Covalent Binding of (+)- and (−)-*trans*-7,8-Dihydroxy-*anti*-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene to B and Z DNAs†

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ABSTRACT: Circular dichroism (CD) as well as absorption spectral measurements reveals that poly(dG-m^{5}dC)·poly(dG-m^{5}dC) suffers more extensive covalent modification by (+)-dihydroxy-*anti*-epoxybenzo[*a*]pyrene [(+)-*anti*-BPDE] than its unmethylated counterpart and that the covalently attached pyrenyl moiety exhibits stronger stacking interactions with the bases in the methylated polymer as suggested by the much larger pyrenyl spectral red shifts, most likely the consequence of intercalation. Stereoselective binding properties of these polymers are evidenced by the much reduced preference for the (−) enantiomer. Modifications due to (+)-*anti*-BPDE on the 50 μM hexaamminecobalt induced Z DNAs are much less pronounced and much less stereoselective, with the pyrenyl spectral characteristics being distinct from those of the B form. Salt titrations on the (+)-*anti*-BPDE modified poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) indicate much reduced cooperativity on the B to Z transition when compared to the unmodified counterparts. Evidence also suggests that covalent modification by *anti*-BPDE inhibits the B to Z conversion of base pairs in its immediate vicinity, presumably through intercalative stabilization of the B conformer at high salt. In contrast to stabilizing the B conformation for the proximal base pairs, covalent lesion by (+)-*anti*-BPDE appears to destabilize distal base pairs with the consequence of kinetic facilitation of B to Z transformation for these regions. Interesting differential effects on the reverse Z to B transforming abilities of these two enantiomers are observed with the covalent binding of the (−) isomer showing higher potency for inducing such conversion.

It is generally believed that covalent modification of DNA is the critical initial event in the carcinogenesis of some polycyclic aromatic hydrocarbons (PAHs). Such covalent lesions are made possible by enzymatic conversion of these relatively inert PAHs into reactive metabolites (Harvey, 1981). Benzo[*a*]pyrene is the most widely studied PAH and there is strong evidence to suggest that (7*R*,8*S*,9*R*,10*R*)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [(+)-*anti*-BPDE] is its ultimate carcinogenic metabolite (Borgen, et al., 1973; Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Ivanovic et al., 1976). The (−) enantiomer has been shown to possess little or no carcinogenic activities (Wood et al., 1977; Buening et al., 1978; Slaga et al., 1979; Brookes & Osbornes, 1982; Chang et al., 1983).

Previous binding studies with racemic *anti*-BPDE have revealed that the binding to native DNA is quite stereoselective with the dominant covalent adduct being formed by trans addition of the exocyclic 2-amino group of guanine to the C10 of (+)-*anti*-BPDE (Koreeda et al., 1978; Ivanovic et al., 1978; Meehan & Straub, 1979; Osborne et al., 1981). Such stereoselective covalent binding [i.e., the preference for the (+) enantiomer] has been attributed to the stereospecific physical binding such as intercalation (Meehan & Straub, 1979). The difference in the extent of DNA modification by these two enantiomers, however, cannot completely explain their differential carcinogenic activities (Brookes & Osbornes, 1982; Pelling et al., 1984). Suggestions have thus been made that the basis of such differential effects may reside in the differing spatial orientation of the covalent adducts formed (Brookes & Osbornes, 1982).

The discovery of left-handed Z-DNA conformation in 1979 (Wang et al., 1979) has spurred flurries of activity to find agents that will affect the B–Z interconversions [see the review by Rich et al. (1984)]. The intense interest in the left-handed Z-DNA conformation stems from its possible connection with the gene regulatory mechanism. The most interesting is the case of methylation at the 5-position of cytosine, which has been implicated in the inactivation of genes (Doerfler, 1983). The facilitation of B to Z conversion due to 5-methylation of dC thus makes it plausible that transition to Z conformation may play some roles in the regulation of genes. Effects of covalent binding by carcinogens on the B–Z interconversions are consequently of considerable interest.

In order to explore the possible role of Z conformation on the tumorigenesis, comparative binding studies with (+)- and (−)-*anti*-BPDE were carried out in poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) solutions with particular emphasis on the difference in the extent and spectral characteristics of covalent adducts derived from these two enantiomers and their effects on the B–Z interconversions.

Materials and Methods

Poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) were purchased from P-L Biochemicals and dissolved in 10 mM sodium phosphate buffer solution of pH 7 containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.01 M NaCl. Extinction coefficients of 8400 and 7000 cm^{-1} M^{-1} at 255 nm were used for the unmethylated and methylated polynucleotides, respectively, for their concentration (per phosphate) determination. The melting temperatures of these solutions are slightly above 100 °C as indicated by both the absorbance and CD measurements. Z-DNA formation was induced by the presence of 50 μM hexaamminecobalt (HAC)

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except for the salt titration experiment where NaCl was used.

Racemic as well as optical enantiomers of *anti*-BPDE were purchased from the NCI Chemical Carcinogen Reference Standard Repository, a function of the Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, MD 20205. Covalent modification of DNA was achieved by adding an aliquot of *anti*-BPDE in tetrahydrofuran (THF) to the DNA solution to make either a 25 μM (enantiomers) or a 50 μM (racemic mixture) concentration, which was then left overnight to ensure complete reaction. The mixture was subsequently subjected to 15 double-volume ether extractions to remove tetraols in a Mixxor glass separator. The residual ether in the solution was removed by 40–50 pipet aspirations. The concentrations of covalently bound *anti*-BPDE were determined with an extinction coefficient of 30 000 M^{-1} cm^{-1} around 343 nm. The *anti*-BPDE concentrations of stock solutions were determined by using an extinction coefficient of 48 600 M^{-1} cm^{-1} at 344 nm in 95% ethanol.

All absorption spectral measurements were made with Cary 210-plus spectrophotometric system with temperatures maintained around 24 °C. The CD spectral measurements were carried out with a JASCO-J500A recording spectropolarimeter at room temperatures. Appropriate buffer base lines have been subtracted out in all CD spectra presented. Conversion to molar ellipticities were not made in the CD plots since the adduct concentration determination by absorbance is only qualitative and more information can be obtained by comparison of relative CD intensities. Molar ellipticity can readily be calculated by $[\theta] = 100\theta(lc)^{-1}$, where θ is the observed ellipticity in millidegrees, c the concentration in millimolar, and l the pathlength in centimeters. All CD measurements were carried out in a water-jacketed cylindrical cell of 2-cm path length. All absorption spectra presented have been normalized to zero absorbance at 450 nm.

Results

Spectral Characteristics of Covalent Adducts Derived from (+)-anti-BPDE. (A) Pyrenyl Absorption Spectra. Absorption spectra in the pyrene spectral region for B- and Z-form (+)-*anti*-BPDE-modified poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are shown in Figure 1A. Hexaamminecobalt of 50 μM was used to induce Z DNA for both polymers to facilitate comparison, although much less than 50 μM is required to induce the transition for the methylated polymer (Behe & Felsenfeld, 1981). It is readily apparent from the figure that (1) the B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) binds (+)-*anti*-BPDE about 50% more effectively than the B-form poly(dG-dC)·poly(dG-dC), which in turn is twice as effective as the two Z DNAs of approximately equal reactivity, (2) the pyrenyl absorption maxima in the Z-DNA solutions appear at 343 and 327 nm, unshifted from those exhibited by the benzo[*a*]pyrene tetraols, and (3) the (+)-*anti*-BPDE bound to the B form of these polymers, on the other hand, exhibits red shifts of 3 nm for poly(dG-dC)·poly(dG-dC) and 7 nm for poly(dG-m^{5}dC)·poly(dG-m^{5}dC).

The 7-nm bathochromic shift for the (+)-*anti*-BPDE attached to poly(dG-m^{5}dC)·poly(dG-m^{5}dC) is quite interesting, as it suggests extensive stacking (likely the consequence of intercalation) with the bases in this methylated polynucleotide and has thus far not been observed as a major component in *anti*-BPDE- (racemic) modified duplex DNAs or polydeoxynucleotides (Chen, 1985). The magnitudes of the long-wavelength maxima coupled with the amount of (+)-*anti*-BPDE added (25 μM) and the concentrations of polynucleotide solutions (0.1 mM) enable the qualitative estimation of the percentage reacted to DNA and the fraction of bases modified. These results, along with those of the (−) enantiomer, are in Table I.

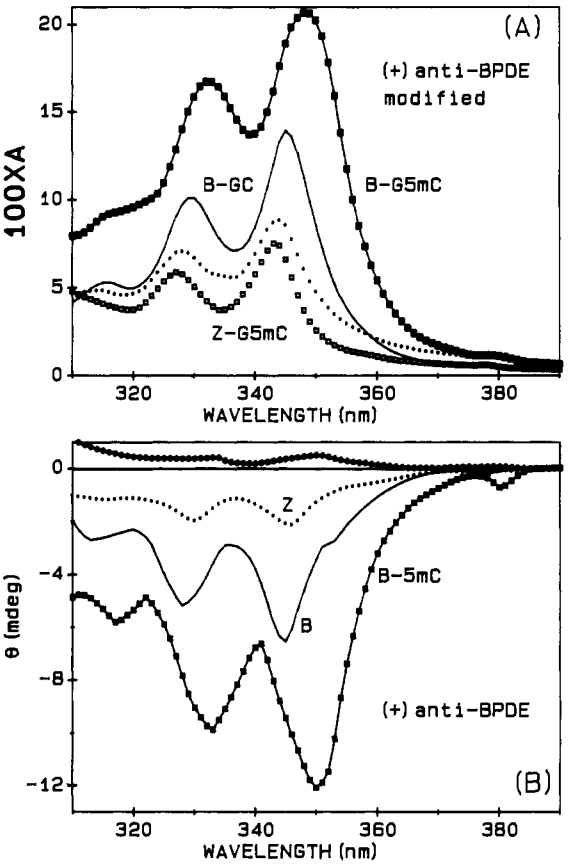

FIGURE 1: Pyrenyl absorption (A) and CD (B) spectra for (+)-*anti*-BPDE-modified 0.1 mM polynucleotide solutions: B-Form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (connected solid square), B-form poly(dG-dC)·poly(dG-dC) (solid line), Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) [open square, in (A) only], and Z-form poly(dG-dC)·poly(dG-dC) (dotted line). The unlabeled curve in (B) is that of (−)-*anti*-BPDE-modified B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC). Initially added enantiomer concentrations are 25 μM.

Table I: Extent of Covalent Modification by (+)- and (−)-*anti*-BPDE[a]

polynucleotide[b]	concn (mM)	covalently bound concn: (+)-*anti*-BPDE (μM) (nm)[c]	covalently bound concn: (−)-*anti*-BPDE (μM) (nm)[c]
B-form poly(dG-dC)·poly(dG-dC)	0.1	4.5 (345.5)	1.8 (343.5)
Z-form poly(dG-dC)·poly(dG-dC)	0.1	2.5 (344.0)	2.7 (343.5)
B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC)	0.1	6.7 (349.5)	1.4 (344.0)
Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC)	0.1	2.4 (343.5)	2.9 (343.5)

[a] A concentration of 25 μM *anti*-BPDE added to the polynucleotide solutions. [b] B form refers to solution in 10 mM sodium phosphate/10 mM NaCl/1 mM EDTA pH 7.0 buffer. Z conformation is induced by the presence of 50 μM hexaamminecobalt in the solution. [c] Concentration estimated with the absorbance maximum indicated inside the parentheses. These are single-experiment estimates, but their qualitative trends have been collaborated by separate experiments with different initial *anti*-BPDE concentration.

(B) CD in the Pyrene Spectral Region. The pyrenyl CD spectra of the (+)-*anti*-BPDE-modified B and Z forms of poly(dG-dC)·poly(dG-dC) as well as the B form of poly(dG-

m^{5}dC)·poly(dG-m^{5}dC) are shown in Figure 1B. No detectable pyrenyl Cotton effects are observed for the (+)-*anti*-BPDE-modified Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC). The CD spectrum of the (−)-*anti*-BPDE-modified B form of this polymer is thus included in the same figure for comparison instead.

It is evident from the figure that (1) the CD amplitudes for the (+)-*anti*-BPDE bound to B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are roughly twice those of poly(dG-dC)·poly(dG-dC) with location of maxima closely matching those of absorbance (350 vs. 345 nm; see Figure 1A), (2) aside from the close correspondence between the extrema of CD and absorbance, a CD shoulder is clearly discernible around 352 nm for the (+)-*anti*-BPDE-modified poly(dG-dC)·poly(dG-dC), (3) although the reactivities of (+)-*anti*-BPDE toward the Z forms of poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are only $^1/_2$ and $^1/_3$ of their B counterparts (Figure 1A), the corresponding CD amplitudes are roughly $^1/_3$ and less than $^1/_{30}$, respectively, and (4) (−)-*anti*-BPDE bound to the B form of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) exhibits a CD sign opposite from that of the (+) enantiomer but with much reduced intensity (about 20 times), in contrast to a mere 3-fold reduction in the extent of covalent modification as judged by the absorbance measurements.

Addition of 50 μM HAC to the (+)-*anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) solution shifts the 345-nm CD maximum to 350 nm (results not shown but see the similar results for the racemic mixture in Figure 6A). In contrast, no spectral shift is observed for the (+)-*anti*-BPDE-modified B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC), which already exhibits a 350-nm maximum at low salt. Similar results are obtained with progressive NaCl titration (not shown). These results possibly suggest that the *anti*-BPDEs bound to the B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are already intercalated in the low-salt condition whereas those covalently bound to B-form poly(dG-dC)·poly(dG-dC) shift from "external" in low salt to "intercalation" at high ionic strength.

(*C*) *CD in the DNA Spectral Region.* CD characteristics in the DNA spectral region for the (+)- and (−)-*anti*-BPDE modified and unmodified B-form poly(dG-dC)·poly(dG-dC) as well as B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are shown in Figure 2. In agreement with the absorbance and pyrenyl CD results, the CD spectra in the DNA region are little affected by the (−) modifications but are greatly altered by the (+)-*anti*-BPDE lesions. Consistent with the higher extent of modification, such spectral alteration is most apparent for the (+)-*anti*-BPDE-modified poly(dG-m^{5}dC)·poly(dG-m^{5}dC) and can be seen clearly from the difference spectra obtained by subtracting the unmodified from the (+)-*anti*-BPDE-modified polynucleotides. The difference spectra due to (+)-*anti*-BPDE modifications on poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are presented in Figure 3. Aside from the much larger amplitude for the poly(dG-m^{5}dC)·poly(dG-m^{5}dC), the gross features (positive CD maxima around 280 and 250 nm) of these difference CD, along with the negative pyrenyl Cotton effects (Figure 1B), are in general accord with the previously recorded spectrum for the major *anti*-BPDE–poly(G) adduct, which has been identified as N2-dG trans addition at the C10 of (+)-*anti*-BPDE (Moore et al., 1977; Jeffrey et al., 1977).

The sizable negative CD band around 295 nm for the (+)-*anti*-BPDE-modified B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC), evident in Figure 3, raises the question of whether it is due to partial Z conversion as a result of covalent modification. The B to Z transition barrier is much lower for

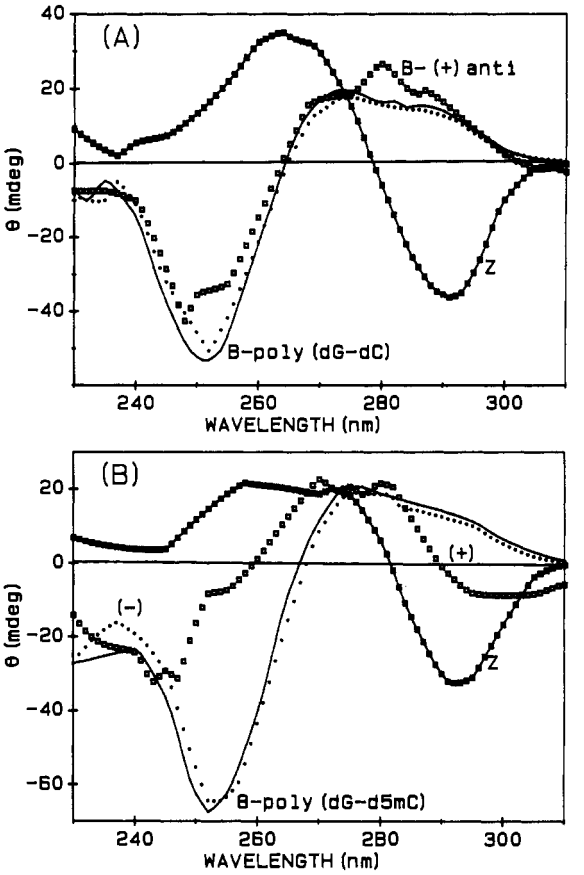

FIGURE 2: Comparison of CD curves in the DNA spectral region for 0.1 mM poly(dG-dC)·poly(dG-dC) (A) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (B) with (+)-*anti*-BPDE modification of B form (open square), (−)-*anti*-BPDE modification of B form (dotted curve), and unmodified B form (solid line). The CD curves for the unmodified Z form (connected square) are included for the sake of comparison.

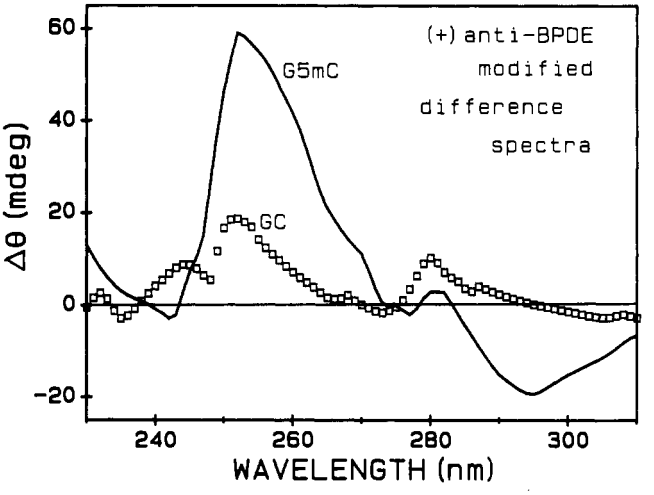

FIGURE 3: Difference CD in the DNA spectral region obtained by subtracting unmodified from that of (+)-*anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) (open square) and B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (solid line).

poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (Behe & Felsenfeld, 1981), and covalent lesions with some carcinogens are known to facilitate this transformation [see the review by Rich et al. (1984)]. To test this possibility, actinomycin D was added to the (+)-*anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) and B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) solutions ([Act D] = 1.9 μM). The CD intensity changes at 295 nm were then compared. Since actinomycin D will convert Z DNA back to the B form (Mirau & Kearns, 1983), CD

changes will be greater for modified poly(dG-m^{5}dC)·poly(dG-m^{5}dC) if Z conformers exist in the solution. No visible difference is observed, however, suggesting that the negative CD band around 295 nm in the modified poly(dG-m^{5}dC)·poly(dG-m^{5}dC) is due only to the pyrenyl Cotton effects rather than partial conversion to the Z form.

Covalent Modification by (−)-anti-BPDE. (A) Spectral Characteristics. Experiments similar to those of (+)-*anti*-BPDE were carried out with (−)-*anti*-BPDE, and the absorbance results are summarized in Table I along with those of the (+) enantiomer. It is interesting to note that the extent of binding of the (−) variety to the Z form of each polymer is roughly equal to or slightly more than that for the (+) enantiomer. In contrast, the B form of these two polymers exhibits much less reactivity toward (−)-*anti*-BPDE than toward the (+) enantiomer; the methylated polymer is now less modified. It is also worth noting that the 3- and 7-nm red shifts exhibited by the (+) enantiomer are no longer apparent. The higher modifications by the (−) isomer for the Z DNA as compared to the corresponding B DNA are unexpected and noteworthy since higher ionic strength is usually accompanied by reduced reactivity (Michaud et al., 1983; Geacintov et al., 1984b).

The CD spectra in the long-wavelength region for the (−)-*anti*-BPDE-modified polynucleotide solutions are about 20-fold smaller than that of (+)-*anti*-BPDE-modified poly(dG-m^{5}dC)·poly(dG-m^{5}dC). Although the reduction in the CD signals is expected from the decreased modification by the (−) enantiomer, such dramatic reductions are surprising. This may be partially due to the heterogeneous adduct formation (less specific binding) as suggested by the appearance of bisignate CD with broad positive bands above 350 nm and negative impressions at 343 nm except for the (−)-*anti*-BPDE-modified poly(dG-m^{5}dC)·poly(dG-m^{5}dC), where no negative impression is apparent [only the latter is included in Figure 1B for comparison with adducts derived from (+)-*anti*-BPDE]. Binding studies of natural DNA with enantiomers of *anti*-BPDE have recently been carried out with linear dichroism to indicate that the (−) isomer exhibits a higher degree of heterogeneity (greater intercalative contribution) than its (+) counterpart (Geacintov et al., 1984a). Our CD results are thus consistent with this observation.

The possibility that the 343-nm negative band may partly be due to binding of BPDE to the phosphate buffer was tested by reacting 25 μM (+)- or (−)-*anti*-BPDE with 10 mM sodium phosphate buffer. About 2 μM *anti*-BPDE is retained in the buffer solution after exhaustive ether extraction, with the (−) enantiomer showing slightly higher modification. Less than 0.5 μM retention is achieved in a control solution without sodium phosphate. The (−)-*anti*-BPDE-modified solution exhibits weak negative CD bands around 343 and 325 nm while the (+) isomer shows little detectable Cotton effects. Although the presence of DNA in the solution will surely compete for the available *anti*-BPDE, the appearance of the 343-nm negative CD impressions in the (−)-*anti*-BPDE-modified DNA solutions is quite suggestive of significant buffer phosphate modification. The low stereoselective binding of buffer phosphate is also consistent with the discrepancy found between the absorption and CD intensities as noted earlier.

(B) Differential Effects of Enantiomeric anti-BPDE on Reverting Z DNA to B Form. The CD in the DNA spectral region for the Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) solution containing 50 μM HAC is not appreciably changed by either the (+)- or (−)-*anti*-BPDE modification and, thus, is included in Figure 2B for comparison with its B form. The CD spectrum of the Z-form poly(dG-dC)·poly(dG-dC), however, is affected by the *anti*-BPDE modification in rather interesting ways. The addition of (−)-*anti*-BPDE to the Z-form poly(dG-dC)·poly(dG-dC) solution results in a sizable decrease in the magnitude of the 290-nm negative CD while the (+) enantiomer exhibits a much smaller effect. The differential effect of the enantiomers on the Z to B transition is more dramatically illustrated by CD measurements after ether extractions, which show a near spectral inversion from that of Z DNA for the (−) but not for the (+) enantiomer modification (Figure 4). These spectra, however, overestimate the efficiency of Z to B transitions upon *anti*-BPDE covalent binding since ether extraction decreases the HAC concentration in the solution, as indicated by a slight 290-nm negative CD amplitude decrease for the unmodified Z-DNA solution after ether extractions. Comparison of the CD spectra before ether extraction (not shown) suggests about 20% conversion to the B form upon 25 μM (−)-*anti*-BPDE addition to Z-form poly(dG-dC)·poly(dG-dC). This estimation coupled with a 2.7 μM modification in a 0.1 mM Z-form poly(dG-dC)·poly(dG-dC) solution (see Table I) suggests that at least about four base pairs can be converted back to the B form by binding covalently to one (−)-*anti*-BPDE molecule. This rough estimate probably is too low since significant modification to the buffer phosphates occurs (thus overestimating the extent of base modification) as mentioned earlier. It is interesting to note that Walker et al. (G. T. Walker, M. P. Store, and T. R. Krugh, private communication) have estimated that about 25 base pairs are being converted from Z to B for one noncovalently bound ethidium bromide or actinomycin D in poly(dG-dC)·poly(dG-dC) solutions containing 40 μM HAC.

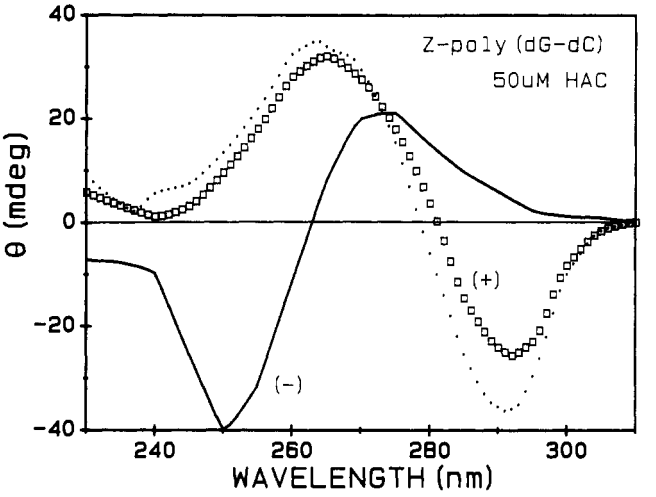

FIGURE 4: Effect of covalent lesion on the CD spectra of 0.1 mM Z-form poly(dG-dC)·poly(dG-dC): unmodified and without ether extraction (dotted curve), (+)-*anti*-BPDE modified and ether extracted (open square), and (−)-*anti*-BPDE modified and ether extracted (solid line). The spectrum for the unmodified Z DNA after ether extraction is nearly identical with the open square curve.

The differential effects of enantiomeric *anti*-BPDE are quite surprising since the extent of modification is roughly equal as judged by the absorbance measurements (Table I). The absence of similar effect for the (−) enantiomer binding on Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) most likely is due to the stabilization of the Z conformation in the presence of high HAC concentration (50 μM), which is about 10 times the concentration of the B–Z transition midpoint for this polymer (Behe & Felsenfeld, 1981). Indeed, Z-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) induced by 10 μM HAC can partially be reverted to B form by binding to (−)-*anti*-BPDE (results not shown). A smaller effect due to the (+)-*anti*-BPDE

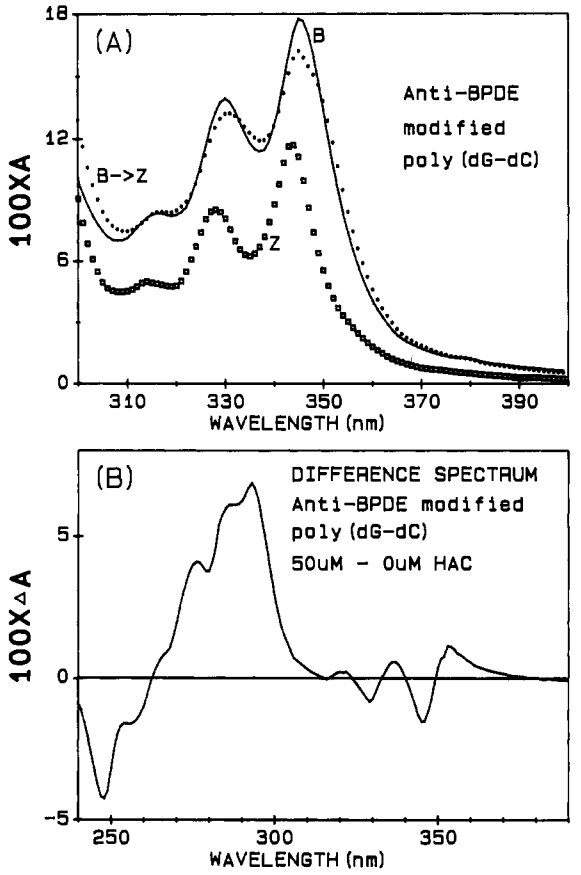

FIGURE 5: (A) Absorbance in the pyrene spectral region for 0.1 mM poly(dG-dC)·poly(dG-dC) solutions covalently modified under B (solid) and Z (open square) conditions by the addition of 50 μM racemic *anti*-BPDE. The noncovalently bound tetraols were removed by exhaustive ether extraction. The B condition refers to a solution in 10 mM sodium phosphate buffer of pH 7.0 containing 1 mM EDTA/0.01 M NaCl, and Z signifies the formation of Z DNA induced by the presence of 50 μM hexaamminecobalt (HAC). The dotted line represents *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) under Z condition (50 μM HAC added afterward). (B) Difference spectrum, including both pyrene and DNA spectral regions, obtained by subtracting spectrum of *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) from that of post 50 μM HAC addition.

modification as compared to its (−) counterpart has also been observed.

Covalent Binding of Racemic anti-BPDE to Poly(dG-dC)·Poly(dG-dC). Although spectral difference exhibited by the *anti*-BPDE- (racemic) modified B and Z DNAs has previously been noted in conjunction with our polynucleotide studies (Chen, 1985), a more detailed presentation on polymer solutions with higher covalent modification is described here in order to facilitate comparison with the enantiomeric results.

The absorption spectra for the racemic *anti*-BPDE- (50 μM added) modified B and Z forms of poly(dG-dC)·poly(dG-dC) in the pyrene and DNA spectral regions are presented in Figure 5A. In agreement with the stereoselective covalent binding toward the B DNA and nonstereoselective lesion toward the Z form with enantiomers exhibiting identical spectra, the spectral characteristics for adducts derived from racemic *anti*-BPDE are very similar to the ones presented for (+)-*anti*-BPDE in Figure 1A. The extent of modification (5.6 and 3.8% for the B and Z, respectively) is, however, somewhat less than the combined (+)- and (−) enantiomeric contributions (Table I). This is consistent with the notion that, for the most part, the isomers are competing for the same reacting sites.

In order to investigate the possible difference in adducts formed by B vs. Z DNA, 50 μM HAC was added to the *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) solution, and the resulting spectrum is included in Figure 5A for comparison. It is seen that the 50 μM HAC addition results in the lowering of the 346-nm intensity with a concomitant amplitude increase in the long-wavelength wing. The effect due to the HAC addition is most readily seen by subtracting spectra of pre- from the post-HAC addition to the modified B-form poly(dG-dC)·poly(dG-dC) solution, and the resulting difference spectrum is shown in Figure 5B. Positive maxima at 353, 337, and 322 nm and negative extrema at 346, 328, and 316 nm are apparent in the pyrene spectral region. The locations of the negative maxima (indicating absorbance reduction upon HAC addition) correspond closely to the absorption maxima of the modified B form at low salt. The observed sizable red shifts (~7 nm) of the positive maxima from those of negative is consistent with the notion that the covalently bound BPDE moieties become intercalated at high salt. B to Z conformational transition of poly(dG-dC)·poly(dG-dC) is evidenced by the large positive difference band at 293 nm, indicating absorbance enhancement around this wavelength. This increase in the A_{295}/A_{260} ratio (characteristic of B to Z transition) (Pohl & Jovin, 1972) due to the HAC addition is, however, only half as large in the *anti*-BPDE modified as that in the unmodified poly(dG-dC)·poly(dG-dC) solution. This is consistent with the enantiomeric results indicating that B DNA greatly prefers (+)-*anti*-BPDE and its modification stabilizes the B conformation.

The corresponding CD spectra for these solutions are shown in parts A and B of Figure 6 for the pyrenyl and DNA spectral regions, respectively. As is apparent, sizable pyrenyl Cotton effects are observed for the *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) despite the fact that the reacting ligands are racemic. In contrast to a mere 50% increase in the extent of modification, roughly 6 times larger CD intensities are observed for B-form poly(dG-dC)·poly(dG-dC) as compared to the Z form, suggesting that the binding of *anti*-BPDE to the B form is much more stereoselective than the corresponding Z conformation in conformity with our enantiomeric results. These observations are also consistent with the previously observed stereospecific covalent binding of *anti*-BPDE to natural DNA (Meehan & Straub, 1979; Osborne et al., 1981).

Although the CD extrema for the *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) correspond reasonably well to the absorbance maxima, the prominent appearance of the 352-nm CD shoulder, which has previously been noted in the enantiomeric study, is of particular interest as it is located at the region where a mere broadening is observed in the absorption spectrum. The 9-nm red shift from that of tetraol absorbance is consistent with the fact that this shoulder results from an intercalative binding as opposed to the majority of adducts exhibiting only 3-nm red shifts, which most likely are residing at the external sites (Geacintov et al., 1978; Lefkowitz & Brenner, 1982). The CD results are thus in agreement with those of linear dichroic studies, suggesting some heterogeneity in *anti*-BPDE binding to DNA (Undeman et al., 1983; Geacintov et al., 1984a).

CD spectral comparison among the *anti*-BPDE-modified Z- and B-form poly(dG-dC)·poly(dG-dC) solutions in the absence and presence of 50 μM HAC is most revealing. In agreement with alteration in the absorption spectra (Figure 5), the addition of 50 μM HAC to the modified B-form poly(dG-dC)·poly(dG-dC) solution results in pyrenyl CD now showing maxima at 350 and 332 nm (correspond roughly to the positive maxima of the difference spectrum in Figure 5B)

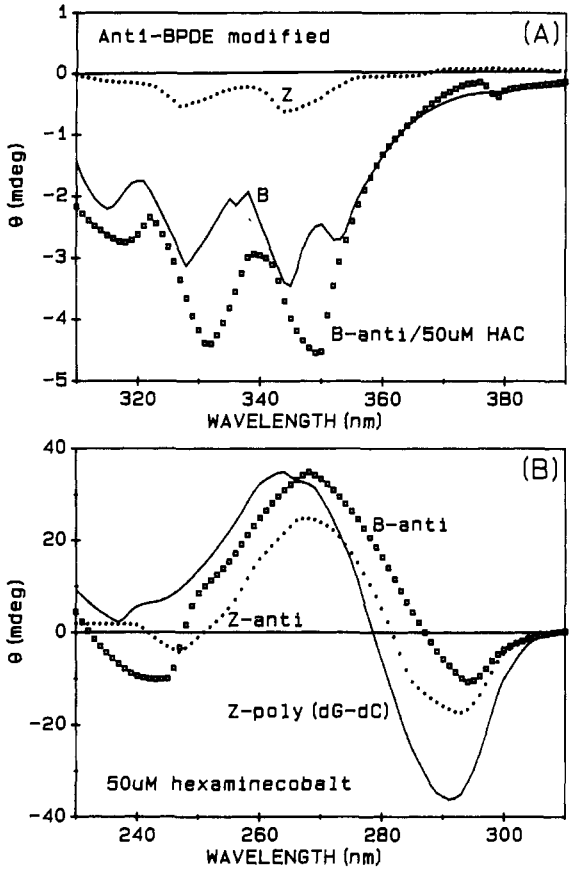

FIGURE 6: (A) CD spectra for *anti*-BPDE covalently bound to 0.1 mM poly(dG-dC)·poly(dG-dC) of B form (solid line) and Z form (dotted line) and 50 μM HAC added to modified B (open square). Covalent modification was accomplished by the presence of 50 μM racemic *anti*-BPDE. (B) CD in the DNA spectral region for unmodified Z-form poly(dG-dC)·poly(dG-dC) without ether extraction (solid line), *anti*-BPDE modified Z-form poly(dG-dC)·poly(dG-dC) after ether extraction (dotted line), and *anti*-BPDE modified under B condition (and ether extracted) and then changed to Z condition (open square).

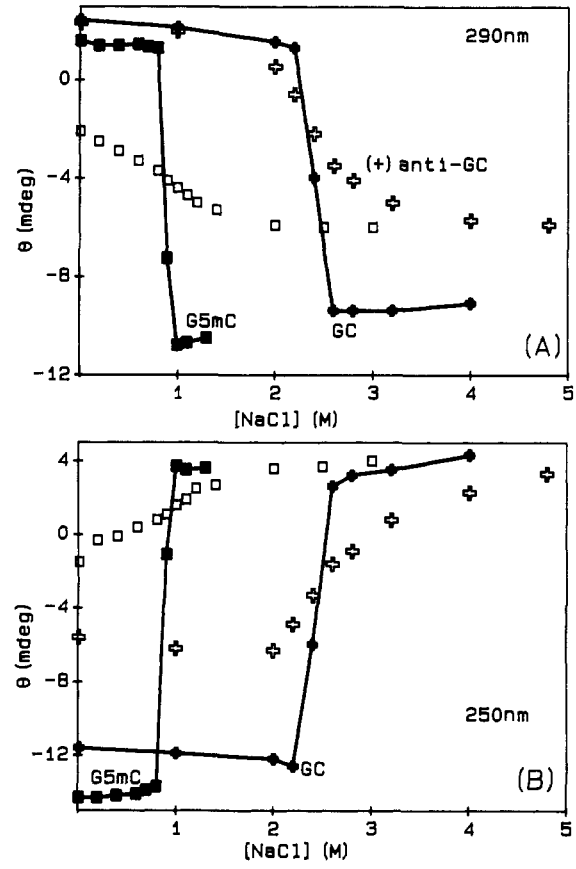

FIGURE 7: Effect of (+)-*anti*-BPDE covalent modification on the B–Z salt-titration curves with dichroic monitoring. Polynucleotide concentrations of 20 μM are used in the titration. Unmodified poly(dG-dC)·poly(dG-dC) (solid cross) at 290 (A) and 250 nm (B), (+)-*anti*-BPDE-modifed poly(dG-dC)·poly(dG-dC) (open cross) at 290 (A) and 250 nm (B), unmodified poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (solid square) at 293 (A) and 252 nm (B), and (+)-*anti*-BPDE-modified poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (open square) at 293 (A) and 255 nm (B). Lines are drawn through data points for the unmodified polymers to facilitate visualization.

instead of 345 and 327 nm. Such spectral shifts were also observed in NaCl titration (results not shown), suggesting that most of the dominant "external" pyrene moieties may have now become more intercalated as postulated earlier. The conversion of this modified polynucleotide to the Z form is apparently not complete as judged by the smallness of the 295-nm negative CD band, diagnostic of the Z conformation, and the presence of a negative 245-250 nm maxima, characteristic of the B-form poly(dG-dC)·poly(dG-dC) (Figure 6B). The possibility that this reduction in negative CD intensity at 295 nm is the result of a partial cancellation due to positive pyrenyl Cotton effects can be ruled out by the fact that the CD of dominant adduct does not exhibit positive intensity in this region (see Figure 3). These CD results, coupled with the smaller change in A_{295}/A_{260} mentioned earlier, strongly suggest that the covalent binding of *anti*-BPDE to DNA resists the B to Z conversion. This interpretation is consistent with our earlier speculation, based on the absorbance results, of intercalation enhancement of these bound moieties at higher ionic strength and findings by other to indicate that intercalation stabilizes the B conformation [see the review by Rich et al. (1984)]. The prominence of the 344-nm negative CD maximum (instead of 350 nm) in the modified Z-DNA solution containing 50 μM HAC may thus suggest differing base adducts environments as compared to B-form modification and/or significant binding to the phosphate groups of buffer and DNA backbones (Gamper et al., 1977). Characterization of these adducts must, however, await detailed HPLC analysis of the enzymatically hydrolyzed products.

Although the CD intensity for the *anti*-BPDE-modified Z-form poly(dG-dC)·poly(dG-dC) is not large in the pyrene spectral region, significant difference with the unmodified polymer can be seen in the DNA spectral region as is apparent from Figure 6B. Reduction of the negative CD amplitude at 290 nm with a concomitant appearance of a small negative band at 250 nm suggests partial reversion to the B form. The fact that the efficiency of the racemic mixture for such an effect lies between those of enantiomers (Figure 4) appears to be consistent with their bindings being competitive.

Effect of anti-BPDE Covalent Binding on the B–Z Interconversions. (A) Salt Titration. In order to further investigate the effect of *anti*-BPDE covalent binding on the B–Z interconversions, comparative salt titrations were carried out with (+)-*anti*-BPDE-modified and unmodified B-form poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) solutions. Changes in ellipticities were monitored around 290 and 250 nm yielding very similar results, as can be seen in Figure 7. It is noted that the B to Z transitions for the unmodified polynucleotides are quite cooperative and appear around 2.4 and 0.9 M NaCl for poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC), respectively, in general agreement with earlier observations (Pohl & Jovin, 1972; Behe & Felsenfeld, 1981). The (+)-*anti*-BPDE-modified polymers, however, exhibit much less cooperativity and much smaller

ellipticity changes during salt titration. Such reduction in ellipticity change is more pronounced with poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (70% compared to 40% reduction in unmethylated polymer), in conformity with the more extensive covalent modifications in this polymer (6.7 vs. 4.5% modification). These results taken together suggest that the (+)-*anti*-BPDE modification in the B form resists the conversion (protecting above five base pairs per each covalent attachment) to the Z conformation in a thermodynamic sense as mentioned earlier and indicate that only regions relatively distant from the modified base pair are liable for the B to Z conversion.

(*B*) *Kinetics of B–Z Interconversions.* To investigate the effect of *anti*-BPDE modification on the kinetics of B–Z interconversions, comparative rate studies were carried out with unmodified and *anti*-BPDE-modified poly(dG-dC)·poly(dG-dC). The B to Z conversion was induced by an abrupt increase in HAC concentration from 0 or 10 μM to 50 μM, and the Z to B transformation was accomplished either by HAC dilution from 50 to 10 μM or by actinomycin D addition.

The logarithm of ellipticity change vs. time plots at 290 nm on the B to Z transitions induced by the addition of 50 μM HAC for the unmodified and 5.6% *anti*-BPDE (racemic) modified 0.1 mM poly(dG-dC)·poly(dG-dC) solutions (results not shown) indicate that although the curve for the unmodified polymer cannot be approximated by a single exponential, its deviation from such is not too great. The effect of the *anti*-BPDE covalent binding, however, is to accentuate such a deviation, especially the fast component facilitation. The reduction of total ellipticity change at 290 nm due to covalent lesion is about 50% as judged by the zero time intercepts (in general accord with the results of salt titration), in contrast to a mere 5.6% modification.

Experiments on the B to Z transformation kinetics were also carried out with 20 μM poly(dG-dC)·poly(dG-dC) solutions with 0 and 5.6% *anti*-BPDE modifications by changing the HAC concentration from 10 to 50 μM with the results shown in Figure 8A. Results on 20 μM poly(dG-m^{5}dC)·poly(dG-m^{5}dC) solutions with 0 and 6.7% (+)-*anti*-BPDE modifications by changing the HAC concentration from 0 to 50 μM HAC are also included in Figure 8A. The B to Z transition of the methylated polymer is decidedly not single exponential and its much faster initial rate compared to its non-methylated counterpart most likely is the consequence of the much lower HAC concentration requirement for such transformation. The kinetic facilitation of the fast component and the concomitant reduction in the 290-nm ellipticity change with the increased modification are again quite evident for poly(dG-dC)·poly(dG-dC). Although the greatly reduced dichroic change is apparent upon (+)-*anti*-BPDE modification for the B to Z transition of poly(dG-m^{5}dC)·poly(dG-m^{5}dC) in 50 μM HAC solutions, the decay rate for the fast component does not appear to be greatly affected. This may be the consequence of the presence of 10 times higher HAC concentration than is needed to reach the B–Z transition midpoint so that any effects due to *anti*-BPDE modifications are not felt. The ability for the 5-methylated polymer to undergo the B to Z transition at a lower HAC concentration is illustrated by including in the same figure the transition kinetics induced by 10 μM HAC. It is interesting to note that although a significant slowing down of the fast component is observed, the slow component appears not to be affected despite the lower HAC concentration.

The Z to B transition kinetics were investigated by either HAC concentration reduction (Figure 8B) or by actinomycin D addition (1.9 μM) (not shown). The covalent modification

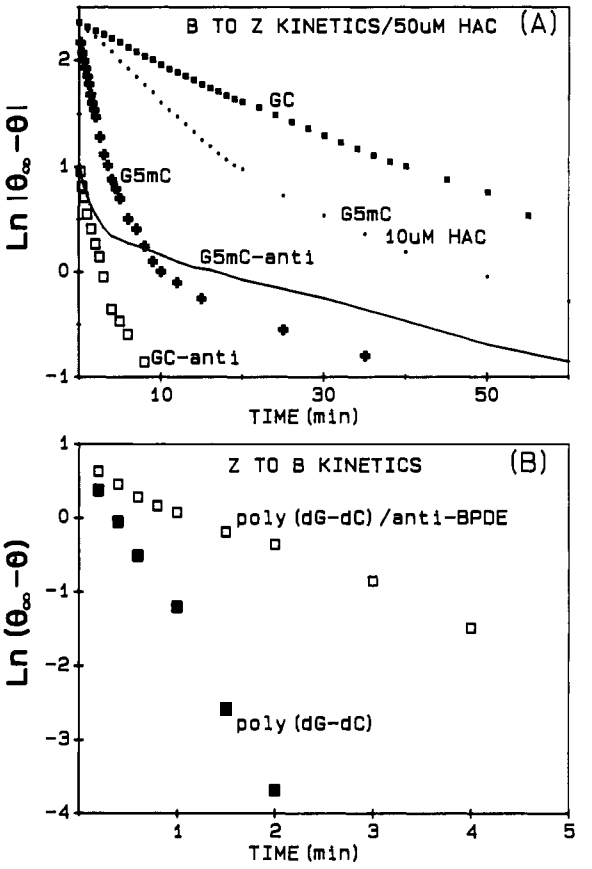

FIGURE 8: Effect of *anti*-BPDE modification on the kinetics of B–Z transitions. All kinetic measurements on B–Z interconversions in this article are made by 290-nm dichroic monitoring. (A) B to Z transition of 20 μM poly(dG-dC)·poly(dG-dC) solution induced by changing the HAC concentration from 10 to 50 μM: unmodified (solid square) and 5.6% *anti*-BPDE (racemic) modified (open square). B to Z transition of 20 μM poly(dG-m^{5}dC)·poly(dG-m^{5}dC): unmodified polymer as induced by 50 (solid cross) and 10 μM (dotted line) HAC and B to Z transition induced by 50 μM HAC for 6.7% (+)-*anti*-BPDE-modified B form (solid line). (B) Effect of *anti*-BPDE (racemic) covalent binding on the kinetics of Z to B transition of 20 μM poly(dG-dC)·poly(dG-dC) solutions. Transition induced by sudden HAC dilution from 50 to 10 μM accomplished through addition of 0.36 mL of 0.1 mM DNA solution containing 50 μM HAC to 1.44 mL of buffer. Unmodified Z DNA (solid square); 5.6% *anti*-BPDE-modified B-form poly(dG-dC)·poly(dG-dC) under Z condition (50 μM HAC added) (open square).

is to slow down the Z to B conversion initiated by HAC dilution, but the effect on the actinomycin D induced Z to B transition appears to be insignificant except for the reduction in ellipticity change as a consequence of incomplete Z formation. Covalent modification on the Z-form poly(dG-dC)·poly(dG-dC) appears to have minimal effect on the actinomycin D induced Z to B transition kinetics.

It is to be noted in passing that the addition of minute amounts of SDS (as low as 0.02%) is very effective in inducing a Z to B transition, presumably the result of cobalt-complex sequestering by the micelles. The formation of a somewhat cloudy solution, however, is a definite disadvantage of this procedure, and no systematic studies have been carried out.

Discussion

Some interesting findings derived from this study are (1) the covalent binding of *anti*-BPDE to B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) is not only more extensive but also more stereoselective [greater preference for the (+) enantiomer] than to B-form poly(dG-dC)·poly(dG-dC), (2) chemical lesions of *anti*-BPDE to the Z-form DNAs (induced by 50 μM HAC) are less extensive and appear to be neither

stereoselective nor methylation dependent, (3) the covalent adducts of (+)-*anti*-BPDE exhibit dominant external binding characteristics in B-form poly(dG-dC)·poly(dG-dC) but appear to be intercalative in B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) as judged by the relative spectral red shifts, (4) salt titration indicates that the "external" pyrenyl moieties attached to B-form poly(dG-dC)·poly(dG-dC) become "intercalated" at high-salt condition, (5) the covalent binding of (+)-*anti*-BPDE appears to protect the bases in the immediate vicinity from transforming into the Z form at high salt on the one hand but kinetically facilitates the distal base pairs in such transition on the other, (6) the general features of the difference CD spectra resulting from the (+)-*anti*-BPDE covalent binding on the B form of unmethylated and methylated poly(dG-dC)·poly(dG-dC) are consistent with the dominant adduct being the trans addition of N2 dG at C10 of (+)-*anti*-BPDE, and covalent binding of (−)-*anti*-BPDE to the Z DNA appears to be more effective in inducing partial Z to B transition than its (+) counterpart.

The greater extent of (+)-*anti*-BPDE modification and stereoselectivity for poly(dG-m^{5}dC)·poly(dG-m^{5}dC), coupled with the inhibition of B to Z transition, may have important implication. As is well-known in eukaryotes, methylation at the 5-position of cytosine in d(CG) sequences in vivo is associated with gene inactivation (Doerfler, 1983). Methylation at the 5-position of cytosine in d(CG) sequences has also been shown to facilitate the B to Z transformation (Behe & Felsenfeld, 1981). If gene inactivation is indeed associated with conversion to the Z conformation, the inhibition of Z formation by (+)-*anti*-BPDE binding may thus be one of the mechanisms for cancerous action. This, however, is pure speculation and further works are clearly needed.

Inhibition of salt-induced B to Z transition due to covalent binding similar to the one observed here has previously been observed for aflatoxin B1 (Nordheim et al., 1983), but the basis for such an effect has been attributed to its bulkiness and possible interaction with components of the B-DNA duplex in the major groove. As the two moieties (large number of potential hydrogen bonding groups in aflatoxin but not in BPDE) and the points of covalent attachment (N7 dG vs. N2 dG) are distinctly different, their mechanisms for B-DNA stabilization are not expected to be the same. Intercalative stabilization of the B form most likely is the basis for the BPDE action in this respect since spectral evidence indicates that the (+)-*anti*-BPDE covalently bound to poly(dG-m^{5}dC)·poly(dG-m^{5}dC) appears to be intercalated and the "externally" bound moieties in poly(dG-dC)·poly(dG-dC) become intercalated under high-salt condition.

There has been controversy on whether the *anti*-BPDE covalently bound to native DNA is intercalated between the bases or residing at the external site (Drinkwater et al., 1978; Geacintov et al., 1978; Camper et al., 1980; Hogan et al., 1981). Spectroscopic evidence strongly suggests that the pyrene moiety resides at the external site (Geacintov et al., 1980; Lefkowitz & Brenner, 1982), most likely in the minor groove. As there is also evidence to indicate that physical binding is intercalative and precedes the covalent attachment (Geacintov et al., 1981; Meehan et al., 1982), it is rather puzzling that the covalent adducts should reside at the external sites. One possible mechanism is that covalent binding does indeed occur at the intercalative site and subsequently the pyrene moiety slides out of the intercalative cage due to some reasons. Such a mechanism appears to be energetically rather unlikely at first sight. Our finding that those covalently attached pyrene moieties residing at the external sites under low ionic strength can slide into the intercalative sites at higher salt condition, possibly to avoid the cation clustering at the phosphate backbones, seems to suggest that the energy barrier between the intercalative and external sites is probably not as high as one assumes. Although our results do not prove the correctness of the notion that covalent binding occurs initially at the intercalative sites (Meehan & Bonds, 1984; Geacintov et al., 1984b), they do make this mechanism appear to be rather plausible. It is also interesting to note that the higher extent of modification and stereoselectivity as well as the spectral evidence of intercalated (+)-*anti*-BPDE in poly(dG-m^{5}dC)·poly(dG-m^{5}dC) are also consistent with this model. Although the basis for the predominant intercalative binding in this polymer is not clear, it may have its origin in the greater flexibility of this duplex as evidenced by the lower barrier for the B to Z transition (Behe & Felsenfeld, 1981).

The difference CD spectra obtained by subtracting B-form poly(dG-dC)·poly(dG-dC) and B-form poly(dG-m^{5}dC)·poly(dG-m^{5}dC) from their (+)-*anti*-BPDE-modified counterparts are qualitatively similar (Figure 3) and are consistent with major adducts being formed by trans addition of N2 dG at the C10 of (+)-*anti*-BPDE (Moore et al., 1977; Jeffrey et al., 1977). The adduct characterization of *anti*-BPDE-modified Z DNAs by CD spectra is, however, complicated by the reduced extent of modification and possible binding to the residual B form. Detailed characterization of hydrolysis products by HPLC and other techniques will be of interest.

Although the increased ionic strength due to the presence of 50 μM HAC may be partly responsible for the decreased modification of the Z DNAs, the other reason most likely is due to the fact that in Z conformation the 2-amino group of guanine now resides in the deep groove (Wang et al., 1979) and may become less accessible for ligand attack. These adducts appear to be nonintercalative and much less stereoselective as judged from CD intensities and locations of the spectral maxima. These characteristics, however, are also consistent with significant buffer phosphate modification and may suggest that the base modification of Z DNA is much less than is apparent from this study.

The differential effect exhibited by the enantiomers of *anti*-BPDE on the Z to B transition is rather interesting. Although the basis for such a differential effect is not understood, it most likely is related to the possible difference in stereogeometry of the covalent adducts. In this connection, it is interesting to note that recent covalent binding studies of these two enantiomers with natural DNA have indicated that although N2 dG is the dominant adduct due to the (+)-*anti*-BPDE modification, significant O6-dG (31%) and N7-dG (18%) products are derived from reaction with the (−) isomer (Osborne et al., 1981). The N7-dG derivative, however, is unstable and undergoes either spontaneous release of the substituted guanine or imidazole ring opening. It is thus tempting to implicate the O6-dG binding for such interesting phenomenon. Further studies, however, are needed to clarify this point.

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Registry No. (+)-*anti*-BPDE, 63323-31-9; (−)-*anti*-BPDE, 63323-30-8; poly(dG-dC), 36786-90-0; poly(dG-m^{5}dC), 51853-63-5.

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