+)- and *anti*-(−)-BPDE isomers were found to be distinctly nonrandom with respect to DNA sequence. The most and least reactive guanines, defined in terms of the upper and lower 10 percentiles of reactivity, differed on average by a factor of 17. This range of guanine reactivities was correlated with distinct sequence preferences, which differed in part for the two isomers. The strongest determinant for preferred reaction of *anti*-(−)-BPDE to form a labile adduct at a guanine was the presence of a 3′-flanking guanine, but a thymine 5′-flanking a guanine also generally enhanced reactivity. The triplets containing central guanines most preferred by *anti*-(−)-BPDE were AGG, CGG, and TG(G>T>C,A). *anti*-(+)-BPDE also formed labile adducts preferentially at AGG and CGG triplets, but not at TGN triplets. Significant effects of next-nearest-neighbor bases on guanine reactivities were also noted.

Benzo[*a*]pyrene (B[a]P)[1] is a prototypical carcinogen of the polycyclic aromatic hydrocarbon class and was one of the first verified examples of a chemical carcinogen [reviewed by Phillips (1983) and Gräslund and Jernström (1989)]. B[a]P is not direct acting but is metabolically activated in vivo to the ultimate carcinogenic forms, the benzo[*a*]pyrene diol epoxides (BPDE's). Of the many possible stereoisomers of BPDE, the (7*R*,8*S*)-dihydroxy-(9*S*,10*R*)-epoxy-7,8,9,10-tetrahydro-B[a]P isomer [anti-(+)-BPDE] is both the most abundant product formed in vivo in most tissues [Yang & Gelboin, 1976; Thakker et al., 1977; Yang et al. 1977; Deutsch et al., 1978; Harvey, 1981; Conney, 1982; isomer named according to Gräslund and Jernström (1989)] and the most potent carcinogen in mammalian systems (Thakker et al., 1976; Buening et al., 1978; Slaga et al., 1979; Harvey, 1981; Conney, 1982).

Both the *anti*-(+)- and *anti*-(−)-BPDE isomers bind covalently to DNA via predominantly trans addition of base nucleophiles at the 10-position of the highly reactive BPDE epoxide ring (Weinstein et al., 1976; Jeffrey et al., 1976; Osborne et al., 1976, 1978). Addition occurs mainly at the guanine N^2-amino group and is stereoselective: *anti*-(+)-BPDE reacts much more readily at this position than the anti-(−)-isomer (Weinstein et al., 1976; Jeffrey et al., 1976, 1977; King et al., 1976; Meehan & Straub, 1977; Osborne et al., 1981; Brookes & Osborne, 1982). BPDE adducts at guanine N^2 are stable to alkali. Additional, *alkali-labile* adducts are formed on other base sites, however, particularly on the guanine N7 ring nitrogen (Osborne et al., 1978; 1981; King et al., 1979; Sage & Haseltine, 1984). Sage and Haseltine (1984) recently showed that alkali-labile adducts are formed in larger yields than previously suggested, accounting for up to 40% of the total adducts formed by racemic (±)-*anti*-BPDE. About 60% of the alkali-labile products occurred on G's, and formation of alkali-labile adducts was found to be nonrandom with respect to base sequence.

Linear dichroism and fluorescence studies have been able to differentiate between two distinct conformations of BPDE adducts termed types I and II [reviewed by Gräslund and Jernström (1989)]. The pyrene moieties of type I adducts are more perpendicular than parallel to the DNA helix axis and are protected from solvent. In type II adducts the pyrenes are more exposed and more parallel than perpendicular to the helix axis. *anti*-(+)-BPDE–DNA adducts consist almost entirely of the type II conformers, but *anti*-(−)-BPDE–DNA adducts are a nearly equal mixture of type I and type II conformers.

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[1] Abbreviations: *anti*-(+)-BPDE, 7(*R*),8(*S*)-dihydroxy-9(*S*),10(*R*)-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; *anti*-(−)-BPDE, the enantiomer of *anti*-(+)-BPDE; B[a]P, benzo[*a*]pyrene; BPDE, *trans*-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; DMSO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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Molecular mechanics calculations for adducts at either the N7 (Pearl & Neidle, 1986) or exocyclic N^2 position of guanine (Aggarwal et al., 1983; Taylor et al., 1983; Hingerty & Broyde, 1985; Anderson et al., 1987; Rao et al., 1989) have suggested that the conformations of the covalent *anti*-(+)-BPDE–DNA and *anti*-(−)-BPDE–DNA adducts should be different at a given position.

Since the reactions of BPDE isomers are stereoselective, and the conformations of the final covalent complexes appear distinct, the sequence preferences for covalent binding of the two isomers to DNA may be significantly different. Although limited data have been reported on the sequence preferences of adduct formation by the *anti*-(±)-BPDE racemic mixture (Boles & Hogan, 1984; Sage & Haseltine, 1984; Boles & Hogan, 1986; Lobanenkov et al., 1986; Geacintov et al., 1988), the preferences of the individual isomers have not been compared in detail. We have used techniques analogous to the chemical method of DNA sequencing to examine the influence of DNA sequence on the stereoselectivity of covalent DNA binding by specific BPDE isomers. Our initial studies have focused on the sequence preferences of the *anti*-(+)- and *anti*-(−)-BPDE isomers for formation of *alkali-labile* adducts of guanines.

Data accumulated on over 1200 DNA base pairs (including >300 G's) showed that the reactions of both isomers with guainines are distinctly nonrandom. Both isomers exhibited a strong preference for reaction at the 5′-G of GG doublets, but the flanking sequences strongly influenced the degree of preference. In particular, the triplet sequences most preferred by *anti*-(+)-BPDE were CGG > AGG, while the triplets most preferred by *anti*-(−)-BPDE were AGG, TGG, CGG, and TGX. A significant difference between isomer reactivities was noted in that a 5′-flanking T generally increased the reactivity of a guanine toward *anti*-(−)-BPDE, but not *anti*-(+)-BPDE. These preferences contrast with recent measurements showing that *noncovalent* association of racemic (±)-*anti*-BPDE is more favored for alternating pyrimidine–purine polymers [poly[d(AT)]·poly[d(AT)], poly[d(GC)]·poly[d(GC)] than for nonalternating polymers [poly[d(A)]·poly[d(T)], poly[d(G)]·poly[d(C)]] (Geacintov et al., 1988).

Materials and Methods

Visualization of Piperidine-Labile Sites. DNA restriction fragments were obtained from plasmid pUC9 containing the *Strongylocentrotus purpuratus* early H2a and H3 genes (of known sequence; Sures et al., 1978) and were labeled on a single 3′-end by using [α-^{32}P]dXTP and reverse transcriptase by standard procedures (Veal & Rill, 1988). The pure *anti*-(+)- and *anti*-(−)-BPDE isomers were obtained from the NCI Chemical Carcinogen Repository as solutions in tetrahydrofuran–triethylamine (19:1) and were dried, then dissolved at the original concentration in dry DMSO, and stored at −20 °C. Labeled samples of DNA were mixed with >10-fold excess carrier DNA in 25 mM MOPS buffer (pH 7.5, adjusted with NaOH) containing 50 mM NaCl and 1 mM EDTA and then equilibrated with varying amounts (1 BPDE per 2–50 base pairs) of *anti*-(+)- or *anti*-(−)-BPDE for 30 min at 37 °C. Reactions were carried out in dark brown Eppendorf tubes, and other procedures were conducted in the dark to prevent photolysis of the diol epoxides. Samples were extracted with 1-butanol once to remove excess BPDE and precipitate DNA and then treated with 0.25 M piperidine at 90 °C for 15 min to cause cleavage at adduct sites. Electrophoresis was performed on 8% polyacrylamide DNA sequencing gels (Veal & Rill, 1988). Samples subjected to standard DNA sequencing reactions were electrophoresed in parallel with those treated with BPDE to establish the precise sequence positions of cleavages caused by piperidine treatment of BPDE adducts.

Quantitative Representation of Cleavage Patterns. Autoradiograms were scanned with a Bio-Rad Model 620 videodensitometer. Band intensities were quantitated by integration of densitometer tracings in regions where peaks were sufficiently resolved to permit accurate integration. Data were quantitated under conditions that corresponded to or closely approximated "single-hit" kinetics; that is, only a very small fraction of the total molecules were cleaved, and on average few or no molecules were cleaved more than once. Such conditions were considered met for a sample if the parent bands in control and sample lanes were of equal intensity, within 10%, and there was no obvious general increase in the band intensities from the top to the bottom of the gel. The latter condition was checked by determining the slope of a linear least-squares fit through all the data points. Under "single-hit" conditions the intensities of bands reflect the relative rates of reaction at specific sites.

Merging and Normalizing Data. Since several densitometer scans were required to represent each sequence, and data were obtained from many sequences, methods for normalizing and subsequently merging data sets are required for a quantitative analysis of sequence preferences. Pascal programs (Borland Turbo Pascal for MS-DOS microcomputers) were developed to carry out these procedures, as well as subtraction of background indicated by control lanes (samples treated with piperidine alone), if necessary. The purposes of these programs are briefly summarized below, and the algorithms will be detailed in a subsequent paper (Rill, Marsch, and James Veal, in preparation). To properly merge band intensities from a series of scans of a single lane, data were taken so that at least three bands from the end of one scan overlapped bands from the beginning of the next scan. The averages of the intensities of the overlapping bands from the two scans were then compared and used to normalize intensities of the second scan to that of the first. This process was repeated for successive scans on the same lane. A similar process was used to merge data from lanes corresponding to successive loadings on the DNA sequencing gel. Treatment with hot piperidine alone caused slight chain cleavage. To correct for background, the intensities measured from control lanes (samples treated with piperidine, but not BPDE) were subtracted from the sample data prior to normalization and merging. This correction was usually small and was minimized by keeping the piperidine concentration low (0.25 M).

An additional normalization step was required to merge data from different restriction fragments. Normalization factors were calculated from the average intensities of *all* bands from each fragment. This procedure introduces biases if a particular fragment is fortuitously rich or poor in preferred sequences. Quantitative comparisons of highly preferred or avoided sequences indicated by analyses of the total, merged data sets with those indicated by examination of individual data sets showed that uncertainties of this nature did not affect the major trends. Simple considerations show that most sources of uncertainty in this normalization procedure tend to *reduce* the distinctions between the reactivities of specific sequences (e.g., triplets), provided each sequence is represented several times in the data set. Preferred sequences will be weighted *less* than appropriate if they are unusually abundant in a particular fragment; conversely, nonpreferred sequences will be weighted *more* than appropriate if they predominate in a fragment. In the analyses below we have not attempted to draw conclusions from small differences in preferences or

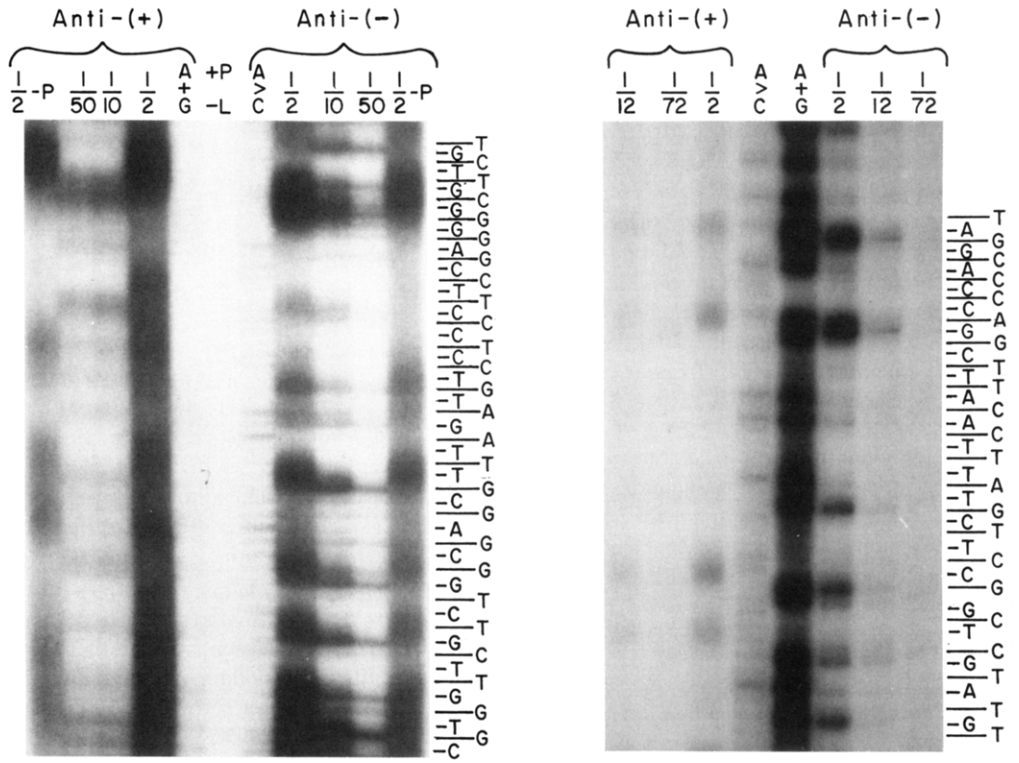

FIGURE 1: Autoradiograms illustrating the introduction of piperidine-labile sites into DNA by *anti*-BPDE isomers. Numbers indicate the ratio of mol of anti-BPDE/mol of DNA base pairs. "A>C" and "A+G" indicate standard sequencing reactions. Lanes labeled "–P" were reacted with *anti*-BPDE but heated in the absence of piperidine. Lanes labeled "+P – L" were not reacted with BPDE but were heated with piperidine.

average intensities or in cases where a sequence was represented by one or two occurrences.

Two additional programs were written to extract statistical information from the complete data set. One sorts the intensity data for a chosen base (e.g., guanine) in order of increasing intensity, calculates and plots the number distribution of normalized intensities, and reports characteristics of the intensity distribution (see Table I and Figure 3 below). Another program searches for each occurrence of the selected base type, notes the sequence context of the base, and updates accumulators corresponding to each possible doublet, triplet, and quartet containing the target base. These accumulators are used to calculate and report in tabular form the number of occurrences of the base in a particular sequence, the average intensities of bands corresponding to cleavage at the base in the specified sequence, and the propensities for high or low cleavage rates at the target base in the specified sequence (see Tables II–V below).

Results

Formation of Alkali-Labile Base Adducts by BPDE Isomers. Hot piperidine treatment of DNA reacted with either isomer caused cleavage at all base types detectable as fragments migrating faster than the parent on DNA sequencing gels, as noted by Sage and Haseltine (1984) (Figures 1 and 2). Examination of samples not treated with piperidine indicated that some adducts were particularly labile and were spontaneously lost, causing chain scission during handling and storage. The most intense bands, with or without piperidine treatment, usually corresponded to cleavages occurring at guanines, with the exception that a few cytosines in specific sequences appeared unusually reactive. The order of *overall* reactivity was G ≫ C ≈ A > T. Here we describe in detail only the effects of sequence context on reactivities of guanine residues.

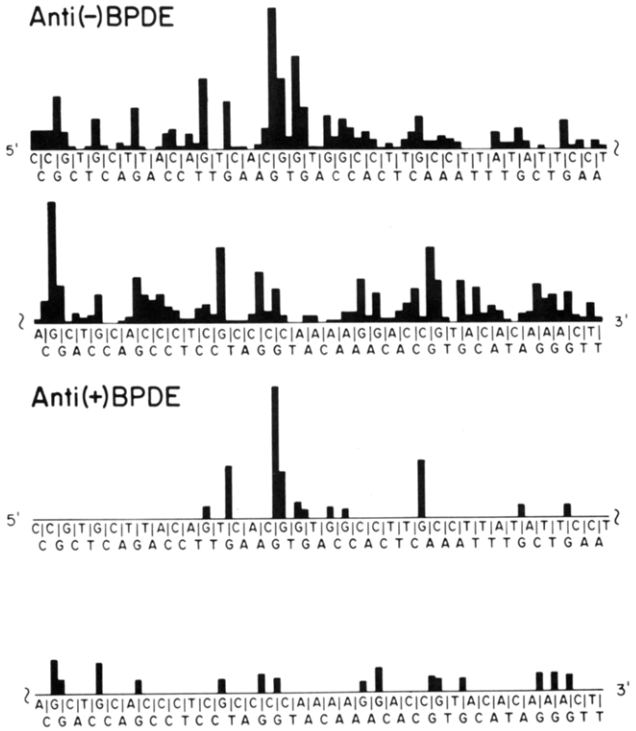

FIGURE 2: Histogram showing the normalized, merged intensities of bands corresponding to cleavage at specific bases caused by hot piperidine after reaction of the DNA with *anti*-(–)-BPDE (top) and *anti*-(+)-BPDE (bottom). Intensities for bands resulting from *anti*-(+)-BPDE treatment were plotted for guanines only. The positions of these bands were adjusted by +1 nucleotide relative to those obtained for *anti*-(–)-BPDE to correct for the effects of nonlabile adducts as described in the text. No data were available for the 22 nucleotides on the extreme 5′-end.

Bands corresponding to cleavage products of DNA reacted with the *anti*-(–)-BPDE isomer were approximately as sharp

as those observed with normal DNA sequencing reactions and comigrated with the bands of traditional sequencing lanes except at the highest levels of total DNA modification. By contrast, bands derived from DNA treated with anti-(+)-isomer were usually broader and lagged behind the corresponding bands on sequencing lanes by the equivalent of one to two nucleotides (Figure 1). We attribute this greater breadth to the greater propensity of *anti*-(+)-BPDE to form *stable* adducts at exocyclic amino groups, particularly of guanine (Meehan & Straub, 1977; Weinstein et al., 1976; Jeffrey et al., 1977; Osborne et al., 1981; Brookes & Osborne, 1982). Examination of short exposures of autoradiograms obtained with short DNA fragments or synthetic oligonucleotide duplexes showed that BPDE adducts significantly retarded the electrophoretic mobilities of DNA single strands. Samples treated with *anti*-(+)-BPDE yielded much larger amounts of material migrating behind the parent band, both before and after piperidine treatment, than those treated with an equal amount of *anti*-(−)-BPDE (data not shown), as expected from the greater overall reactivity of the anti-(+)-isomer at the guanine N^2 position (Meehan & Straub, 1977; Weinstein et al., 1976; Jeffrey et al., 1977; Osborne et al., 1981; Brookes & Osborne, 1982). By contrast, *anti*-(−)-BPDE appeared to produce somewhat more alkali-labile adducts than *anti*-(+)-BPDE at equal doses (Figure 1). The greater breadth of bands from DNA treated with *anti*-(+)-BPDE can in this case be attributed to the much greater probability of occurrence of a stable adduct on a strand also containing a labile adduct.

The effects of stable *anti*-(+)-BPDE adducts on DNA fragment mobility and bandwidth caused uncertainties of 1–2 nucleotides in assignments of the chain cleavage positions and intensities. Since the most intense bands obtained from samples treated with *anti*-(−)-BPDE corresponded to guanines, the most intense bands from samples treated with *anti*-(+)-BPDE were also assigned to guanines (provided a guanine was expected one to two positions further down the gel). Band intensities were assigned by dropping perpendiculars at increments corresponding to the intervals between nucleotides as indicated by neighboring sequencing lanes. Because of these uncertainties the sequence preferences of *anti*-(+)-BPDE could not be unequivocally established at the precise nucleotide level, but differences in the preferences of the two isomers were clearly evident and the predominant triplet sequences preferred by *anti*-(+)-BPDE could be identified. Examination of the band intensities in Figures 1 and 2 illustrates qualitatively the finding that reactions of guanines yielding alkali-labile products were distinctly nonrandom and that the two isomers differed in sequence preferences.

Inspection of autoradiograms suggested that formation of alkali-labile products by *anti*-(−)-BPDE was preferred if the 3′-flanking base was a second G, or the 5′-flanking base was a T. Several examples of strongly reacting AGG, TGG, CGG, and TGC sequences are illustrated in Figures 1 and 2. By contrast, guanines in sequences such as AGA, AGC, and CGA and the last two G's in GGGG appeared relatively less reactive. *anti*-(+)-BPDE also preferentially formed labile adducts at GG sites but did not exhibit a preference for TG sites (Figure 1). These trends were confirmed by quantitative analyses of band intensities described below.

Range of Reactivity of Guanines with BPDE Isomers. The normalized intensity of a band represents the relative frequency of reaction at a specific site to form an alkali-labile adduct. A plot of the number distribution of band intensities provides a visual representation of the range of reactivities and is useful

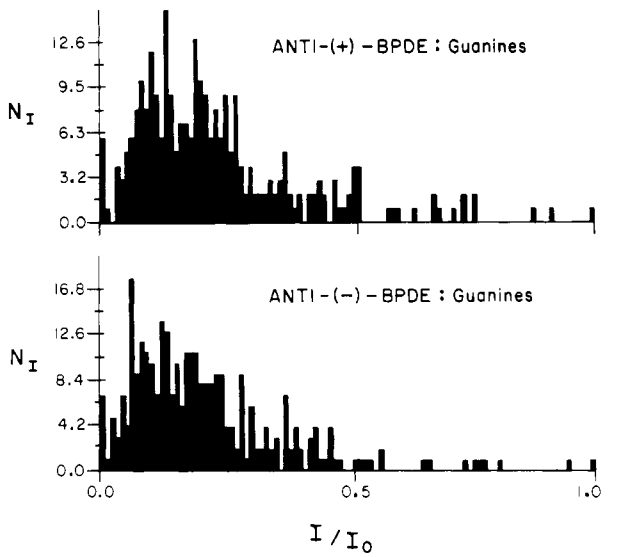

FIGURE 3: Number distribution of band intensities on DNA sequencing gels corresponding to cleavage at guanines after treatment with *anti*-(+)-BPDE (top) and *anti*-(−)-BPDE (bottom). The number of bands with a given intensity are plotted against intensity (normalized to the most intense band = 1.0). The number of guanine bands represented are 303 for *anti*-(−)-BPDE and 275 for *anti*-(+)-BPDE (see Table I).

Table I: Quantitative Measures of the Range of Reactivities of Guanines with BPDE Isomers[a]

	anti-(−)-BPDE
no. of intensities sorted	303
range of intensities	0–1779
median intensity	316
10–90 percentile range	109–726
25–75 percentile range	173–479
mean intensity of the mid-80 percentile	334 ± 132 (SD)
mean intensity of the upper 10 percentile	1005
mean intensity of the lower 10 percentile	58
ratio of mean intensities of the upper and lower percentile	17
ratio of mean intensity of upper 10 percentile to median intensity	3.2
	anti-(+)-BPDE
no. of intensities sorted	275
range of intensities	0–1527
median intensity	302
10–90 percentile range	111–730
25–75 percentile range	176–433
mean intensity of the mid-80 percentile	362 ± 194 (SD)
mean intensity of the upper 10 percentile	950
mean intensity of the lower 10 percentile	64
ratio of mean intensities of the upper and lower 10 percentile	15
ratio of mean intensity of upper 10 percentile to median intensity	3.2

[a] Relative reactivities of guanines to produce piperidine-labile adducts are represented by the intensities of bands on DNA sequencing gels.

for comparing the reactivities of different ligands. The distributions obtained for reaction of guanines with both BPDE isomers to yield piperidine-labile adducts were similar and very broad (Figure 3). Both distributions peaked at a relatively low intensity and were skewed to the high-intensity side. Skewing of the distribution suggests that the intrinsic reactivity of a guanine is dependent on flanking sequences and that some sequences are highly preferred.

A quantitative measure of the relative BPDE reactivities of guanines is provided by comparing the *ratios* of the band intensities of the "high" and "low" reactivity groups for the two ligands (Table I). Definition of the high and low groups is arbitrary, but the upper and lower 10 percentile ranges are commonly used in population statistics and were chosen here

for comparison. For example, the most and least reactive guanines, defined in terms of the upper and lower 10 percentiles, differed by a factor of ≈17 in reactivity toward both BPDE isomers. The most reactive guanines were about 3–3.2 times as reactive as the average guanine.

Methods of Identifying Preferred Sequences. Sequence preferences of both isomers were examined at the levels of doublets, triplets, and quartets including the target G. Sequences surrounding G's that were highly preferred were identified by two criteria: the propensity for occurrence of the sequence in the highly preferred group (defined as the upper quartile of band intensities) and the band intensity of the target guanine of the sequence *averaged over all occurrences of the sequence.* The former criterion, termed the "reaction propensity", is defined as in the following example. For a triplet XGY with the target base (G) in the middle position, the propensity of occurrence of the sequence XGY within the high-intensity range = P_{XGY}, where

$$P_{XGY} = (N_{XG^*Y}/N_{G^*})/(N_{XGY}/N_G) = (N_{XG^*Y}/N_{XGY})/(N_{G^*}/N_G)$$

= [probability that a preferred G (G*) occurs in sequence XGY]/(probability that any G occurs in sequence XGY)

= [probability that sequence XGY is in the preferred group (XG*Y)]/[probability that any G is in the preferred group (G*)]

The limiting values of a propensity are as follows: P_{XGY} = 0, if no XGY sequences are in the preferred group; P_{XGY} = 1.0, if XGY sequences are in the preferred group with statistical frequency; $P_{XGY} = N_G/N_{G^*}$, if all XGY sequences are in the preferred group; and $P_{XGY} \approx 4$, if the preferred group = upper quartile.

These two criteria are useful because they measure different aspects of the sequence preferences. The average band intensity for cleavage at a guanine in a particular sequence is a quantitative measure of the degree of preference for reaction (to form a piperidine-labile adduct) at that sequence, relative to other sequences. If formation of the noncovalent BPDE–DNA complex were rate limiting, then the reaction rate would reflect the equilibrium population of noncovalently bound ligand at a particular sequence. The ratio of the average intensity of a band corresponding to cleavage at a specific sequence to the average intensity for all sequences, e.g., $\langle I_{XGY}\rangle/\langle I\rangle$, would then measure the binding affinity of the specific sequence relative to an "average" site and could be related to the difference in free energy for binding at the preferred site relative to an "average" site. Ideally, $\Delta G_{0,\text{preferred}} - \Delta G_{0,\text{average}} = RT \ln [\langle I_{\text{pref}}\rangle/\langle I\rangle]$. In this case differences in reactivity of 5, 10, or 20 correspond to approximately 1.0, 1.4, and 1.8 kcal/mol differences in standard free energies of binding, respectively. Noncovalent binding appears *not* to be the rate-limiting step in BPDE adduct formation, however. As described by Geacintov et al. (1988), the ratios of adducts formed at different sites are a complex function of reaction rates, noncovalent binding constants, and the efficiency of DNA addition, as opposed to hydrolysis, of the activated BPDE.

The propensity for occurrence of a specific sequence in the preferred group is a measure of the *sufficiency* of that sequence for determining a high reactivity. For example, below we show that the G's most reactive with both BPDE isomers are those followed by a second G (Table II). However, a GG sequence is not *sufficient* to guarantee high reactivity of the 5′-G (Table III), as indicated by the failure of the propensity for reaction at GG doublets to approach the limit expected if the 5′-G's of *all* GG doublets were highly reactive. In fact, the specificities of the two BPDE isomers appear to be determined most stringently in terms of quartet to pentamer sequences.

Sequences Preferred by anti-(−)-BPDE: Nearest-Neighbor Effects on Guanine Reactivity. Data were collected for reactions of *anti*-(−)-BPDE with >1200 DNA base pairs, including 303 guanines. These data provided a statistically firm basis for establishing the sequence preferences of *anti*-(−)-

Table II: Dependence of Guanine Reactivity with *anti*-(−)- and *anti*-(+)-BPDE on Nearest-Neighbor 3′- and 5′-Flanking Bases

	A	T	G	C
anti-(−)-BPDE, 5′-Flanking Base				
propensity,[a] $P_{\underline{XG}}$	0.6	**1.8**	0.2	1.0
av intensity,[b] $\langle I\rangle$	329	**478**	240	419
no. of occurrences, N	55	**102**	81	63
anti-(−)-BPDE, 3′-Flanking Base				
propensity, $P_{\underline{GY}}$	0.6	1.2	**1.7**	0.5
av intensity, $\langle I\rangle$	304	364	**531**	302
no. of occurrences, N	55	69	**81**	94
anti-(+)-BPDE, 5′-Flanking Base				
propensity, $P_{\underline{XG}}$	1.1	1.1	0.5	**1.6**
av intensity, $\langle I\rangle$	367	350	268	**504**
no. of occurrences, N	50	91	76	**56**
anti-(+)-BPDE, 3′-Flanking Base				
propensity, $P_{\underline{GY}}$	0.5	0.8	**1.8**	0.7
av intensity, $\langle I\rangle$	298	297	**532**	304
no. of occurrences, N	51	62	**76**	83

[a] The propensity for occurrence of the guanine in this sequence in the group of most reactive guanines. The target guanine is underlined. The most reactive group is defined here as the upper quartile—see text. Unusually high values are indicated in boldface. [b] The average intensity of bands corresponding to cleavage at the target G residue in this sequence, averaged over *all N* occurrences of this sequence.

Table III: Relative Preferences for Triplets with Target Guanine Centered (X$\underline{G}$Y)

		3′-flanking base			
5′-flanking base		A	T	G	C
	anti-(−)-BPDE				
A	$P_{\underline{AGY}}$[a]	0.0	0.0	**2.1**	0.2
	$\langle I\rangle$[b]	229	214	**620**	260
	N	10	8	**13**	23
T	$P_{\underline{TGY}}$	1.3	**2.5**	**2.4**	1.3
	$\langle I\rangle$	442	**500**	**586**	404
	N	19	**24**	**27**	31
G	$P_{\underline{GGY}}$	0.0	0.2	0.4	0.1
	$\langle I\rangle$	160	237	308	239
	N	12	20	19	28
C	$P_{\underline{CGY}}$	0.6	1.1	**1.8**	0.0
	$\langle I\rangle$	294	385	**602**	269
	N	14	15	**22**	12
	anti-(+)-BPDE				
A	$P_{\underline{AGY}}$	0.0	0.5	**2.6**	0.8
	$\langle I\rangle$	296	212	**623**	279
	N	7	8	**14**	21
T	$P_{\underline{TGY}}$	0.7	1.1	1.3	1.0
	$\langle I\rangle$	334	293	404	358
	N	17	22	25	27
G	$P_{\underline{GGY}}$	0.0	0.3	1.0	0.4
	$\langle I\rangle$	237	225	353	264
	N	12	15	19	27
C	$P_{\underline{CGY}}$	0.9	1.2	**2.9**	0.5
	$\langle I\rangle$	294	412	**828**	321
	N	14	16	**18**	8

[a] The propensity for occurrence of the guanine in this sequence in the group of most reactive guanines. The target guanine is underlined. The most reactive group is defined here as the upper quartile. Unusually high values are indicated in boldface. [b] The average intensity of bands corresponding to cleavage at the target G, averaged over all N occurrences of this sequence.

BPDE at the triplet level, or higher in many cases. The doublet intensities and propensities reflect the influence of a base either 5′-flanking *or* 3′-flanking a guanine on the rate of alkali-labile adduct formation at that guanine (Table II). Guanines flanked on the 3′-side by a second guanine exhibited higher than average reactivity, about 1.7 times the median reactivity. No special effects were noted for other 3′-flanking bases. By contrast, reactivity was moderately enhanced if the 5′-flanking base was T or C (T > C) and diminished if the 5′-flanking base was G.

The effects of 3′- and 5′-flanking bases were roughly additive in the above cases; thus although preferred guanines were followed by a second guanine, the reactivity of the first G of a GG doublet depended on the 5′-flanking base and was enhanced if preceded by an A, T, or C and diminished if preceded by a G (Table III). The reactivities of the central G's in these triplets were approximately twice the median G reactivity and 10 times the reactivity of the least reactive 10 percentile of G's. The effects of nearest-neighbor bases were not additive in all cases, however. For example, a 5′-flanking T generally enhanced the reactivity of a G in any TGY sequence (Y = any base), but CGY sequences were usually reactive only if Y = G. Conversely, G's with a 5′-flanking A were not unusually reactive in general, but the central G in AGG triplets was highly reactive. No triplets other than TGG, TGT, AGG, and CGG exhibited a propensity approaching or exceeding 2.0 or average intensity greater than 1.5 times the median. A propensity >2.0 indicates that ≥50% of the central G's in the sequences TGT, TGG, AGG, and CGG occurred in the upper quartile of reactivity. The fact that these sequences did not universally occur in the upper quartile suggests that the surrounding sequences also influence reactivity.

Sequences Preferred by anti-(−)-BPDE: Next-Nearest-Neighbor Effects on Guanine Reactivities. Examination of quartet intensities and propensities showed that the reactivities of the central G's in preferred triplets were moderately to strongly influenced by the bases 5′-flanking and 3′-flanking the triplet (Tables IV and V). The following quartet sequences were more preferred than the included preferred triplets (target base underlined):

TGG: CTGG, TGG(A>C)
TGT: CTGT
TGA: (A≈G)TGA
AGG: (C≈T)AGG, AGG(A>T)
CGG: (A>C)CGG, CGG(C>T)

The preferences listed above are less well established than those at the triplet level because the data set is not of sufficient size to represent all possible quartets with statistical significance. Nonetheless, most sequences related to the above preferences were represented at least three times in the data set and therefore appear reliable (Tables IV and V). For example, there were 27 cases of TGG triplets reacted with *anti*-(−)-BPDE and at least four examples of each possible quartet containing this triplet with the target guanine in positions 2 or 3. Reaction at this triplet clearly was most favored if the 5′-flanking base was C (in CTGG) and the 3′-flanking base was A (in TGGA). Reaction at several preferred triplets appeared enhanced if the 5′-flanking base was C (e.g., CTGG, CTGT, CAGG, CCGG). Curiously, however, this and other next-nearest-neighbor preference trends were not universal. For example, reaction of the G in TGA sequences was clearly favored only if the 5′-flanking base was a purine.

Sequences Disfavored by anti-(−)-[BPDE. Examination of the data for triplets (Table III) shows that although AGG and PyGG triplets were most preferred, the least preferred guanines usually were either preceded by a purine (i.e., GGX and AGY sequences, where X = any base, and Y = any base but G) or followed by an A (i.e., XGA, where X = any base but T). The guanines least preferred in quartets were generally those preceded by two purines, particularly in the sequences GAGX, GGGX, and AGG(A or T) (Table IV). There were no occurrences of these sequences in the upper reactivity quartile, and the reactivities of the guanines (underlined) in these sequences were on average about one-third the median reactivity, and $^1/_5$ or less of the reactivities of the most preferred guanines in other quartet sequences.

Reaction Preferences of anti-(+)-BPDE. Fewer data were available for *anti*-(+)-BPDE because of the ambiguities caused by broad bands as described above. Data are therefore reported only for doublet and triplet preferences (Tables II and III). Nonetheless, the similarities and differences in sequence preferences between the *anti*-(+)- and *anti*-(−)-BPDE isomers

Table IV: Relative Preferences of *anti*-(−)-BPDE for Quartets with Target Guanine in Position 3 (XYGZ)[a]

5′-flanking doublet		3′-flanking base A	T	G	C
AA	*P*	0.0	0.0	4.0	0.0
	⟨*I*⟩	344	205	593	361
	N	3	2	2	3
TA	*P*	0.0	0.0	**2.7**	0.0
	⟨*I*⟩	258	150	**727**	379
	N	3	1	**3**	5
GA	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	137	301	212	136
	N	3	2	2	9
CA	*P*	0.0	0.0	**2.0**	0.0
	⟨*I*⟩	77	185	**712**	236
	N	1	3	**6**	5
AT	*P*	**2.7**	2.3	1.3	**2.7**
	⟨*I*⟩	**563**	470	569	**516**
	N	**6**	7	6	**6**
TT	*P*	0.0	2.2	2.0	0.9
	⟨*I*⟩	218	424	396	358
	N	5	9	6	13
GT	*P*	**1.3**	2.7	2.8	1.3
	⟨*I*⟩	**552**	411	558	398
	N	**6**	6	7	6
CT	*P*	0.0	**4.0**	**3.0**	0.7
	⟨*I*⟩	305	**1215**	**765**	400
	N	2	**2**	**8**	6
AG	*P*	0.0	0.0	0.0	0.7
	⟨*I*⟩	291	196	398	341
	N	2	2	2	6
TG	*P*	0.0	0.0	2.0	0.0
	⟨*I*⟩	146	217	412	174
	N	4	9	4	9
GG	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	144	167	241	115
	N	3	3	7	6
CG	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	108	314	287	253
	N	3	6	6	7
AC	*P*	0.0	0.0	**3.2**	0.0
	⟨*I*⟩	219	76	**995**	404
	N	2	1	**5**	2
TC	*P*	0.8	**2.0**	0.0	0.0
	⟨*I*⟩	419	**501**	429	259
	N	5	**4**	1	2
GC	*P*	0.0	0.8	0.5	0.0
	⟨*I*⟩	161	372	395	225
	N	6	5	8	7
CC	*P*	**4.0**	0.8	2.5	0.0
	⟨*I*⟩	**622**	367	585	332
	N	**1**	5	8	1

[a] See legend to Figure 3. Values are given in boldface only if the intensities exceed those observed in the included XGY triplets.

Table V: Relative Preferences of *anti*-(−)-BPDE for Quartets with Target Guanine in Position 2 (XGYZ)[a]

triplet		3′-flanking base A	T	G	C
AGA	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	351	92	272	199
	N	1	2	5	2
AGT	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	118	251	266	193
	N	2	3	2	1
AGG	*P*	**4.0**	**4.0**	2.0	1.3
	⟨*I*⟩	**1068**	**712**	399	547
	N	**2**	**2**	2	6
AGC	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	200	289	300	168
	N	3	9	7	4
TGA	*P*	0.0	1.0	1.6	1.5
	⟨*I*⟩	251	393	517	466
	N	2	4	5	8
TGT	*P*	**3.2**	**3.0**	1.8	2.0
	⟨*I*⟩	**523**	**530**	471	453
	N	**5**	**8**	9	2
TGG	*P*	**3.0**	2.2	1.0	**3.1**
	⟨*I*⟩	**878**	529	440	**605**
	N	**4**	9	4	**9**
TGC	*P*	1.4	0.6	1.6	1.5
	⟨*I*⟩	442	347	439	381
	N	11	7	5	8
GGA	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	229	110	187	119
	N	3	5	2	1
GGT	*P*	0.0	0.0	0.6	0.0
	⟨*I*⟩	191	230	300	164
	N	4	6	7	3
GGG	*P*	0.0	0.0	0.6	0.7
	⟨*I*⟩	240	204	360	332
	N	3	3	7	6
GGC	*P*	0.0	0.0	0.0	1.3
	⟨*I*⟩	279	161	277	301
	N	6	10	9	3
CGA	*P*	0.0	1.6	0.0	0.0
	⟨*I*⟩	263	438	140	242
	N	3	5	3	3
CGT	*P*	2.0	0.0	0.8	**4.0**
	⟨*I*⟩	341	278	413	**682**
	N	2	6	5	**2**
CGG	*P*	0.0	**2.0**	2.0	**2.3**
	⟨*I*⟩	275	**658**	483	**796**
	N	3	**6**	6	**7**
CGC	*P*	0.0	0.0	0.0	0.0
	⟨*I*⟩	133	215	295	326
	N	1	3	5	3

[a] See legend Figure 3. Values are given in boldface only if the average intensity exceeds that of the induced XGY triplet.

indicated by examination of autoradiograms (Figures 1 and 2) are apparent in the tabulated data. Reaction of *anti*-(+)-BPDE, like that of *anti*-(−)-BPDE, was clearly most favored by a 3′-flanking G and by a 5′-flanking A or C (Table II). On the other hand, only the triplets C*G*G and A*G*G were strongly preferred by *anti*-(+)-BPDE. By contrast, the triplets T*G*G and T*G*T, which were strongly preferred by *anti*-(−)-BPDE, were not as preferred by the anti-(+)-isomer. Like *anti*-(−)-BPDE, reaction of *anti*-(+)-BPDE at C*G*G triplets was significantly enhanced if the 5′-flanking base was A or C (in AC*G*G and CC*G*G); and reactions of guanines preceded by two purines was generally low (i.e., in sequences AA*G*X, GA*G*X, and GG*G*Y, where X = any base but G, and Y = any base) (data not shown).

Discussion

Both *anti*-BPDE isomers form piperidine-labile adducts predominantly with guanines. Individual guanines differed significantly in their intrinsic reactivity toward both BPDE isomers to form piperidine-labile products. The most reactive guanines were about 17 times as reactive as the least reactive guanines, where the terms most and least are defined as the upper and lower 10 percentiles of reactivity. Differences in reactivities cannot be linked to a specific process because the rate of covalent addition at a specific site depends on a combination of binding affinities, rate of active intermediate [perhaps carbonium ion formation (Hulbert, 1975; Jerina & Lehr, 1978)], and the efficiency of covalent addition relative to hydrolysis of the active intermediate (Geacintov et al., 1981, 1988; Chen, 1984; Meehan & Bond, 1984). Nonetheless, this combination of factors leads to a significant degree of discrimination between guanines in the formation of piperidine-labile adducts.

Discrimination between guanines was clearly linked to sequence context. Guanines followed by a second guanine were most reactive toward both isomers, but GG doublets were not equally reactive. The reactivities of GG doublets were strongly affected by the 5′-flanking base and moderately to strongly affected by the 3′-flanking base. The influence of the 5′-flanking base on reactivity is particularly interesting because *anti*-(+)-BPDE and *anti*-(−)-BPDE isomers differed in reaction preference. *anti*-(−)-BPDE most strongly preferred the triplets 5′-CGG ≈ 5′-AGG ≈ 5′-TGG > 5′-TGT; while *anti*-(+)-BPDE most strongly preferred CGG and AGG triplets but did not strongly prefer TGG, TGT, or other TGN triplets. Formation of a piperidine-sensitive adduct at guanine is therefore facilitated by a 5′-flanking T in the case of *anti*-(−)-BPDE, but not *anti*-(+)-BPDE.

The different sensitivities of the (+)- and (−)-BPDE isomers to the bases flanking the covalent binding site must reflect steric/electronic influences of the unsaturated diol epoxide ring and may be related to the observation of different conformers for the DNA adducts of *anti*-(+)- and *anti*-(−)-BPDE on "random sequence" DNA (Kolubayev et al., 1987; Eriksson et al., 1988; Gräslund & Jernström, 1989; Kim et al., 1989). It should be noted, however, that such measurements and related molecular mechanics calculations (Aggarwal et al., 1983; Taylor et al., 1983; Hingerty & Broyde, 1985; Anderson et al., 1987; Rao et al., 1989) pertain most directly to the adduct at the guanine N^2 group, whereas our data reflects binding at a different site, predominantly the guanine N7 position. At the present time we do not know if there is a direct relationship between specificities of binding at the two guanine positions. Boles and Hogan examined the sequence preferences of addition of racemic *anti*-(±)-BPDE by utilizing 355-nm laser light to cleave DNA at all adduct sites (Boles & Hogan, 1986). Data were obtained on only ≈200 DNA bases; hence a statistical analysis was not performed. Very strong cleavage was observed at guanines in guanine tracts, including several CGG and AGG triplets, and less strong cleavage was observed at G's with a 5′-flanking T. These data are in good agreement with ours for *anti*-(+)-BPDE except that we did not observe strong reaction of *all* G's in oligo-G tracts. Since the guanine N^2 adduct of *anti*-(+)-BPDE is expected to be the predominant product of reaction of racemic *anti*-(±)-BPDE, it appears that the sequence preferences for formation of labile and stable guanine adducts by *anti*-(+)-BPDE are very similar.

The site of most intimate *noncovalent* association between DNA and BPDE, prior to adduct formation, cannot be easily inferred from our data. The fact that both isomers prefer to react at guanines in a pyrimidine–guanine (TG or CG) doublet is consistent with the preference of intercalating agents for pyrimidine–purine steps (Reinhardt & Krugh, 1978; Krugh

& Reinhardt, 1975; Patel & Canuel, 1976, 1977; Patel, 1977). However, the strongest requirement appears to be a GG sequence, and both isomers also exhibit a strong preference for reaction at the first G of an AGG sequence. These preferences are less easily reconciled with an intercalation intermediate. Spectral properties of the noncovalently bound BPDE complex with DNA are highly consistent with formation of an initial intercalation complex (Geacintov et al., 1981, 1984; Meehan et al., 1982; Chen, 1984). It is also clear, however, that noncovalent binding is significantly more rapid than covalent adduct formation and that intercalation alone is not sufficient for covalent addition. The latter point is particularly evident from the fact that *noncovalent* association of racemic *anti*-(±)-BPDE is more favored for poly[d(AT)]·poly[d(AT)] than for poly[d(GC)]·poly[d(GC)], but covalent addition occurs much more readily to the latter duplex (Geacintov et al., 1988; Shimer et al., 1988). Likewise we observed that PyGG and AGG sequences were more reactive than PyPuPy sequences toward both isomers (Py = pyrimidine, Pu = purine), although duplexes of alternating purines and pyrimidines noncovalently bind BPDE more tightly than duplexes of homopolymers (Geacintov et al., 1988). Covalent addition may require rearrangement of the intercalation complex or migration of the activated BPDE out of the intercalation site and thereby involve participation of the bases both 5′- and 3′-flanking the modified base.

The reactivities of the central G residues in preferred triplet sequences are also influenced by flanking bases, i.e., the next-nearest neighbors of the modified guanine. The strongest *pentamer* preferences suggested by our data are

anti-(+)-BPDE:	5′-(C,A)C	G̲G(C,G)
	5′-(T,C)A	G̲GA
anti-(−)-BPDE:	5′-AC	G̲G(C>T)
	5′-(T,C)A	G̲G(A,C)
	5′-(C>A)T	G̲G(A>T)
	5′-CT	G̲TN (N = any base)

(Note that we do not have statistically significant data on these pentamers. The above preferences are deduced from data on quartets and will be valid if the effects of the 5′- and 3′-terminal bases are approximately additive.) The reactivities of the most preferred quartets were, on average, about 3 times the median reactivity—significantly above the reactivities of the preferred triplets—and 10–15 times the reactivity of the least reactive 10 percentile of G's. The influences of next-nearest-neighbor bases are complex in many cases and cannot be easily explained in terms of direct interactions with the ligand since BPDE cannot interact physically across five base pairs. Presumably these influences are exerted at the level of the DNA conformation or base pair stabilities at the binding site.

The carcinogenicities of BPDE isomers are determined by multiple factors that are incompletely understood. Although addition of the highly carcinogenic *anti*-(+)-BPDE occurs predominantly at the guanine N^2 position to form a piperidine-stable product, 20% or more of the total DNA guanine adducts may be piperidine-labile. The fact that some guanines exhibit much higher than average propensities for formation of piperidine-labile BPDE adducts than others argues that the contribution of these adducts to overall carcinogenicity cannot be ignored. In fact, there is good evidence that apurinic sites, expected from spontaneous loss of labile adducts, makes a contribution to the overall carcinogenicity of *anti*-BPDE (Sage & Haseltine, 1984; Vousden et al., 1986). In this context, the differences in sequence preferences noted for piperidine-labile adduct formation by *anti*-(+)- and *anti*-(−)-BPDE may also contribute to the differences in potency of these two carcinogens.

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Registry No. *anti*-(+)-BPDE, 63323-31-9; *anti*-(−)-BPDE, 63323-30-8.

References

Aggarwal, A. K., Islam, S. A., & Neidle, S. (1983) *J. Biomol. Struct. Dyn. 1*, 873–881.

Anderson, R. W., Whitlow, M. D., Teeter, M. M., & Mohr, S. C. (1987) *J. Biomol. Struct. Dyn. 5*, 383–404.

Boles, T. C., & Hogan, M. E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5623–5627.

Boles, T. C., & Hogan, M. E. (1986) *Biochemistry 25*, 3039–3043.

Brookes, P., & Osborne, M. R. (1982) *Carcinogenesis 3*, 1223–1226.

Buening, M. E., Wislocki, P. G., Levin, W., Yagi, H., Thakker, D. R., Akagi, H., Koreeda, M., Jerina, D. M., & Conney, A. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5358–5361.

Chen. F.-M. (1984) *Carcinogenesis 5*, 753–758.

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

Deutsch, J., Leutz, J., Yang, S. K., Gelboin, H. V., Chiang, Y. L., Vatsis, K. P., & Loon, M. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3123–3127.

Eriksson, M., Nordën, B., Jernström, B., & Gräslund, A. (1988) *Biochemistry 27*, 1213–1221.

Geacintov, N. E., Yoshida, H., Ibanez, V., & Harvey, R. G. (1981) *Biochem. Biophys. Res. Commun. 100*, 1569–1577.

Geacintov, N. E., Yoshida, H., Ibanez, V., Jacobs, S. A., & Harvey, R. G. (1984) *Biochem. Biophys. Res. Commun. 122*, 33–39.

Geacintov, N. E., Shahbaz, M., Ibanez, V., Moussaoui, K., & Harvey, R. G. (1988) *Biochemistry 27*, 8380–8387.

Gräslund, A., & Jernström, B. (1989) *Q. Rev. Biophys. 22*, 1–37.

Harvey, R. G. (1981) *Acc. Chem. Res. 14*, 218–226.

Hingerty, B. E., & Broyde, S. (1985) *Biopolymers 24*, 2279–2299.

Hulbert, P. B. (1975) *Nature 256*, 146–148.

Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Weinstein, I. B., Beland, F. A., Harvey, R. G., Kasal, H., Miura, I., & Nakanishi, K. (1976) *J. Am. Chem. Soc. 98*, 5714–5715.

Jeffrey, A. M., Weinstein, I. B., Jennette, K. W., Grzeskowiak, K., Nakanishi, K., Harvey, R. G., Autrup, H., & Harris, C. (1977) *Nature 269*, 348–350.

Jerina, D. M., & Lehr, R. E. (1978) in *Microsomes and Drug Oxidation* (Ulrich, V., Roots, I., Hilderbrandt, A., & Estabrook, R. W., Eds.) pp 709–720, Pergamon Press, Elmsford, NY.

Kim, S. K., Geacintov, N. E., Brenner, H. C., & Harvey, R. G. (1989) *Carcinogenesis 10*, 1333–1335.

King, H. W., Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2679–2681.

King, H. W., Osborne, M. R., & Brookes, P. (1979) *Chem.-Biol. Interact. 24*, 345–353.

Kolubayev, V., Brenner, H. C., & Geacintov, N. E. (1987) *Biochemistry 26*, 2638–2641.

Krugh, T. R., & Reinhardt, C. G. (1975) *J. Mol. Biol. 97*, 133–162.

Lobanenkov, V. V., Plumb, M., Goodwin, G. H., & Grover, P. L. (1986) *Carcinogenesis 7*, 1689-1695.
Meehan, T., & Straub, K. (1977) *Nature 277*, 410-412.
Meehan, T., & Bond, D. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2635-2639.
Meehan, T., Gamper, H., & Becker, J. R. (1982) *J. Biol. Chem. 257*, 10479-10485.
Osborne, M. R., Beland, F. A., Harvey, R. G., & Brookes, P. (1976) *Int. J. Cancer 18*, 362-368.
Osborne, M. R., Harvey, R. G., & Brookes, P. (1978) *Chem.-Biol. Interact. 20*, 123-130.
Osborne, M. R., Jacobs, S., Harvey, R. G., & Brookes, P. (1981) *Carcinogenesis 2*, 553-558.
Patel, D. J. (1977) *Biopolymers 16*, 2739-2754.
Patel, D. J., & Canuel, L. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3343-3347.
Patel, D. J., & Canuel, L. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2624-2628.
Pearl, L. H., & Neidle, S. (1986) *FEBS Lett. 209*, 269-276.
Phillips, D. H. (1983) *Nature 303*, 468-472.
Rao, S. N., Lybrand, T., Michaud, D., Jerina, D. M., & Kollman, P. A. (1989) *Carcinogenesis 10*, 27-38.
Reinhardt, C. G., & Krugh, T. R. (1978) *Biochemistry 17*, 4845-4854.
Sage, E., & Haseltine, W. A. (1984) *J. Biol. Chem. 259*, 11098-11102.
Slaga, T. J., Bracken, W. J., Gleason, G., Levin, W., Yagi, H., Jerina, D. M., & Conney, A. H. (1979) *Cancer Res. 39*, 67-71.
Sures, I., Lowry, J., & Kedes, L. H. (1978) *Cell 15*, 1033-1044.
Taylor, E. R., Miller, K. J., & Bleyer, A. J. (1983) *J. Biomol. Struct. Dyn. 1*, 883-904.
Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., & Jerina, D. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3381-3385.
Thakker, D. R., Yagi, H., Akagi, H., Koreeda, M., Lu, A. Y. H., Levin, W., Wood, A. W., Conney, A. H., & Jerina, D. M. (1977) *Chem.-Biol. Interact. 16*, 281-300.
Veal, J. M., & Rill, R. L. (1988) *Biochemistry 27*, 1822-1827.
Vousden, K. H., Bos, J. L., Marshall, C. J., & Phillips, D. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1222-1226.
Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H., & Nakanishi, K. (1976) *Science 193*, 592-595.
Wu, R. S., Kohn, K. W., & Bonner, W. M. (1981) *J. Biol. Chem. 256*, 5916-5920.
Yang, S. K., & Gelboin, H. V. (1976) *Biochem. Pharmacol. 25*, 2221-2225.
Yang, S. K., Roller, P. P., & Gelboin, H. V. (1977) *Biochemistry 16*, 3680-3687.

Kinetics of Interaction of Nucleotides with Nucleotide-Free H-ras p21

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ABSTRACT: A method is described for the convenient preparation of substantial quantities of nucleotide-free p21 or of 1:1 complexes with nucleotides other than GDP. The nucleotide-free protein has been used for kinetic studies of the binding of GDP and GTP, making use of the fluorescent analogues 3'-(methylanthraniloyl)-2'-deoxy-GDP and -GTP. Stopped-flow studies have led to the formulation of a two-step binding mechanism for both GDP and GTP, involving initial rapid but weak binding of the nucleotide followed by a relatively slow (10–20 s^{-1} at 25 °C; 3–5 s^{-1} at 5 °C) quasi-irreversible isomerization reaction. By use of a nonequilibrium competition method, guanosine and GMP have been shown to interact weakly but significantly with p21 (dissocation constants of 153 and 29 μM, respectively). The presence of guanosine or GMP at the active site of p21 leads to a marked stabilization of p21 against spontaneous denaturation when compared with the nucleotide- and nucleoside-free protein.

The protein product of the H-ras protooncogenes (p21)[1] is known to be a GTPase with very low GTPase activity in the isolated state [for review, see Barbacid (1987)]. The role of p21 is not known, but overexpression or mutations at certain characteristic sites lead to oncogenic transforming properties. Although earlier work indicated that transforming properties were associated with a reduction of the GTPase rate, a clear correlation of these two factors could not be demonstrated. Recently, a protein has been identified that activates the rate of GTP cleavage on the protein, leaving the rate of nucleotide (GDP) release unchanged and rate controlling in the turnover of GTP (Trahey & McCormick, 1987; Vogel et al., 1988; McCormick, 1989). This activation does not occur with transforming mutant forms of the protein. Thus, in the presence of the activating protein (GAP), whereas the cellular form of p21 exists for only a short time in the GTP bound state, transforming forms have a long-lived GTP-bound state that decays only slowly to the GDP form. According to current thinking, the GTP form of p21 is responsible for activating a process important for cell growth or differentiation, and mutants leading to a long-lived GTP-bound state result in loss of control over this process.

Since p21, like most guanine nucleotide binding proteins, binds nucleotides with an association constant of $>10^8$ M^{-1}, it is isolated with 1 equiv of nucleotide bound. With the protein

[1] Abbreviations: p21, protein product of the human c-Ha-ras protooncogene; GTP(β,γ-CH_2), guanosine 5'-(β,γ-methylenetriphosphate); GPPNP, guanosine 5'-(β,γ-imidotriphosphate); mantdGDP/GTP or mdGDP/GTP, 3'-*O*-(*N*-methylanthraniloyl)-2'-deoxyguanosine di/triphosphate; EDTA, ethylenediaminetetraacetic acid; DTE, dithioerythritol.

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