Articles

A-Tract and (+)-CC-1065-Induced Bending of DNA. Comparison of Structural Features Using Non-denaturing Gel Analysis, Hydroxyl-Radical Footprinting, and High-Field NMR†

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ABSTRACT: (+)-CC-1065 is a biologically potent DNA-reactive antitumor antibiotic produced by *Streptomyces zelensis*. In a previous study we have reported that (+)-CC-1065 produces bending of DNA that has similarities to that intrinsically associated with A-tracts [Lin, C. H., Sun, D., & Hurley, L. H. (1991) *Chem. Res. Toxicol. 4*, 21–26]. In this article we provide evidence using a combination of non-denaturing gel analysis, hydroxyl-radical footprinting, and high-field NMR for both distinctions between the two types of bends and the importance of junctions in both types of bends. For A-tracts we demonstrate that the locus of bending is at the center of an A-tract and that upon modification of the 3′ adenine with (+)-CC-1065 this locus is moved less than 1 base pair to the 3′ side, and the bending magnitude is significantly increased. For drug bonding sequences such as 5′-AGTTA* or 5′-GATTA* (where * denotes the drug bonding site), the locus of bending is found to be between the two thymines, and the bending is focused over a 2-base-pair sequence rather than a 5-base-pair sequence, as is the case for the A-tract. An important distinction between an A-tract intrinsic bend and a (+)-CC-1065-induced bend is the effect of temperature. While, as shown previously, the magnitude of A-tract bending increases with decrease in temperature, for drug-induced bending of 5′-AGTTA* the bending magnitude increases with increased temperature. Hydroxyl-radical footprinting of the drug-modified 5′-AGTTA* sequence shows a decrease in cleavage centered around the TT sequence, which is presumably associated with a decrease in minor groove width. In a parallel study, the non-self-complementary 12-mer duplex (5′-GGCGGAGTTA*GG-3′)·(5′-CCTAACTC-CGCC-3′) (Figure 2B) and the corresponding (+)-CC-1065-modified duplex adduct were examined thoroughly by one- and two-dimensional ^{1}H NMR and NOESY restrained molecular mechanics and dynamics calculations. Both the 12-mer duplex and the (+)-CC-1065-12-mer duplex adduct maintain an overall B-form DNA with the anti base orientation throughout in aqueous solution at room temperature. The 18C nucleotide of both the 12-mer duplex and its drug-modified adduct has an average C3′-endo sugar pucker. The 12-mer duplex exhibits a unique internal motion at the 16A nucleotide, which is located to the 3′ side of the complementary partner of the covalently modified adenine, and a major kink at the 18C–19T step. Following covalent bonding with (+)-CC-1065, the discontinuity around 18C is entrapped and further exaggerated. In addition, the 12-mer duplex adduct displays a compression of the minor groove at the 8T to 9T step and widening on both sides, but especially abruptly at the covalent modification site. Structurally, the 12-mer duplex adduct bears many similarities to a bent DNA structure, which is intrinsically associated with A-tracts. The major drug-induced distortion on DNA is localized at the 9T and 10A step of the covalently modified strand. A truncated junction model for the drug-entrapped/induced bending of DNA is proposed, and a comparison to intrinsic A-tract bending is made.

(+)-CC-1065 (Figure 1) is an extremely potent antitumor antibiotic produced by *Streptomyces zelensis* (Hanka et al., 1978). (+)-CC-1065 reacts with double-stranded DNA through N3 of a reactive adenine, forming a covalent adduct that overlaps a 5-bp[1] region in the minor groove (Swenson et al., 1982; Hurley et al., 1984; Scahill et al., 1990). (+)-CC-1065 consists of a CPI subunit (subunit A in Figure 1) and two repeating pyrroloindole subunits (subunits B and C in Figure 1) attached via amide linkages that are approximately 15° out of plane, providing the drug molecule with a right-handed twisted banana shape (Chidester et al., 1981). The CPI subunit contains the DNA-reactive cyclopropane ring that alkylates N3 of adenine when it binds within certain

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[1] Abbreviations: A-tract, adenine tract; bp, base pair; CPI, cyclopropylpyrroloindole; DDW, double-distilled water; DQF-COSY, phase-sensitive double quantum filtered correlated spectroscopy; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE correlated spectroscopy; ppm, parts per million; Pu, purine; Py, pyrimidine; ss, single strand; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; TSP, 3-(trimethylsilyl)propionic acid; ROE, rotating frame NOE.

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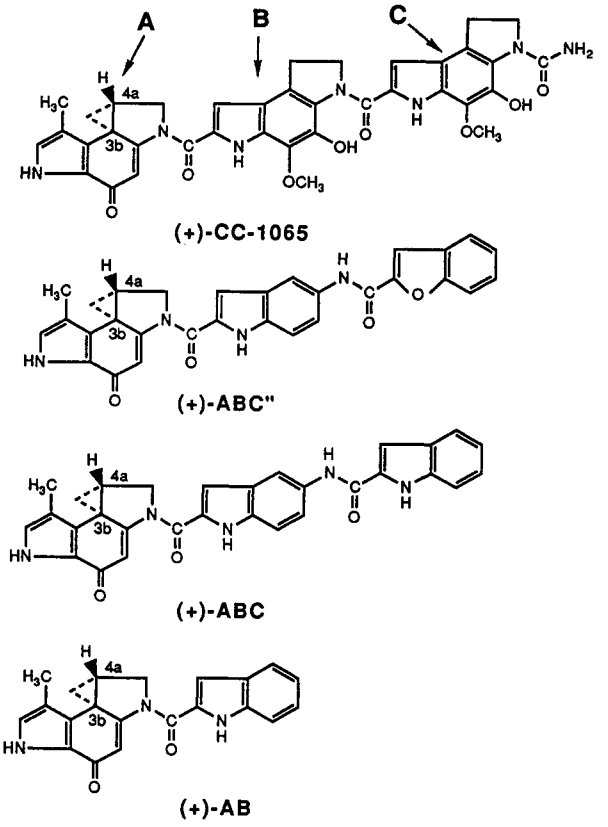

FIGURE 1: Structures of (+)-CC-1065, (+)-ABC", (+)-ABC, and (+)-AB. Arrows point to subunits A, B, and C in (+)-CC-1065.

reactive sequences (Reynolds et al., 1985).

The covalent linkage sites between (+)-CC-1065 and DNA have been determined (Hurley et al., 1984; Scahill et al., 1990), and the predominant tautomeric species of the covalently modified adenine is the doubly protonated 6-amino form with the additional positive charge delocalized over the entire adenine molecule (Lin & Hurley, 1990). A consensus sequence analysis of the (+)-CC-1065 bonding sites on DNA reveals that there are two subsets of DNA sequences, 5′-PuNTTA* [2] and 5′-AAAAA*, that are highly specific (Reynolds et al., 1985). Surprisingly, the alkylating subunit alone contains sufficient structural information to encode the primary molecular basis for sequence selectivity (Hurley et al., 1988), and this subunit is also essential for antitumor activity (Warpehoski et al., 1988; Warpehoski & Hurley, 1988). However, as we have previously demonstrated, the noncovalent binding interactions of the B and C subunits with DNA can modulate or fine-tune this sequence selectivity (Hurley et al., 1988) and, in the case of (+)-CC-1065, produce winding of DNA (Lee et al., 1991).

The covalent bonding reaction of (+)-CC-1065 with DNA has been proposed to involve catalytic activation of the covalent reaction between (+)-CC-1065 and DNA and to be at least partially responsible for the molecular basis for sequence-selective recognition of DNA by the alkylating subunit of (+)-CC-1065 (Warpehoski & Hurley, 1988; Lin et al., 1991a). In addition to catalytic activation, we have also proposed that DNA conformational flexibility is an important component of sequence recognition in the (+)-CC-1065 bonding reaction (Hurley et al., 1988).

As a possible means of defining the molecular basis of the potent cytotoxicity shown by (+)-CC-1065 and its analogues, the structural changes occurring in DNA molecules as a result of covalent adduct formation with drug molecules have been extensively studied (Lin et al., 1991b; Lee et al., 1991; Sun & Hurley, 1992a,b). Using a combination of gel electrophoresis and high-field NMR, we have previously shown that, following the covalent reaction with (+)-CC-1065 and the synthetic analogues shown in Figure 1, the DNA helix becomes bent, with the locus of bending being between the two thymines within the bonding sequence 5′-AGTTA* (Lin et al., 1991b). The magnitude of this bend is about 17–22°, and the direction is in toward the minor groove of DNA; structurally it bears many similarities to the bent DNA structure that is intrinsically associated with A-tracts (Lin et al., 1991b; Lee et al., 1991). The purpose of this study was twofold. First, because of the biological significance of A-tract bending [reviewed in Travers (1990)], it was important to make a more rigorous structural and dynamic comparison of the drug-induced bend to an intrinsic A-tract bend. Second, an understanding of the unique reactivity of 3′ adenines in A-tracts to (+)-CC-1065 might provide additional insight into both the structure of A-tracts and the molecular basis for the sequence selectivity of (+)-CC-1065.

Non-denaturing polyacrylamide gel analysis was used to monitor the mobility shifts of T4-ligated oligomers containing A_5-tracts and the (+)-CC-1065 reactive bonding sequence 5′-AGTTA* with and without drug modification. Hydroxyl-radical footprinting was used to estimate variation in minor groove width. The results of these studies were combined with the results of one- and two-dimensional ^{1}H NMR experiments and NOESY constrained molecular mechanics and dynamics calculations to define the solution structure of a 12-mer duplex containing the highly reactive (+)-CC-1065 sequence 5′-AGTTA* and its drug-modified adduct. Overall, we conclude that the 12-mer duplex adduct bears many similarities to the bent DNA structure intrinsically associated with A-tracts. A junction bend model is proposed for the (+)-CC-1065-entrapped/induced bend in DNA, which is a truncated version of that proposed for A-tract bending (Koo et al., 1986).

MATERIALS AND METHODS

Chemicals and Enzymes. (+)-CC-1065 and its analogues used in this study were obtained from the Upjohn Company and used without further purification. Electrophoretic reagents [acrylamide, TEMED, ammonium persulfate, and bis-(acrylamide)] were purchased from Bio-Rad. T4 polynucleotide kinase and T4 DNA ligase were from United States Biochemical Co. [γ-^{32}P]ATP was from ICN. X-ray film, intensifying screens, and developing chemicals were from Kodak. Reagents used to prepare NMR buffer and to prepare and purify the 12-mer duplex and the corresponding (+)-CC-1065–12-mer duplex adduct have been reported previously (Lin & Hurley, 1990; Lin et al., 1991a,b).

Preparation of Oligonucleotides. The oligonucleotides (Figure 2) were synthesized on an automatic DNA synthesizer (Applied Biosystems 381A) by the phosphoramidite method (Gait, 1984). The deprotection was performed as described previously (Lee et al., 1991).

DNA Bending Experiments. Approximately 5 μg of each oligonucleotide of the complementary sequences was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and hybridized together, and the resulting duplex was gel-purified by 12% non-denaturing polyacrylamide gel electrophoresis as described previously (Lee et al., 1991). Duplexed oligomers were modified with (+)-CC-1065, (+)-ABC", or (+)-ABC (see

[2] Here and throughout the manuscript, an asterisk denotes the covalently modified adenine.

A

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21-C        5'GAGGACCGGAAAAACGGATTC3'
               3'CTGGCCTTTTTGCCTAAGCTC5'

AT-1        5'AAAAAGGGCCCTTTTTCCGGG3'
               3'TTCCCGGGAAAAAGGCCCTTT5'

AT-2        5'AAAAAGGCCCTTTTTCCCGGG3'
               3'TTCCGGGAAAAAGGGCCCTTT5'

AT-3        5'AAAAAGGGCCCTTTTTCCGG3'
               3'TTCCCCGGGAAAAAGGCCTTT5'

SP-A1       5'AAAAAGCGGAGTTAGGGGCGG3'
               3'TTCGCCTCAATCCCCGCCTTT5'

SP-A2       5'AAAAAGGCGGAGTTAGGGGCG3'
               3'TTCCGCCTCAATCCCCGCTTT5'

SP-A3       5'AAAAAGGGCGGAGTTAGGGGC3'
               3'TTCCCGCCTCAATCCCCGTTT5'

21-SP       5'TGGGCGGAGTTAGGGGCGGGA3'
               3'CGCCTCAATCCCCGCCCTACC5'

41MER       5'TCCCGCCCCTAACTCCGCCCACAGTGTTCGATTACAGGCAT3'
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B

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   1 2 3 4 5 6 7 8 9 10 11 12
5'GGCGGAGTTAGG3'
3'CCGCCTCAATCC5'
  24 23 22 21 20 19 18 17 16 15 14 13
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FIGURE 2: Sequences of oligomers used in this study (A) and the 12-mer duplex (B).

Figure 1) (final concentration: 28 μM) at room temperature for 3 days. Unbound drug molecules were removed by phenol/chloroform extraction, and drug-modified duplexes were further purified by ethanol precipitation. Drug-modified and unmodified duplexes were self-ligated in 20 μL of ligation buffer with 1 unit of T4 DNA ligase at room temperature overnight to produce multimers, which were electrophoresed on 8% polyacrylamide gel, and the bands were located by autoradiography.

Hydroxyl-Radical Footprinting. A partial duplex of DNA containing a unique drug bonding sequence within the duplex region was prepared by annealing the 41-mer and the 21-SP-mer (top strand) shown in Figure 2. This partial duplex was modified with either (+)-CC-1065, (+)-AB, or (+)-ABC″, as described above, and the site-directed drug-modified ss 21-mer was separated by denaturing polyacrylamide gel electrophoresis, as described previously (Sun & Hurley, 1992b). Purified drug-modified ss DNA was annealed to the 5′-end-labeled 21-SP(−)-mer and radio-labeled with [γ-^{32}P]-ATP, and the resulting duplex was purified, as described above. Hydroxyl-radical cleavage of the drug-modified and unmodified oligomers was carried out at room temperature for 5 min with the same reagents as previously published (Burkoff & Tullius, 1988) except for changes in the concentration of hydrogen peroxide (Lin et al., 1991b).

Strand Breakage Assay and DNA Sequencing. Drug-modified duplexes were subjected to thermal treatment for 30 min at 95 °C in DDW to locate the drug bonding site and determine the extent of reaction at the desired sequence on the oligmer duplex. Heat-treated samples were dried, redissolved in formamide (80%)-NaOH (10 mM) dye solution, and then subjected to denaturing 20% polyacrylamide gel electrophoresis run in parallel with the DNA sequencing reaction. Purine- and pyrimidine-specific sequencing reactions were carried out according to the methods of Maxam and Gilbert (1980).

Preparation and Purification of the 12-mer Duplex and the (+)-CC-1065–12-mer Duplex Adduct. The non-self-complementary 12-mer duplex (Figure 2) for NMR studies was synthesized in-house on a 10-μmol scale, as described above. The general procedures for synthesis, deprotection, drug bonding, HPLC, and chromatography purification of the 12-mer duplex and the (+)-CC-1065–12-mer duplex adduct have been reported previously (Lin & Hurley, 1990; Lin et al., 1991a,b).

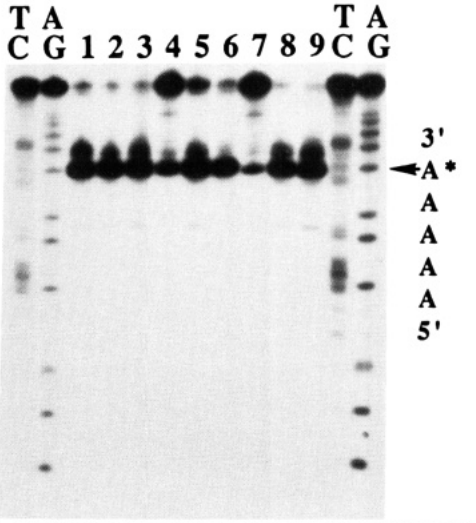

FIGURE 3: Autoradiogram of the thermally induced strand breakage assay on oligomer 21-C after covalent modification with (+)-CC-1065, (+)-ABC, and (+)-ABC″. Outside lanes are AG- and TC-specific reactions. Lanes 1–3, 4–6, and 7–9 contain oligomers modified with (+)-CC-1065, (+)-ABC, and (+)-ABC″, respectively. Drug concentrations used in this experiment were 2.8 μM in lanes 1, 4, and 7; 28 μM in lanes 2, 5, and 8; and 280 μM in lanes 3, 6, and 9. The appearance of more than one band following thermal cleavage is due to incomplete chemical degradation of the deoxyribose at the 3′ end of the fragment (Reynolds et al., 1985).

High-Field NMR Spectroscopy. One- and two-dimensional 500-MHz ^{1}H NMR data sets in 90% H_2O/10% D_2O or 99.96% D_2O buffered solution containing 10 mM sodium phosphate and 100 mM sodium chloride at pH 6.85 were recorded on a General Electric GN-500FT NMR spectrometer. Proton chemical shifts were recorded in parts per million (ppm) and referenced relative to external TSP (1 mg/mL) in D_2O (HOD signal was set to 4.751 ppm). Two-dimensional NMR data sets were recorded according to the procedures described previously (Lin et al., 1992).

RESULTS

Unique Reactivity of the 3′ Adenine in A-Tracts to (+)-CC-1065, (+)-ABC″, and (+)-ABC. (+)-CC-1065 reacts preferentially at two concensus sequences, 5′-PuNTTA* and 5′-AAAAA* (Reynolds et al., 1985). Significantly, in A-tracts the almost exclusive bonding site for (+)-CC-1065 is the 3′ adenine, which suggested to us and others (Koo et al., 1986) that the 3′ adenine of an A-tract has unique structural features that make it especially reactive to (+)-CC-1065. It is well known that the 3′ terminal adenine junction in A-tracts has a unique structure (Koo et al., 1986; Koo & Crothers, 1988; Nadeau & Crothers, 1989; Katahira et al., 1990). Confirmation of this unique reactivity of the 3′ adenine in an A_5-tract toward (+)-CC-1065, (+)-ABC″, and (+)-ABC is shown in Figure 3. Overall, this result supports the junction model (Koo et al., 1986; Koo & Crothers, 1988) that emphasizes the presence of a unique structure at the 3′ junction of the A-tract. In addition, this implies a high level of sequence selectivity of (+)-CC-1065 and its analogues in order for them to be able to discriminate between the subtle differences in reactivity of the different adenines in an A_5-tract.

Determination of the Locus of Bending in an A_5-Tract and in (+)-ABC-Modified 21-mers Containing the Drug Bonding

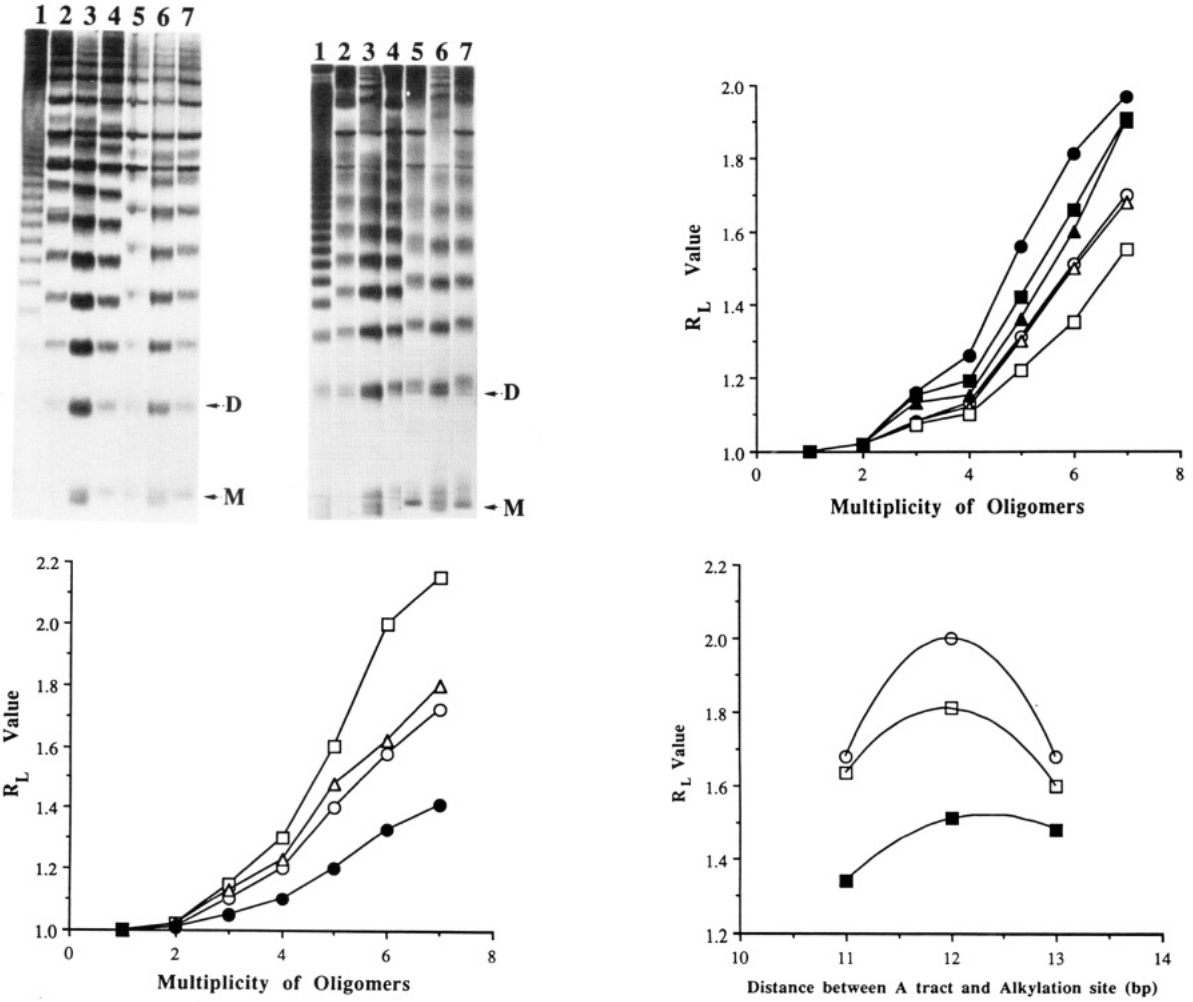

FIGURE 4: Panel A (top left): (I, left) Autoradiogram of the ligation products of oligomers AT-1, AT-2 and AT-3 and (+)-ABC-modified oligomers AT-1, AT-2, and AT-3. Lane 1 is control containing ligation products of oligomer 21-SP as a size marker, and lanes 2–4 contain oligomers AT-1, AT-2, and AT-3, respectively. (II, right) Lanes 5–7 contain ligation products of oligomers AT-1, AT-2, and AT-3 modified with (+)-ABC respectively. (II) Lanes 1–7 are the same ligated products as lanes 1–7 in panel (I), respectively, but electrophoresis was carried out in the presence of Mg^{2+} (10 mM). M and D correspond to monomer and dimer. Panel B (top right): Plot of R_L values vs total length of ligated multimers in multiplicity of oligomers from the experiment shown in panel A. The plots are shown for ligation products of oligomer AT-1 (○), oligomer AT-1 modified with (+)-ABC (●), oligomer AT-2 (△), oligomer AT-2 modified with (+)-ABC (▲), oligomer AT-3 (□), and oligomer AT-3 modified with (+)-ABC (■). Panel C (bottom left): Plot of R_L values of the ligated products of (+)-ABC-modified oligomers SP-A1 (○), SP-A2 (□), and SP-A3 (△) and unmodified oligomer SP-A1 (●). Panel D (bottom right): Effect of distances in bp between the center of the A_5-tract and the (+)-ABC covalent modification site on R_L values of the six multimers of unmodified AT1, AT2, and AT3 (■); of (+)-ABC-modified AT1, AT2, and AT3 (□); and of (+)-ABC-modified SP-A1, SP-A2, and SP-A3 (○).

Sequences 5′-AAAAA and 5′-AGTTA**. The helical in-phase bending of A-tracts contained in multimers produced by ligation of oligomers each containing A-tracts results in overall curvature of the resulting molecule, and if sufficient oligomers are ligated, circular DNA is formed. Overall curvature of the multimer results in retardation of the electrophoretic gel mobility of the DNA molecules, which is maximal when the bends are in helical phase (i.e., in B-form DNA spaced every 10.3 bp). In subsequent sections we describe experiments in which we determine the locus or center of bending of drug-induced bending. The locus of bending of A-tracts is presumed to be at the center of the run of A's, i.e., at A_3 in an A_5 tract. To verify this assumption, three different 21-mers (AT-1, AT-2, and AT-3 in Figure 2) were designed. The center of the T-tract is positioned 11, 10, and 12 bp from the center of the A-tract in AT-1, AT-2, and AT-3, respectively. By *inverting* the T- and A-tracts we test whether the center of bending occurs at the central A or T. In contrast, if the A-tracts were on the same strand, then whether the locus of bending is at the center or at either end of the A-tract would produce the same result. If the local bending center of the A_5-tract is precisely at the center of the five adenines, or in other words, if the A_5-tract and the T_5-tract are identical in both the direction and the center of bending, then we expect that the ligated multimers of both oligomers AT-1 and AT-2 should show nearly identical electrophoretic mobilities with more retardation compared to that of oligomer AT-3. However, the ligated multimer of oligomer AT-2 *or* AT-3 should show the greatest retardation in mobility if the bending locus of the A-tract is biased toward the 5′ or 3′ side of the A-tract, respectively.

Non-denaturing gel analysis of the T4-ligated 21-bp oligomers (AT-1, AT-2, and AT-3) was used to evaluate the relative phasing of the A_5-tracts in these three oligomers; the gel results are shown in Figure 4A, and the calculated R_L values of the multimers are plotted in Figure 4B (open symbols). R_L values for AT-1 and AT-2 are essentially identical, confirming that the center of bending corresponds to the adenine in the middle of the A_5-tract, while the R_L value for AT-3 is decreased relative to those of AT-1 and AT-2, indicating that the bends corresponding to these A_5-tracts are relatively out of phase. When the T_5-tract in the

middle of the duplex was replaced with an A_5-tract, R_L values of the multimers were nearly identical between AT-1 and AT-2 (Sun & Hurley, unpublished results). To determine the effect of drug modification of the 3′ adenine in an A_5-tract on both the magnitude and the locus of bending, AT-1, AT-2, and AT-3 were modified at the 3′ adenine with (+)-ABC. (+)-ABC was used rather than (+)-CC-1065 since the latter compound also produces winding of DNA (Lee et al., 1991), the equivalent of about 1 bp in addition to bending, which complicates the analysis of the results. The gel results in Figure 4A-I and the R_L values plotted in Figure 4B (filled symbols) show that in all three cases the R_L values are increased upon (+)-ABC bonding, relative to the unmodified A_5-tracts, but the R_L value of drug-modified AT-1 is *increased* more than that of AT-2, and that of AT-3 is intermediate between those of AT-1 and AT-2. Taken together, these results reveal that after drug modification the magnitude of bending is significantly increased, and the locus of bending of the A_5-tract is moved about 0.5 bp to the 3′ side of the central adenine in the drug-modified A_5-tract. When non-denaturing gel electrophoresis was carried out in the presence of Mg^{2+} (10 mM), the overall bending magnitude of both A-tract and drug-induced DNA bends was only slightly decreased, but the bending locus was not changed (see Figure 4A-II).

To determine the locus of bending induced by (+)-ABC in the 5′-AGTTA* bonding sequence, similar ligation experiments using the 21-mers SP-A1, SP-A2, and SP-A3 shown in Figure 2 were carried out. The results plotted in Figure 4C show that SP-A2, in which the bonding site is 12 bp removed from the center of the A-tract, produced the maximum increase in R_L. Thus, the locus of bending is between the two A·T base pairs to the 5′ side of the covalently modified adenine. A summary of the comparison of R_L values of the six multimers for the A_5-tract and for the (+)-ABC-modified A_5-tract and 5′-AGTTA* is shown in Figure 4D. Significantly, the curve that links together the (+)-ABC-modified 5′-AGTTA* and A_5-tract (open symbols) is steeper than that associated with the unmodified A_5-tract (filled symbols), suggesting that drug-induced bending is more focused than the A-tract intrinsic bending (see later).

Comparison of the Temperature Dependency of the Mobility Shifts of Ligated 21-mers Containing an A_5-Tract, the Drug-Modified A_5-Tract, and the 5′-AGTTA Bonding Sequence.* It has already been well established that the gel mobility is retarded, and the corresponding magnitude of intrinsic bending of A_5 tracts is increased, at lower temperatures (Koo et al., 1986; Koo & Crothers, 1988; Abagyan et al., 1990), presumably because lower temperature favors the conformation associated with the bent DNA structure. This expectation was confirmed for the A_5-tract contained within oligomer 21-C, as shown in Figure 5. At 7 °C the bending magnitude is increased for oligomer 21-C relative to that at 20 °C [compare open (7 °C) and filled (20 °C) symbols]. After drug modification of the 3′ adenine in the A_5-tract, the temperature dependency of bending is greatly decreased, and only in multimers of 7 and 8 oligomers does the lower temperature produce an increased R_L value (Figure 5). In sharp contrast to these results, *increasing* the temperature of the ligated 21-mer containing the drug-modified sequence 5′-AGTTA* produced an *increase* in bending magnitude (Figure 5). This is presumably because at the higher temperatures the DNA becomes more flexible, allowing the drug molecule that is conformationally restrained by DNA to now bend the DNA even more. In addition, these results support the notion that these drug molecules do not nullify

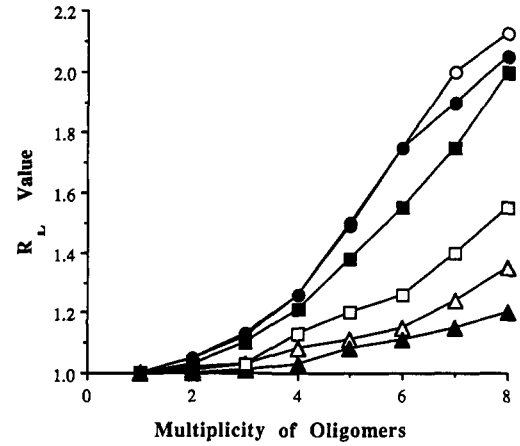

FIGURE 5: Temperature dependency of electrophoretic mobility of ligated multimers of oligomers of an A_5-tract (21-C), of (+)-ABC-modified oligomer 21-C, and of (+)-ABC-modified oligomer 21-SP. Open and filled symbols represent the experiment at 7 and 20 °C, respectively. R_L plots are of the ligated multimers of 21-C (△, ▲), (+)-ABC-modified 21-C (○, ●), and (+)-ABC-modified oligomer 21-SP (□, ■).

the preexisting bent structure of A-tract DNA upon drug modification of the 3′-side adenine of the A-tract DNA, but take advantage of the intrinsic bent structure to achieve a drug-entrapped bent DNA structure.

Hydroxyl-Radical Footprinting of the Noncovalently Modified Strand of a 21-mer with Either an Unmodified or a Drug-Modified 5′-AGTTA Sequence.* A_5-tracts show a pattern of inhibition of hydroxyl-radical cleavage within the bending region (Burkhoff & Tullius, 1987, 1988). The locus of bending occurs at the sites of greatest inhibition, which presumably correspond to the region of maximum narrowing of the minor groove. In comparison to the unmodified 21-SP duplex (panel A in Figure 6), the drug-modified 21-mers (panels B, C, and D, corresponding to (+)-CC-1065, (+)-ABC″, and (+)-AB, respectively) all show an increased inhibition of cleavage to the 3′ side of the covalently modified adenine. In general, inhibition occurs maximally 1–3 bp to the 3′ side of this covalently modified base, which is in good agreement with the locus of bending determined by non-denaturing gel analysis (see before).

High-Field NMR Studies on the 12-mer Duplex and Its (+)-CC-1065-Modified Adduct. As an adjunct to our macroscopic structural data on the (+)-CC-1065-induced bending of DNA obtained by non-denaturing gel analysis and hydroxyl-radical footprinting (see before), high-field NMR studies on a 12-mer duplex adduct containing the same 5′-AGTTA* bonding sequence were carried out. This data, in comparison to high-field NMR studies on A-tracts (Katahira et al., 1990), provides us with more definitive information with which to propose a model for the (+)-CC-1065-entrapped/induced bending of DNA and make a comparison with A-tract bending.

The non-self-complementary 12-mer duplex sequence (Figure 2B) is part of the early promoter region of SV40 DNA and is contained within the 21-bp repeat region. This 21-bp repeat region contains the Sp1 protein binding sites (Kadonaga et al., 1986; Courey et al., 1990) as well as two identical highly reactive (+)-CC-1065 bonding sequences, 5′-AGTTA* (Reynolds et al., 1985). This 12-mer duplex has been thoroughly characterized by one- and two-dimensional ^1H and ^{31}P NMR, hydroxyl-radical footprinting, and molecular dynamics calculations and exists as an overall right-handed B-form DNA duplex (Lin et al., 1992).

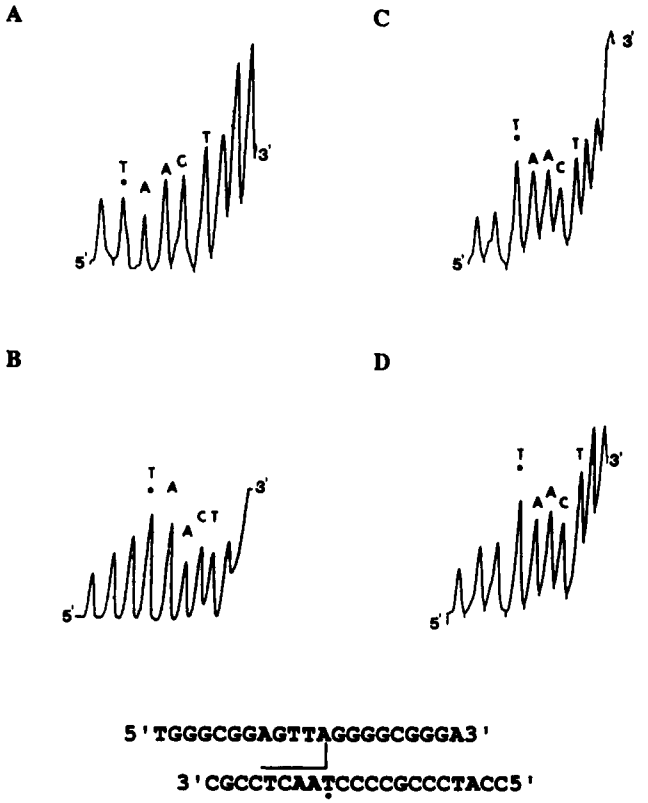

FIGURE 6: Comparison of the laser densitometer tracings of the hydroxyl-radical footprinting of the noncovalently modified strand of oligomer 21-SP without modification (A) and with drug modification by (+)-CC-1065 (B), (+)-ABC″ (C), and (+)-AB (D).

Bonding of (+)-CC-1065 to the 12-mer Duplex Entraps the Transient Kink at the 17A–18C and 18C–19T Steps and Freezes the Internal Motion at 16A. Although the 12-mer duplex exists as an overall right-handed B-form DNA duplex, it also has a number of unique features that are unusual for B-form DNA. In particular, the 12-mer duplex has transient kinks around 18C, internal motion at 16A, and a C3′-endo-like geometry for 18C and is propeller twisted within the 5′-TAA sequence (Lin et al., 1992). A comparison of the two-dimensional NOESY (120 ms) expanded contour plot of the 12-mer duplex and its (+)-CC-1065–12-mer duplex adduct demonstrating the two NOE connectivities for the aromatic protons to the H1′ sugar proton for nucleotide 18C is plotted in Figure 7A,B. For the (+)-CC-1065–12-mer duplex adduct, the internucleotide NOE connectivity for 18CH6 to the H1′ proton of its 5′ neighbor, 17A (cross peak B′ in Figure 7B), is *much stronger* than the intranucleotide NOE connectivity for 18CH6 to its own H1′ proton (cross peak A′ in Figure 7B). When the intensities of these cross peaks are compared with the equivalent NOE intensities between 18CH6 and 18CH1′ and between 18CH6 and 17AH1′ for the 12-mer duplex (cross peaks A vs A′ and B vs B′ in Figure 7A,B), adduct formation appears to not only entrap but also exaggerate the C3′-endo sugar geometry for the 18C nucleotide in the duplex. In addition, the scalar couplings between the H1′ and the H2′ and H2″ sugar protons of nucleotide 18C in the (+)-CC-1065–12-mer duplex adduct are of the same weak intensity (weak couplings are denoted with an arrowhead in Figure 8B) and are even weaker than those for the unmodified 12-mer duplex (compare panels A and B of Figure 8). Furthermore, the propeller twist angle for the (+)-CC-1065–12-mer duplex adduct is also exaggerated by the presence of (+)-CC-1065, as shown by comparison of the relative NOE intensities between 16AH2–10AH2 and 16AH2–17AH2 in the 12-mer duplex and the corresponding NOEs in the (+)-CC-1065–12-mer duplex adduct (S and T in the supplementary material). In summary, after the covalent reaction with (+)-CC-1065, the sugar geometry for the 18C nucleotide of the 12-mer duplex sequence behaves even more C3′-endo-like, and the A·T-rich region is more propeller twisted than in the unmodified 12-mer duplex.

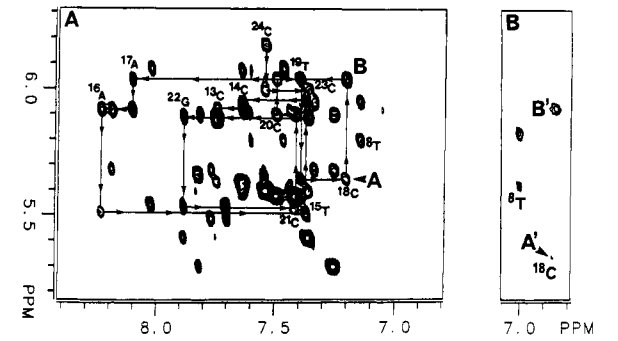

FIGURE 7: Two-dimensional phase-sensitive NOESY (250 ms) expanded contour plots of (A) the 12-mer duplex and (B) the (+)-CC-1065–12-mer duplex adduct in D_2O buffered solution, pH 6.85, as 23 °C, demonstrating NOE connectivities for the PuH8/PyH6 (6.8–8.4 ppm) protons to their own sugar H1′ protons (4.8–6.3 ppm) (denoted with nucleotide units and numbers corresponding to the positions in the 12-mer duplex sequence shown in Figure 2B) and to the H1′ protons of their 5′ neighbors. Only the noncovalently modified strand in both the 12-mer duplex and the (+)-CC-1065–12-mer duplex adduct is shown. Cross peaks A to B′ are assigned as follows: A&A′, 18C(H6)–18C(H1′); B&B′, 18C(H6)–17A(H1′).

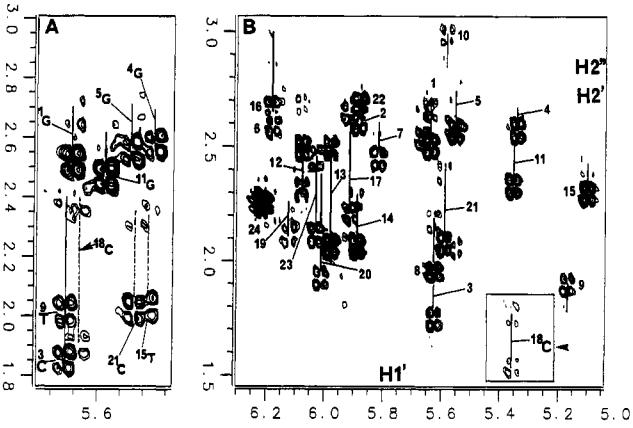

FIGURE 8: Two-dimensional expanded contour plots of the phase-sensitive DQF-COSY of (A) the 12-mer duplex and (B) the (+)-CC-1065–12-mer duplex adduct in D_2O buffer at 23 °C, establishing the scalar couplings for the H1′ to the H2′ sugar protons and the H1′ to the H2″ sugar protons. Weak H1′–H2′ and H1′–H2″ scalar couplings of nucleotide 18C are denoted with arrowheads.

Bonding of (+)-CC-1065 to the 12-mer Duplex Induces a Discontinuity at the 9T–10A Step and Produces an Overall Narrowing of the Minor Groove of DNA in the 8T/17A,9T/16A Region. (+)-CC-1065 creates a new discontinuity in the 12-mer duplex that occurs at the 9T–10A step on the DNA duplex when it bonds to the 12-mer duplex sequence. On the covalently modified strand, the aromatic to H1′ internucleotide NOE connectivity for the 9T–10A step is dramatically reduced, and the aromatic to H2″ internucleotide ROE connectivity for the same step, which is present in the unmodified 12-mer duplex (Lin et al., 1992), is now lost in the adduct (C. H. Lin and L. H. Hurley, unpublished results). In addition, the relative chemical shifts of the H2′ and H2″ protons for both the 9T and 10A nucleotides are reversed with respect to the 12-mer duplex (Figure 8B). These results indicate that the DNA backbone conformation at this step is highly perturbed upon covalent modification by (+)-CC-1065.

DISCUSSION

Previous work from this laboratory has shown that covalent modification of DNA by (+)-CC-1065 results in bending of DNA into the minor groove by 17–22° (Lee et al., 1991). On the basis of non-denaturing gel electrophoresis, hydroxyl-radical footprinting, and the relative intensity of NOE cross peaks for interresidue protons across the minor groove, we have proposed that this bending has structural features that resemble the intrinsic bending associated with A-tracts (Lin et al., 1991b). In this investigation, we have examined in more detail the structural and dynamic features of an A_5-tract modified with (+)-CC-1065 and a highly reactive (+)-CC-1065 bonding sequence (5′-AGTTA*) and its covalent adduct.

The special reactivity of the 3′ adenine in A-tracts to (+)-CC-1065 led us first to consider the possibility that (+)-CC-1065 and related compounds might entrap or induce bending of DNA. We were particularly intrigued by the junction bend model (Koo et al., 1986) and the idea that (+)-CC-1065 might be recognizing a junction site rather than just an adenine at the 3′ end of the sequence lacking a G·C base pair. In this paper we have addressed three questions: (1) What are the structural consequences of modifying an A_5-tract with (+)-CC-1065? (2) What are the similarities and differences between a drug-induced bend at a highly reactive bonding sequence such as 5′-AGTTA* and an A-tract? (3) Can we develop a model for the (+)-CC-1065-induced bend that permits us to gain a greater understanding of the structural features of this drug–DNA adduct and further insight into how (+)-CC-1065 achieves its sequence selectivity?

Structural Consequences of Modifying an A_5-Tract with (+)-CC-1065. Upon modification of an A_5-tract by (+)-CC-1065, the center of bending is shifted from the central adenine to between this adenine and the next one to the 3′ side. The bending magnitude of the A_5-tract modified with (+)-CC-1065 is approximately equal to the summation of its intrinsic bending and that due to (+)-CC-1065 bending of a sequence such as 5′-AGTTA*.[3] The increased magnitude of bending of an A-tract by drug–DNA adduct formation can most plausibly be understood by considering the junction bend model for the structure of DNA containing an A-tract. According to the junction bend model, DNA curvature arises from a change in the direction of the helical axis at or near the junctions of these tracts with flanking base pairs. Therefore, drug–DNA adduct formation at the 3′ junction of the A-tract might be expected to increase the magnitude of discontinuity at the 3′ junction and, consequently, the overall magnitude of bending. The special reactivity of the 3′ adenine to (+)-CC-1065, the consequent shift in the locus of bending of an A_5-tract toward the 3′ side after drug modification, and the additive effect on the magnitude of bending are all in accord with the junction bend model (Koo et al., 1986). Furthermore, the loss of temperature dependency of A_5-tract bending after drug modification is proposed to be due to the presence of a conformationally strained drug molecule within the minor groove of the A_5-tract, which also shifts the equilibrium between the bent and nonbent conformations to favor the bent DNA structure. Presumably, the junction site at the 3′ end of the A_5-tract is stabilized more than the one at the 5′ end, and the contribution of the 3′ junction of the A-tract to overall DNA bending becomes greater after drug modification.

Similarities and Differences between the Intrinsic Bend of an A-Tract and a (+)-CC-1065-Induced Bend in a 5′-AGTTA Bonding Sequence.* Overall, the general features of a bend associated with an A_5-tract and a drug-induced bend in the bonding sequence 5′-AGTTA* are similar, i.e., magnitude, direction of bending, and associated narrowing of the minor groove. However, a more in-depth analysis also reveals some important differences. First, while the intrinsic bend of an A_5-tract appears to be spread over the entire A-tract region, in the drug bonding sequence 5′-AGTTA* it is concentrated over just two base pairs, i.e., 5′-(AA)·(TT) in this sequence (see Figure 4D). Second, it is the torque of the drug molecule that overrides the DNA conformational preference to assume a nonbent DNA structure, a fact clearly revealed by the temperature dependency studies.

Previously, it was reported that the mobilities of A-tract-containing oligomers depend crucially upon whether the A-tracts are arranged in the same orientation or with one or more tracts inverted with respect to the others, as in the case of electrophoresis in the absence of EDTA or in the presence of magnesium ion (Diekmann, 1987; Travers, 1989; Hagerman, 1990). The ligated products of AT-1, AT-2, and AT-3, either unmodified or modified with (+)-ABC, were electrophoresed in the presence of magnesium ion (10 mM) without EDTA, in order to test the effect of magnesium ion on electrophoretic mobility. Somewhat surprisingly, magnesium ion did not have a significant effect on the mobility of ligated multimers of oligomers AT-1, AT-2, and AT-3 or those modified with (+)-ABC (Figure 4A-II), although ligated multimers of AT-1 showed slightly more retardation in electrophoretic mobility than those of AT-2 in the presence of magnesium. Since the relative order of the bending magnitude of each oligomer, and each of the (+)-ABC-modified oligomers, was unchanged in the presence of magnesium ion, this also implies that the bending locus remained the same. The results reported here are in accord with the idea that the direction and magnitude of bending is largely independent of the orientation of the (dA)·(dT) tract (Koo et al., 1986; Koo & Crothers, 1988; Wu & Crothers, 1984). Moreover, upon drug modification of A-tract-containing oligomers, the magnesium ion dependence was insignificant, indicating that the drug-induced DNA bending was not sensitive to the presence of magnesium.

A Truncated Junction Bend Model for the Entrapped/Induced Bending of DNA in the (+)-CC-1065–12-mer Duplex Adduct and as a Mechanism for Sequence Recognition of DNA. When a 21-mer containing the 12-mer duplex used in the NMR studies is ligated and subjected to non-denaturing gel electrophoresis, it does not show anomalous gel migration and therefore is unlikely to be bent (D. Sun and L. H. Hurley, unpublished results). However, hydroxyl-radical footprinting of the same 21-mer sequence shows inhibition of cleavage at 16A and 18C (see Figure 6). In addition, the proton NMR data reported here and elsewhere (Lin et al., 1992) provides further evidence for propeller twisting and corresponding narrowing of the minor groove in the A·T region between 7G·18C and 10A·15T. An unusual feature of this region of the duplex is the rapid local conformational flexibility of 16A.

[3] We had previously noted (Lin et al., 1991) that, in contrast to the results shown here, modification of an A_2-tract by (+)-CC-1065 *did not* markedly increase the overall intrinsic bending of the A_5-tract. We believe that the difference between these two results is due to incomplete drug modification of DNA, in the case of these previous results. In subsequent experiments we have evaluated a series of oligomers containing A-tracts following modification by (+)-CC-1065, and in all cases, including the sequence previously evaluated, the intrinsic bending of the A-tract was significantly increased upon drug modification, as noted in the present paper.

This local conformational flexibility is temperature dependent but is lost upon adduct formation with (+)-CC-1065 (Lin et al., 1992). We propose that this inherent conformational flexibility of the 5′-AGTTA* sequence is a contributing factor to sequence recognition by (+)-CC-1065.

The NOESY restrained molecular mechanics and dynamics calculations on this 12-mer duplex indicate that the DNA sequence contains two major kinks, one located at the 17A to 18C step and the other located at the 18C to 19T step (Lin et al., 1992). In addition, the 18C nucleotide has an average C3′-endo sugar geometry and is displaced into the helix axis toward the major groove, resulting in an unwinding at the 5′-(AC)·(GT) step and a destacking at the 5′-(CT)·(AG) step. This kind of base displacement toward the major groove has previously been reported on the Amsacrine–d$(CGCG)_2$ complex (Graves & Wadkins, 1990). The kink around 18C brings the 18CH5 proton under the shielding cone of the aromatic ring of 17A, which explains the upfield shift of this proton in the 12-mer duplex (Lin et al., 1992).

Shortly after we characterized the unusual conformation around 18C, Bolshoy et al. (1991) published a paper in which they reported that DNA sequences containing 5′-(AC)·(GT) or 5′-(CA)·(TG) steps exhibit anomalous high gel mobility (low R_L values) compared to the predicted computed R_L values. On the basis of these results, it is suggested that a transient kink occurs at the 5′-(AC)·(GT) or the 5′-(CA)·(TG) step. In line with this idea, we propose that the unusual C3′-endo sugar geometry for 18C and the associated conformational and dynamic changes, including narrowing of the minor groove in the A·T region and rapid local conformational flexibility at 16A, are related to a transient kink at the 5′-(AC)·(GT) step in the 12-mer. It has also been suggested that in a right-handed B-form DNA a kink of about 40° is produced by the change of sugar pucker from C2′-endo to C3′-endo and unwinding of adjacent base pairs at the kink site by −10° (Saenger, 1983).

Upon reaction with (+)-CC-1065, the unusual *structural* features of the 12-mer duplex become entrapped and exaggerated. The A·T region of the (+)-CC-1065–12-mer duplex adduct is even more highly propeller twisted than in the 12-mer duplex, and the sugar geometry of the 18C nucleotide moves more toward the C3′-endo region (C. H. Lin and L. H. Hurley, unpublished results). In addition, the kink angle at the 18C to 19T step is dramatically increased when compared with that of the same kink in the unmodified 12-mer duplex (Lin et al., 1992). These kinks create a junction around the 18C nucleotide on the noncovalently modified strand. In addition to this entrapped junction, another junction between 9T and 10A on the covalently modified strand is also induced upon adduct formation. Overall, it appears that (+)-CC-1065 takes advantage of an existing propensity of this 12-mer to bend and, after covalent reaction at A10, consolidates and exaggerates this tendency. Our recent observations that (+)-CC-1065 can form an unusual covalent adduct with the guanine in the sequence 5′-AATTG* and a stable, noncovalent complex with the sequence 5′-GAATTC strongly support this hypothesis (H. J. Park and L. H. Hurley, unpublished observations; M. Hansen and L. H. Hurley, unpublished observations). We propose a "truncated junction bend model", based upon the junction bend model for an A-tract (Koo et al., 1986), for the (+)-CC-1065-entrapped/induced bend in the highly reactive sequence 5′-AGTTA* (see Figure 9). An intriguing observation is that upon binding of Sp1 molecules to either side of this 5′-AGTTA* sequence the DNase I and hydroxyl-radical footprinting data shows

FIGURE 9: Models for bending of DNA. (A) The junction bend model for A-tracts (Koo & Crothers, 1986) and (B) the truncated junction bend model for the (+)-CC-1065-entrapped/induced bend of 5′-AGTTA*.

changes in DNA structure, implicating the possible importance of this same transient kink in protein–DNA interactions (D. Sun and L. H. Hurley, unpublished results).

The possibility has recently been raised of the functional importance of bent DNA, because of the surprising ubiquity of inherently curved DNA segments in biological systems (Koudelka et al., 1988; McAllister & Achberger, 1989; Travers, 1990; Hagerman, 1990). In addition, it was recently reported that DNA–protein interactions can be attenuated by the presence of intrinsic sequence-dependent DNA curvature (Shatzky-Schwarz et al., 1992). It is interesting to speculate whether a (+)-CC-1065-entrapped or -induced bent conformation of DNA might mimic an intrinsic or protein-induced curved DNA found in the regulatory regions of a genome, such as the promoter regions, or in replication origins (i.e., serve as a surrogate protein). Alternatively, (+)-CC-1065 could attenuate a DNA–protein interaction at the region containing a highly reactive (+)-CC-1065 bonding site. Experiments to test these hypotheses are in progress in this laboratory.

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SUPPLEMENTARY MATERIAL AVAILABLE

Two-dimensional phase-sensitive NOESY expanded contour plots of the 12-mer duplex and the (+)-CC-1065–12-mer duplex adduct (3 pages). Ordering information is given on any current masthead page.

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