Strong Sequence-Dependent Polymorphism in Adduct-Induced DNA Structure: Analysis of Single *N*-2-Acetylaminofluorene Residues Bound within the *Nar*I Mutation Hot Spot†

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ABSTRACT: We have used a set of chemical probes to characterize and to compare the structural deformation of double-stranded oligomers bearing a single *N*-2-acetylaminofluorene (AAF) adduct covalently bound to each of the three guanine residues located within the frameshift mutation hot spot sequence -$G_1G_2CG_3CC$- (*Nar*I site). Two classes of chemical probes have been used, probes that sense the geometry of the helix, giving rise to cuts at every nucleotide (for example, 1,10-phenanthroline–copper), and probes that react with specific bases depending on their conformation (e.g., diethyl pyrocarbonate). For all probes that were tested, a distinct pattern of reactivity was observed according to the position of the adduct within the DNA sequence, revealing an important polymorphism in the adduct-induced DNA structure. With 1,10-phenanthroline–copper at least three base pairs 3′ of the AAF-modified guanine were reactive on each strand, showing that the deformation of the DNA helix extends over a region of 4–6 bases pairs centered around the adduct and sensed by the probe in both strands. With the base-specific probes, reactivities were limited to the base complementary to the modified guanine and to adjacent bases. Within this sequence context, the three possible AAF adducts have previously been shown to exhibit strong differences in biological responses such as excision repair [Seeberg, E., & Fuchs, R. P. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 191–194] and mutagenesis [Burnouf, D., Koehl, P., & Fuchs, R. P. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4147–4151]. We propose that the polymorphism in adduct-induced DNA structure that is shown in the present paper plays a major role in the sequence-specific responses seen when these lesions are processed in vivo.

Investigating the fine structure of a chemically modified DNA is essential to understand the processing of DNA lesions by enzymes that give rise to DNA repair or mutagenesis. For example, it is believed that the initial step in excision repair is triggered by the recognition of a structural alteration of the DNA double helix. However, it is not known to what extent the structure of the double helix of DNA has to differ from that of "natural" DNA before it is sensed by the repair enzymes as being a lesion.

N-Acetoxy-*N*-2-acetylaminofluorene (N-Aco-AAF),[1] a model compound for the strong rat liver carcinogen *N*-2-acetylaminofluorene (AAF), has been shown to induce mainly frameshift mutations in bacteria (Fuchs et al., 1981; Koffel-Schwartz et al., 1984). These mutations mostly occur within two types of sequences: runs of guanines and *Nar*I sequences ($G_1G_2CG_3CC$). N-Aco-AAF binds primarily to the C-8 position of guanines. Although the guanine residues of the *Nar*I site are about equally reactive (Fuchs, 1984), the mutation frequency induced by G-AAF residues varies considerably according to the position of the adduct: mutagenesis experiments involving single-adducted plasmids have shown that only binding of AAF to the G_3 position of the *Nar*I site leads to high mutation frequencies (Burnouf et al., 1989; Koehl et al., 1989a). Similarly, UvrABC-mediated incision studies using double-stranded oligomers bearing single AAF adducts within the *Nar*I sequence showed that the incision efficiency varies according to the position of the adduct, with cutting being more extensive for the G_1- and G_3-modified DNA than for the G_2-modified DNA (Seeberg & Fuchs, 1990).

Many studies have attempted to describe the structural alteration that covalently bound AAF residues impose upon DNA. In these studies, the structure of randomly modified double-stranded DNA or synthetic polynucleotides was investigated. Two major modes of binding have been described. (1) Covalent binding of AAF to a random sequence of DNA induces a local denaturation of B-DNA, with the AAF ring being inserted between the base pairs and the guanine being rotated outside the double helix. This has been termed the insertion–denaturation model (Daune et al., 1981; Fuchs & Daune, 1972), also called the base displacement model (Grunberger & Weinstein, 1979). (2) AAF binding to d-$(CpG)_n$ sequences promotes a B → Z conversion of the helix (Sage & Leng, 1980; Santella et al., 1981; Wells et al., 1982). When bound to left-handed Z-DNA, AAF residues are located outside of the helix and the base pairing is maintained.

It should be stressed that both models were deduced from low-resolution structural data and must therefore be considered as average modes of binding. The observations that single AAF adducts exhibit large differences in mutagenicity and repair depending on their position within the DNA sequence suggests that there might be large differences in the respective adduct-induced DNA structures. In order to obtain further

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[1] Abbreviations: N-Aco-AAF, *N*-acetoxy-*N*-2-acetylaminofluorene; AAF, *N*-2-acetylaminofluorene; HPLC, high-performance liquid chromatography; DEPC, diethyl pyrocarbonate; HA, hydroxylamine; BAA, bromoacetaldehyde; OP_2Cu, 1,10-phenanthroline–copper; OsO_4, osmium tetraoxide; NMR, nuclear magnetic resonance; CD, circular dichroism.

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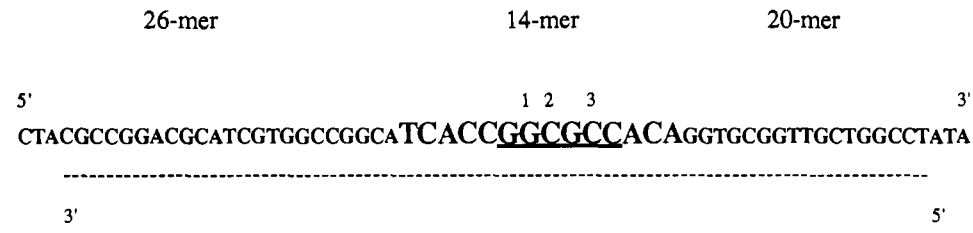
Chart I



Complementary strand (54-mer)

insight into these structures at the nucleotide level, we have used different chemical probes to characterize the structural deformation of double-stranded oligomers bearing single adducts. Chemical probes are reagents that are sensitive to DNA conformation, reacting differently with bases that are not in the usual B-DNA conformation [for complete review, see Nielsen (1990)]. In this paper, we describe a large structural polymorphism depending on the position of the AAF adduct.

Materials and Methods

Construction of Single AAF-Modified 60-mer Oligonucleotides. Oligonucleotides were chemically synthesized (Applied Biosystems, Model 380B) and purified by reverse-phase HPLC on a C18 column (Zymark). The construction of the double-stranded 60-mers bearing a single AAF was achieved by ligation of chemically synthesized oligonucleotides as previously described (Seeberg & Fuchs, 1990) with minor modifications. One of these oligonucleotides (14-mer) contains the AAF-adducted guanine. The synthesis, purification, and characterization of the modified oligonucleotides has been described previously (Koehl et al, 1989a). Briefly, for each construction, the corresponding phosphorylated 14-mer bearing a single AAF residue was mixed with a 26-mer, a 5'-phosphorylated 20-mer, and a complementary 54-mer, yielding a single AAF-modified 60-mer as shown in Chart I. After a heating–reannealing cycle, the oligonucleotides were ligated with T4 DNA ligase.

The AAF-modified oligonucleotides were labeled at either the 3'- or 5'-end with ^{32}P. For the construction of the 5'-end-labeled AAF-containing fragment, the 26-mer was treated with polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol), whereas for 3'-end-labeling the 20-mer was incubated with terminal deoxynucleotidyltransferase and [α-^{32}P]ddATP. After ligation, the DNA fragments were denatured and separated on a 20% polyacrylamide gel to purify the ligated 60-mer. The purified 5'- or 3'-end-labeled 60-mers were reannealed to unlabeled complementary 60-mer. For the construction of fragments labeled in the non-AAF-containing stand, the complementary 60-mer was labeled on either the 5'- or the 3'-end. This strand was purified on a 20% sequencing gel after treatment with 1.0 M piperidine at 90 °C for 30 min and reannealed with the AAF-containing strand. The piperidine treatment is intended to eliminate DNA molecules containing preexisting alkali-labile sites such as AP sites. For each construction, a slight molar excess (1.5×) of the "cold" strand was used in the reannealing step. The complete hybridization of the labeled strand was checked by electrophoresis on a nondenaturing 12% polyacrylamide gel.

Chemical Modifications. Reactions between DNA and the chemical probes were performed as described by Johnston and Rich (1985) for diethyl pyrocarbonate (DEPC) and hydroxylamine (HA) with minor modifications (temperature 20 °C; time of reaction 1 h). Bromoacetaldehyde (BAA) was kindly provided by Philippe Noirot (INRA, Jouy en Jossas). The reaction between BAA and DNA was performed essentially as described by Kohwi-Shigematsu et al. (1987) and Vogt et al. (1988a). DNA (5 fmol) was dissolved in 50 μL of 10 mM Tris-HCl (pH 7) and 0.1 mM EDTA. Following addition of 1 μL of BAA (final concentration 20 mM), the mixture was incubated for 30 min at 20 °C. The sequencing of the DNA was performed by the chemical method of Maxam and Gilbert (1980).

Strand Scission Reaction with 1,10-Phenanthroline–Copper (OP_2Cu). Reaction between DNA and OP_2Cu was performed as described by Yoon et al. (1988) with minor modifications (temperature 10 °C; time of reaction 1 min). The 3'-end-labeled DNAs were used, and the piperidine treatment after the OP_2Cu reaction was omitted. As already indicated, the non-AAF-containing strand was previously purified on a 20% sequencing gel after piperidine treatment. After quenching of the reaction by addition of 2,9-dimethyl-1,10-phenanthroline, the products were lyophilized and resuspended in sequencing gel loading buffer.

Autoradiogram Scanning. For quantification of the OP_2Cu strand scission efficiency, the autoradiograms were scanned by a CS-9000 Shimadzu scanning densitometer.

Results

In the present work we have used three double-stranded 60-mers bearing a single AAF adduct at one of the guanine residues of the *Nar*I site ($G_1G_2CG_3CC$) (Seeberg & Fuchs, 1990). The complete sequence of the 60-mer is given in Chart I. We will refer to these substrates as 60 for the nonmodified oligomer and 60-1, 60-2, or 60-3 for the DNA modified on the G_1, G_2, or G_3 residue, respectively.

Chemical Probing of the Bases. Chemical probing of the structure of nucleic acids at the nucleotide level is based upon the properties of certain chemicals to react with nucleic acids in a way that is dependent upon their three-dimensional structure. Different chemical reagents have been used to study the conformation of the different bases. Diethyl pyrocarbonate (DEPC) reacts strongly with purines in the syn configuration within Z-DNA or in single-stranded DNA (Herr, 1985; Johnston & Rich, 1985; Runkel & Nordheim, 1986). Hydroxylamine (HA) reacts specifically with cytosines in single-stranded DNA and distorted DNA (Johnston & Rich, 1985). It has been used to map B–Z junctions (Johnston & Rich, 1985; Vogt et al., 1988b). Similarly, chloroacetaldehyde (CAA) and bromoacetaldehyde (BAA) react with A and C residues in denatured regions and at the B–Z interfaces (Kohwi-Shigematsu et al., 1987; MacLean et al., 1987). OsO_4 has been used to probe thymine residues (T) in distorted DNA such as B–Z junctions (Nejedly et al., 1985; Vogt et al., 1988b). After reaction with the chemical reagent, the phosphate–sugar backbone is cleaved at the level of the modified bases by piperidine treatment and the resulting fragments are separated on sequencing gels in parallel with a Maxam–Gilbert

sequence ladder (Maxam & Gilbert, 1980).

We also used a nucleolytic agent, 1,10-phenanthroline–copper (OP_2Cu). This compound cleaves the sugar–phosphate backbone by an oxidative mechanism [for complete review, see Sigman (1990)]. OP_2Cu "pseudonuclease" activity has almost exclusively been used by Sigman and co-workers in the analyses of promoter conformation (Spassky et al., 1988; Spassky & Sigman, 1985). The cleavage efficiency was found to be sequence dependent but not base specific (Veal & Rill, 1988), and it has been suggested that it reflects the geometry of the minor groove (Sigman, 1986).

We have used all these chemical reagents to study at the nucleotide level the structural deformations induced by a single AAF adduct in the double-stranded substrates. Control experiments were done with single- and double-stranded nonmodified oligomers.

(*A*) *Diethyl Pyrocarbonate.* DEPC reacts with purines by carbethoxylation of the N7 atom resulting in the ring opening of the imidazole moiety (Vincze et al., 1973). DEPC does not react very efficiently with B-DNA because the N7 atom is buried inside the double helix. However, in left-handed Z-DNA, the N7 position of purines is considerably more exposed because of their syn conformation. DEPC has been used to monitor the B- to Z-DNA transition (Herr, 1985; Johnston & Rich, 1985; Vogt et al., 1988b).

The reactivities of the different monomodified substrates with DEPC are presented in Figure 1 (top). The radioactive label was in the non-AAF-containing strand (complementary strand). Despite the irregular pattern of reactivities observed for purines in single-stranded DNA, we can notice that reactivities are always more important in single-stranded as compared to double-stranded DNA. With the nonmodified double-stranded 60-mer, no particular purine is reactive with DEPC. With G_3-modified substrate (lane 60-3), a hyperreactive band is observed for the guanine 3′ to the cytosine that is paired with G_3. In the 60-1 lane, the guanine immediately 3′ to the cytosine complementary to G_1 is also hyperreactive but to a lesser extent than in the 60-3 oligomer. No hyperreactivity was observed for the 60-mer modified on G_2. These results are represented in Figure 1 (bottom). It can be noted that the G_1 and G_3 bases belong to a $^{5'}CpG^{3'}$ dinucleotide unit, whereas the G2 residue is part of a $^{5'}GpC^{3'}$ dinucleotide. No particular reactivity was observed with purine residues on the AAF-containing strand (data not shown).

(*B*) *Hydroxylamine.* HA is strongly reactive with cytosine in single-stranded DNA, attacking either the C4 position or both C6 and C4 positions (Singer & Grunberger, 1983). The results relative to the complementary strand are shown in Figure 2 (top left). In single-stranded DNA all the cytosine residues are reactive with hydroxylamine. This reagent was reported to be useful for Maxam–Gilbert sequencing when used on denatured DNA (Rubin & Schmid, 1980). Figure 2 (top left) shows the cytosine ladder produced by the reaction of the single-stranded 60-mer; in contrast, no cytosine is reactive with HA in the nonmodified double-stranded DNA. When the 60-mers are monomodified with AAF, intense bands are visible for the cytosines base-paired with the modified G. For the G_1-modified DNA, HA reacts with both the complementary cytosine and the cytosine 5′ to that base, with the latter being more reactive. The same two C residues are also hyperreactive for the G_2-modified oligomer, but in this case they are equally reactive. In the 60-3 lane, only one band is seen corresponding to the cytosine complementary to the AAF-modified guanine. The results are summarized in Figure 2 (bottom). No enhanced reactivity of the cytosines was observed with HA for the AAF-containing strand (data not shown).

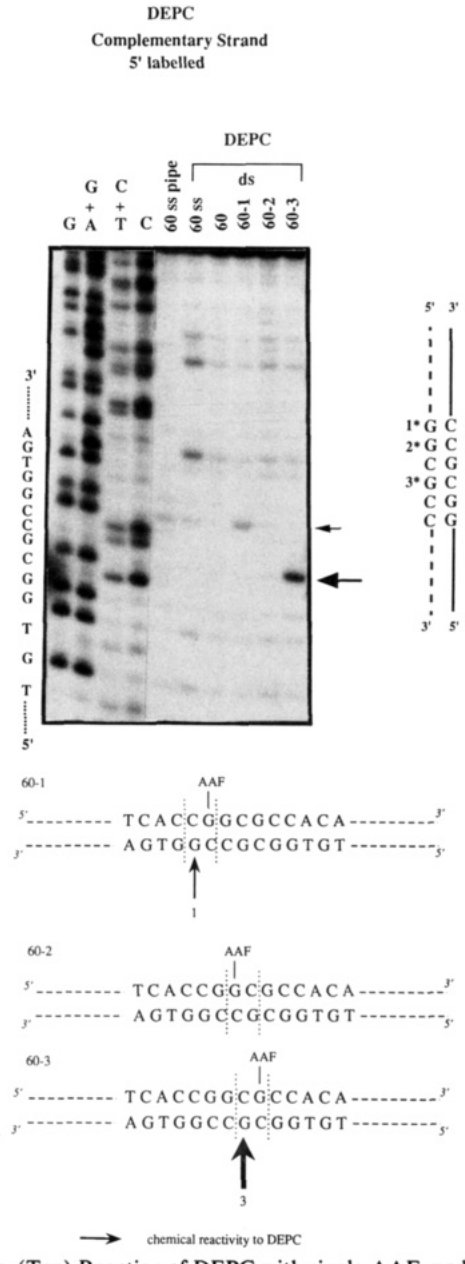


FIGURE 1: (Top) Reaction of DEPC with single-AAF-modified 60-mers. Double-stranded 60-mers are constructed as described under Materials and Methods. Only the non-adduct-containing strand (complementary strand) is 5′-end-labeled with ^{32}P. Lanes G, G + A, C + T, and C refer to the Maxam and Gilbert sequencing reactions. Lane 60 ss pipe corresponds to the complementary single strand treated with piperidine. Lanes 60-1, 60-2, and 60-3 refer to the three AAF-containing double-stranded 60-mers, respectively. The large arrow indicates the DEPC-reactive guanine adjacent to the cytosine paired with the G_3. The small arrow points to the reactive guanine adjacent to the cytosine paired with the G_1. On the right-hand side of the scheme, the labeled complementary strand is represented with a heavy line and the nonlabeled modified strand is represented with a dotted line. (Bottom) Schematic representation of the DEPC-induced cleavages. The arrow indicates the reactive guanine, and the number under the arrow represents the relative cutting efficiency. The dotted lines enclose the $^{5'}CpG^{3'}$ or the $^{5'}GpC^{3'}$ dinucleotides.

(*C*) *Bromoacetaldehyde.* BAA, like CAA, reacts with N1 and N^6 of adenine and N3 and N^4 of cytosine to form an etheno cyclic adduct (Kayasuga-Mikado et al., 1980; Kusmierek & Singer, 1982). These positions are more reactive if they are not engaged in hydrogen bonding. BAA and CAA have been used to detect B-DNA/Z-DNA junctions (Koh-

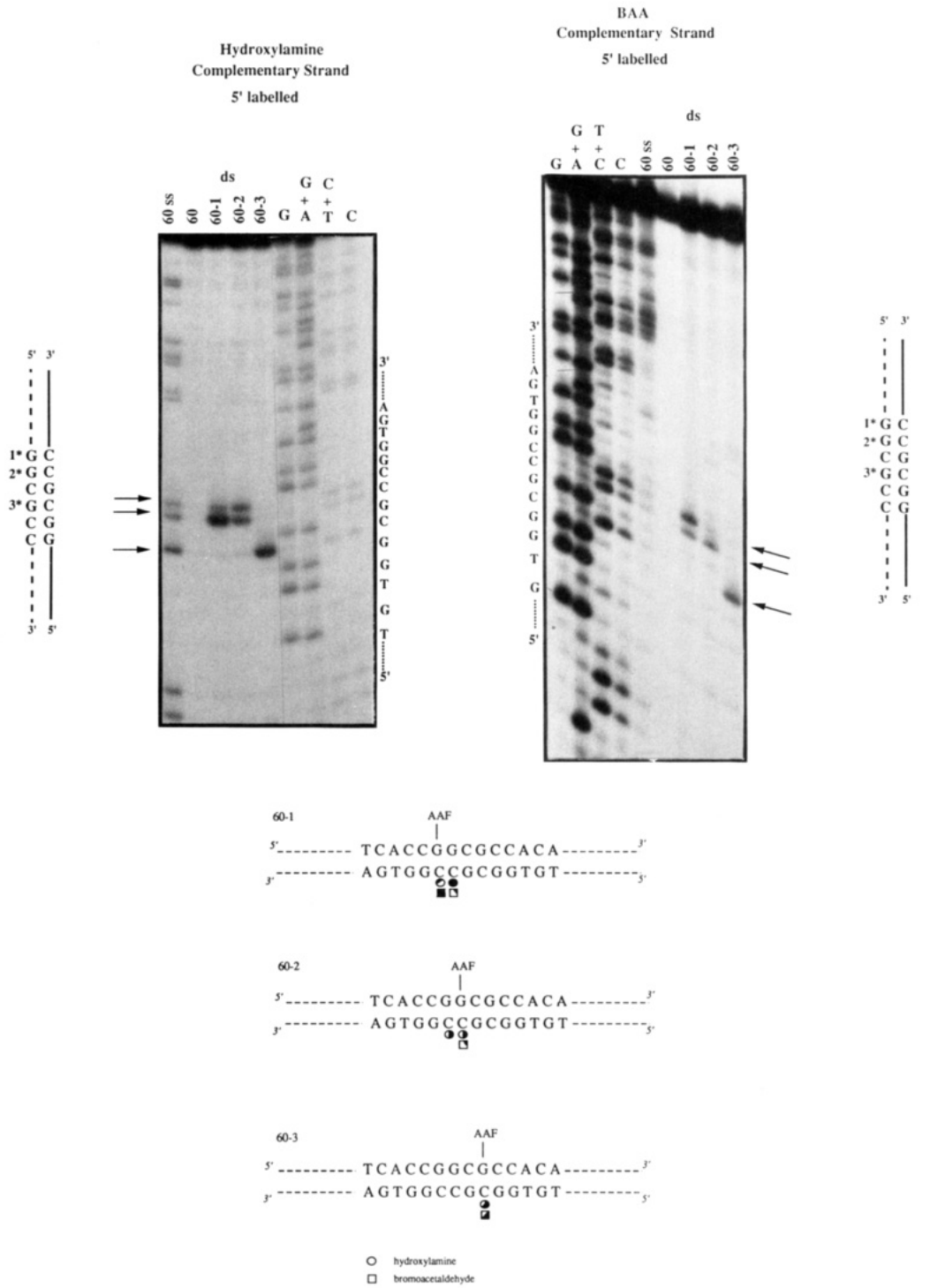


FIGURE 2: (Top left) Reaction of hydroxylamine (HA) with single-AAF-modified 60-mers. Only the non-adduct-containing strand (complementary strand) is 5′-end-labeled with ^{32}P. Lane 60 ss refers to the reactivity of the complementary single strand. Lanes 60-1, 60-2, and 60-3 refer to the three AAF-containing double-stranded 60-mers, respectively. The arrows mark the reactive cytosines. (Top right) Reaction of bromoacetaldehyde (BAA) with single-AAF-modified 60-mers. Only the non-adduct-containing strand (complementary strand) is 5′-end-labeled with ^{32}P. Lane 60 ss refers to the reactivity of the complementary single strand. Lanes 60-1, 60-2, and 60-3 refer to the three AAF-containing double-stranded 60-mers, respectively. The arrows mark the reactive cytosines. (Bottom) Summary of the changes in chemical reactivity toward HA and BAA. Circle symbols refer to reactivity with HA, and square symbols indicate reactivity with BAA. The filling of the symbols is proportional to the reactivity intensities.

wi-Shigematsu et al., 1987) and to study the deformation induced by a single adduct in an oligonucleotide (Marrot & Leng, 1989; Schwartz et al., 1989).

Results relative to the reactivity with the non-AAF-containing strand are shown in Figure 2 (top right). With single-stranded DNA, an irregular ladder of reactivities with cytosine and adenine is seen. With nonmodified double-stranded DNA, no particular base is reactive with BAA. For the three modified double-stranded oligomers, the cytosines complementary to the AAF-modified guanines are reactive toward BAA. Within the 60-1 lane, another band is visible corresponding to the cytosine adjacent (5′) to the C paired with the modified guanine. No reactivity was observed with the AAF-containing strand (data not shown). A summary of the chemical reactivities toward BAA is given in Figure 2 (bottom).

(*D*) *Osmium Tetraoxide.* In the presence of pyridine, osmium tetraoxide (OsO_4) binds covalently to thymine residues through addition across the 5,6 double bond (Nejedly et al., 1985). These positions are not involved in the base pairing; therefore, OsO_4 is capable of probing very mild distortion of the DNA structure. No hyperreactivity of thymine bases on either strand of the AAF-modified oligomers was observed with OsO_4, indicating that the induced structural distortion does not extend to the thymine residues.

Chemical Probing of the Sugar–Phosphate Backbone: Phenanthroline–Copper Digestion. The OP_2Cu complex cleaves DNA by a chemical mechanism related to Fenton chemistry: hydroxyl radicals are generated in the presence of hydrogen peroxide and attack the C1 position of the sugar (Sigman, 1986), following deoxyribose oxidation and strand cleavage. Binding of the complex to the minor groove (Kuwabara et al., 1986) positions the copper ion near the deoxyribose hydrogen that is abstracted. Therefore, cutting of the DNA backbone is less random than with free hydroxyl radicals (Tullius et al., 1987) and will depend upon the minor-groove geometry. This compound has been used to perform protein footprinting (Spassky & Sigman, 1985), but it can also be used to study structural variations of the double helix (Veal & Rill, 1988).

(*A*) *Autoradiography Analysis.* The cleavage patterns of the AAF-modified oligomers following reaction with OP_2Cu are presented in Figure 3. In these experiments, the DNA fragments are all end-labeled on the 3′-side to avoid a smearing effect of the bands on the gel due to the presence of metastable products [for details, see Kuwabara et al. (1986)]. The unmodified double-stranded oligomer shows irregular cutting of the DNA backbone. Therefore, we compared the cleavage pattern of the three modified DNA substrates relative to the unmodified substrate by computing the normalized difference in reactivity for each band. For the complementary strand [Figure 3 (top left)], the observed hyperreactivity extends over several bases, from across the modified guanine toward the 3′ side for all three AAF-modified oligomers. However, these hyperreactivities are much stronger in the case of the oligomer modified at G_3 (lane 60-3).

For the AAF-containing strand [Figure 3 (top right)], the cleavage patterns are more difficult to interpret because of the decreased mobility of the DNA fragments modified with AAF. As can be seen from the top of the autoradiogram, the modified DNA fragments migrate slightly more slowly than the corresponding nonmodified fragments. Consequently, within the same lane, the migration of the OP_2Cu-cleaved fragments changes suddenly at the position of modification. Thus, the little footprint seen for the modified samples corresponds to this difference in migration. In fact, all the bands are present and can be attributed. Hyperreactivity is seen for several bases 3′ to the modified guanine. Stronger bands are visible for the G_1-modified oligomer.

To interpret these results more easily, the efficiency of OP_2Cu digestion has been quantitatively represented on a histogram after autoradiogram scanning [Figure 3 (bottom)]. The different bands have been normalized by subtraction with the unmodified oligomer to visualize the structural changes induced by the AAF moiety.

(*B*) *Three-Dimensional Representation of the OP_2Cu Digestion Patterns.* It has been previously suggested (Drew & Travers, 1984; Veal & Rill, 1988) that the OP_2Cu attack site is located in the minor groove, probably mediated through partial intercalation. The strongest cleavage sites observed for the monomodified oligomers are staggered to the 3′ side of the adduct on both strands [Figure 3 (bottom)], which is indicative of a minor-groove mode of interaction. Indeed, the closest phosphates facing one another across the minor groove are displaced by three bonds in the 3′ direction relative to any base-pair step (Drew & Travers, 1984) as visualized on a three-dimensional model of B-DNA (Figure 4). The normalized digestion patterns have been represented by using a color code (Figure 4). The color intensity is proportional to the cleavage efficiency, ranging from red for strong cleavage to blue for weak cleavage. From this color representation, it can be seen [especially in Figure 4 (left)] that the hyperreactive sites are located across the minor groove on both sides of the AAF-modified base and that the AAF-induced distortion is centered around the modified base.

Discussion

We have constructed a set of three double-stranded oligonucleotides, 60 base pairs long, containing a single AAF residue covalently bound to the C8 position of one of the three G residues present within the *Nar*I site ($G_1G_2CG_3CC$), in order to investigate at the nucleotide level the conformational change that the binding of the carcinogen residue imposes upon the double helix of DNA. This study was prompted by the observation that AAF residues as well as other C8-guanine binding carcinogens induce −2 frameshift mutations at a very high frequency within the *Nar*I site and related sequences (Bintz & Fuchs, 1990). Surprisingly, single-adduct mutagenesis studies revealed that −2 frameshift mutations were solely induced by the binding of AAF to G_3 (Burnouf et al., 1989). Moreover, in vitro studies using the purified UvrABC excinuclease and single-adducted substrates showed large differences in the incision efficiency according to the position of the bound AAF residue (Seeberg & Fuchs, 1990). Taken together, these observations suggested very strongly that the AAF residue induces different alterations of the DNA structure according to its position of binding within the *Nar*I sequence. Using short double-stranded oligonucleotides (12-mers) containing the *Nar*I sequence, we observed large differences in the circular dichroism signal, indicating conformational differences among the three substrates (Koehl et al., 1989b).

In order to investigate the nature of these differences more precisely at the nucleotide level, we decided to probe the conformation of the carcinogen-modified double-stranded oligonucleotides by means of so-called chemical probes for nucleic acid structure. Two classes of chemical probes can be distinguished, probes that sense the geometry of the helix, giving rise to cuts at every nucleotide, and probes that react with specific bases. The first class of probe is sensitive to parameters such as the width of the helix grooves, while the second class detects bases that belong to distorted regions of the helix.

Some general features emerge from our analysis of the results: (i) the size of the perturbation induced by the covalent binding of a single AAF residue extends over a region of about 4–6 base pairs (determined by use of OP_2Cu, a probe sensitive to the geometry of the helix; see Figure 4), (ii) although the perturbation is present in both strands, the non-adduct-containing strand is more extensively distorted (Figure 4), and (iii) for the different probes that we have tested, distinct patterns of reactivity are observed according to the position of the adduct within the DNA sequence, revealing a polymorphism in the adduct-induced DNA structure.

The results obtained with the different probes for each of the three modified substrates allow us to propose different structural models. We will first discuss the G_2-modified ol-

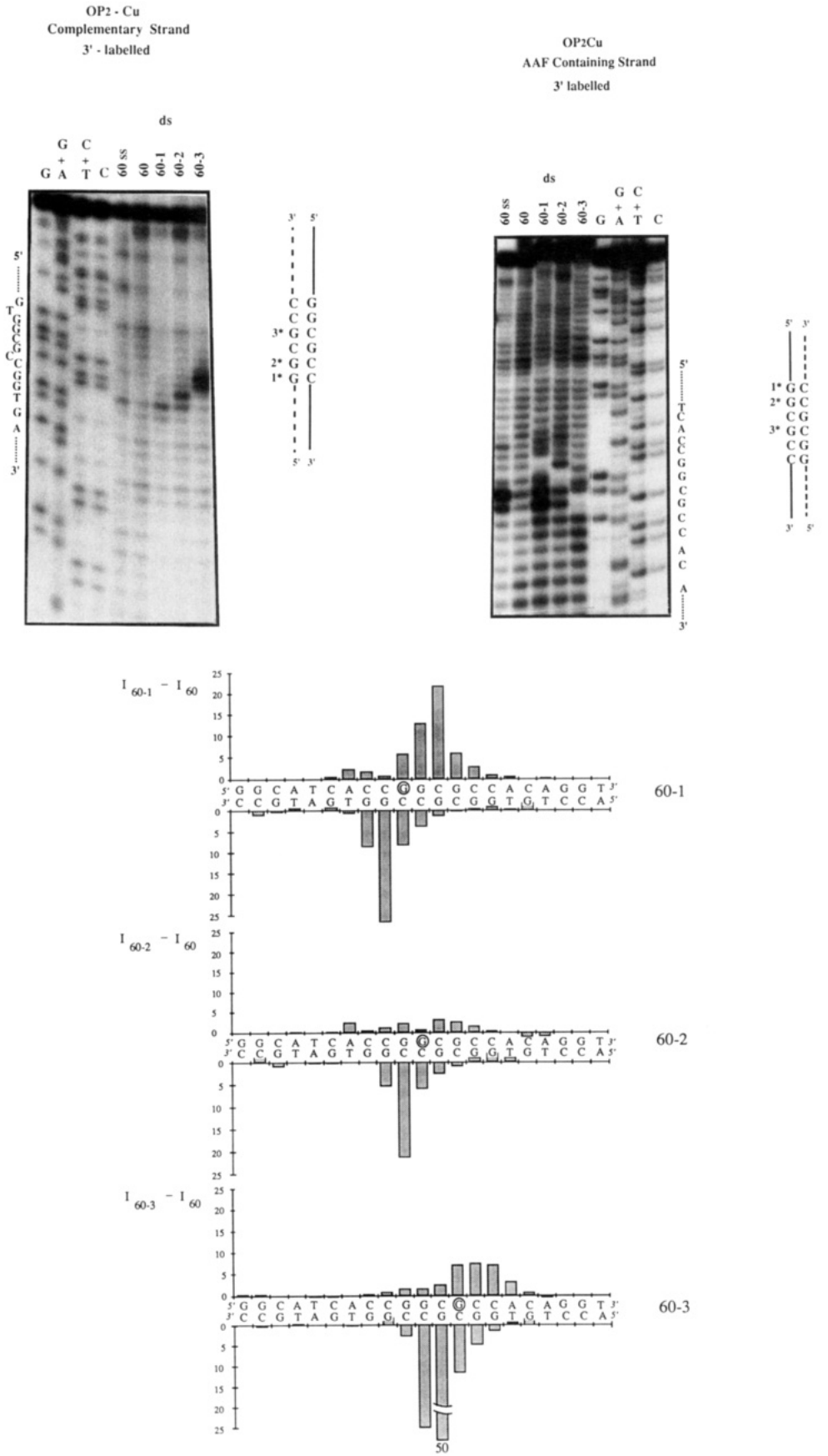


FIGURE 3: (Top left) Reactivity of OP_2Cu with the 3′-end-labeled complementary strand of the 60-mer oligonucleotides. The lane 60 ss refers to the reactivity of the single strand. Lanes 60-1, 60-2, and 60-3 refer to the three AAF-containing double-stranded 60-mers, respectively. Lanes G, G + A, C + T, and C refer to the Maxam and Gilbert sequencing reactions. (Top right) Reactivity of OP_2Cu with the 3′-end-labeled AAF modified strand. Labeling of the lanes is the same as just described. (Bottom) Quantitative representation of the OP_2Cu digestion efficiencies for the three single-modified 60-mers. The autoradiograms have been scanned and the normalized intensities of the different bands of the unmodified oligomer have been subtracted from the corresponding band intensities of the modified substrates (ΔI values). The histogram is given for 20 base pairs surrounding the modified guanine. The modified guanine residues are circled.

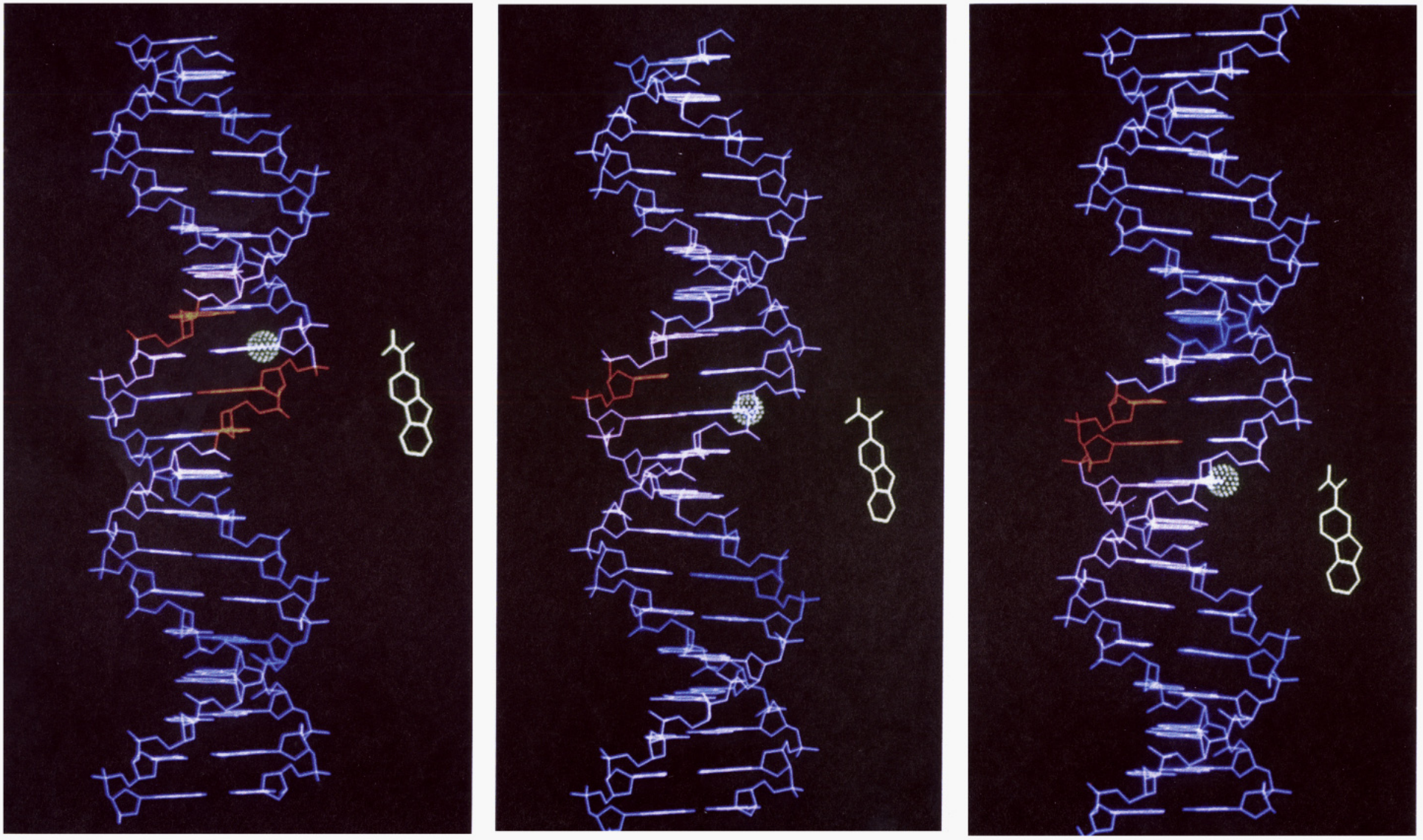


FIGURE 4: Normalized digestion patterns from Figure 3 (bottom) have been represented on a usual right-handed helix model of 20 bp. For each representation, the color intensity is proportional to the cleavage efficiency, ranging from red for strongly enhanced cleavage ($\Delta I \approx 25$) to blue for no enhanced cleavage ($\Delta I = 0$). The C8 position of the AAF-modified guanine is represented in green with its van der Waals sphere. An AAF residue is displayed on the side in order to visualize its size. All views are from the minor groove. Three-dimensional representations of the OP_2Cu digestion efficiencies for the 60-mers are as follows: (Left) 60-1. It can be seen that the amount of cleavage is similar in the two strands. (Center) 60-2. The amount of cleavage is asymmetrical, with the nonmodified strand being more severely cut. (Right) 60-3. As for 60-2, the amount of cleavage is asymmetrical between the two strands.

igomer, which seems to correspond to an insertion–denaturation model type of interaction (Fuchs & Daune, 1972). Then, we will discuss and compare G_3- and G_1-modified oligomers, which induce a conformational change possibly involving a local Z-DNA type of structure.

G_2-Modified Oligomer. For the G_2-modified oligomer, we suggest that the DNA structure is locally unstacked/denatured over at least two base pairs, as shown by the reactivity of the cytosine paired with the G-AAF and the adjacent cytosine toward HA [Figure 2 (bottom)]. The base pairing of G_2 with its partner cytosine is broken or weakened, as shown by the reactivity of this cytosine residue with BAA [Figure 2 (top right)]. No other reactivity with either HA or BAA was observed on the modified strand. DEPC did not show any particular reactivity with this substrate. When compared to the two other substrates, the enhancement in reactivity of the G_2-modified substrate with the 1,10-phenanthroline–copper (OP_2Cu) nuclease is only moderate, especially on the modified strand [Figure 3 (bottom)]. This could mean that the structure of the double helix around the AAF adduct is less distorted for the G_2-modified substrate than for the G_1- and G_3-modified oligomers.

This distortion induced by a single *N*-2-acetylaminofluorene bound to a guanine residue surrounded by two thymines within a homopyrimidine strand was investigated by Schwartz et al. (1989). These authors concluded that the AAF residue induces a local region of denaturation of about three base pairs. These features ressemble the conformation found for the G_2-modified oligomer.

We propose that the adduct-induced DNA structure in the G_2-modified oligomer is best described by an insertion–denaturation type of model (Fuchs & Daune, 1972) or base displacement model (Broyde & Hingerty, 1987; Levine et al., 1974). The AAF moiety is inserted between the base pairs, in front of the cytosine, causing local unstacking and denaturation as revealed by the chemical probes. Shapiro et al. (1989) recently proposed a variation of the insertion–denaturation model called the wedge model. In this model, suggested by the results of NMR studies on *N*-2-aminofluorene-adducted oligonucleotides (Norman et al., 1989), the conversion of the adducted guanine into the syn conformation leads to a small deformation of the helix (no more than two base pairs), with the fluorene moiety being located or "wedged" in the minor groove and the modified G·C base pair being disrupted. Preliminary NMR studies seem to indicate that, for the G_2-modified DNA, the AAF ring might indeed be located inside the helix (J.-F. Lefèvre, personal communication).

G_1 and G_3 Oligomers. Depending upon the accessibility of the N7 position, diethyl pyrocarbonate reacts with purine bases and represents an effective probe for the syn conformation in double-stranded DNA. In the complementary strand, the strong reactivity of DEPC toward the guanine 3′ to the C paired with G_3 suggests that this guanine is in the syn conformation [Figure 1 (bottom)]. It is also possible that DEPC reactivity may be due to local unwinding of the helix induced by the AAF modification. However, DEPC has been used to probe Z-DNA in plasmids containing a $d(CpG)_{16}$ insert (Johnston & Rich, 1985), and in these studies it can be seen that guanine bases in the syn conformation are more reactive than guanine bases in a partially unwound structure such as a B/Z junction. Therefore, we favor the hypothesis of a syn conformation for the observed DEPC-reactive guanine in the 60-3 oligomer. A similar pattern of reactivity is observed for the G_1 adduct where the guanine 3′ to the C paired with G_1 is also reactive, although to a lesser extent. We suggest that this particular guanine may transiently assume the syn conformation or display a particularly distorted conformation that makes the N7 accessible to DEPC.

For both the G_1 and G_3 substrates the cytosine complementary to the G-AAF moiety is reactive towards HA and BAA [Figure 2 (bottom)]. In the complementary strand, enhanced reactivity for the HA and BAA probes was also observed on the cytosine adjacent to the C base-paired with G_1-AAF. No cytosine residues on the adduct-bearing strand were reactive toward HA or BAA in either the G_1 or the G_3 oligomers. Together, the DEPC, HA, and BAA results suggest that covalent binding of AAF to both G_1 and G_3 produces a localized deformation of the DNA helix that extends for at least one base on either side of the G_1 adduct but appears to be limited to the ^{5}′CpG$_3$-AAF$^{3'}$ dinucleotide for the G_3 adduct.

The reactivity of the OP_2Cu, with the G_1- and G_3-modified oligomers increases near the AAF residue [Figure 3 (bottom)]. The strongest cleavage sites are staggered 3′ to the adduct on both strands, indicating that the nucleolytic attack takes place in the minor groove and is enhanced near the adducted G residue. The amount of cleavage is similar in the two strands of the G_1 oligomer [see also Figure 4 (left)] but is asymmetrical for the G_3 oligomer, with the nonmodified strand being cut more severely [Figure 4 (right)]. It appears that the structural alteration of the helix, induced by the AAF adduct in the G_1 and G_3 positions is such that the DNA becomes more accessible to OP_2Cu attack near the modified G. For the substrates modified at G_1 and G_3, preliminary NMR data suggest that the AAF residue is exterior to the DNA helix (J.-F. Lefèvre, personal communication). AAF residues that are located in the exterior of the helix might provide preferential binding sites for OP_2Cu via stacking interactions between the two aromatic moieties (i.e., fluorene and phenanthroline), which in turn might account for the increase in OP_2Cu-mediated cleavage that we observe in the vicinity of the AAF residues.

Circular dichroism (CD) studies have been performed recently on small duplexes containing a single AAF adduct within the *Nar*I sequence (Koehl et al., 1989b). These studies have revealed that modification of G_3 causes a major alteration in the local structure of the helix; the interpretation of the CD spectrum suggests the existence of a local Z-like DNA structure. Our present results tend to support these observations. It is already known that, upon binding of the AAF residue to the C8 position, the guanine base undergoes an anti to syn transition (Evans et al., 1980). In the case of the G_3 adduct, the modified G is part of a ^{5}′CpG$^{3'}$ dinucleotide sequence, and using the DEPC probe we have shown that the G on the complementary strand may also display the syn conformation. Therefore, the dinucleotide ^{5}′CpG$^{3'}$ features the preferred conformation found in Z-DNA, i.e., the alternation of the syn and anti conformation of a purine and a pyrimidine base, respectively.

We propose that a possible structure for the G_3-modified oligomer corresponds to a short Z-like structure, with the AAF moiety being rotated outside the double helix. The base pairing is almost maintained except for the G_3·C base pair. In model studies involving AAF-modified $(CpG)_n$ sequences (Broyde & Hingerty, 1985; Hingerty & Broyde, 1986; Shapiro et al., 1989), the binding of AAF to the Z conformation was found to be energetically favored. Whether such a conformation is possible when a single ^{5}′CpG$^{3'}$ dinucleotide unit is embedded in a long B-DNA structure remains to be established. In such a model, the bases that are adjacent to the Z-DNA unit are

of critical importance since they will have to accommodate the B–Z junctions. Arnott and co-workers have shown that a single Z-form dinucleotide can be merged into a B form without extensive deformation and denaturation (Arnott et al., 1982).

In the G_1-modified oligomer it should be noted that the AAF modification is also part of a ^{5}CpG^3 dinucleotide unit. Moreover, the DEPC data suggest a moderate accessibility of the guanine on the complementary strand, which may account for a transient syn conformation, and we have also seen that binding of the AAF adduct to G_1 induces deformation of the helix structure [Figure 2 (bottom)]. However, CD data do not suggest the presence of a local Z structure (Koehl et al., 1989b), and no −2 frameshift mutations have been observed for the G_1-modified plasmid in single-adduct mutagenesis experiments (Burnouf et al., 1989; Veaute and Fuchs, unpublished results). A principal difference between the sequences of the G_1- and G_3-modified substrates is the bases that flank the ^{5}CpG^3 dinucleotide units. In the G_3 context (^{5}GpCpGpC^3), the bases adjacent to the ^{5}CpG^3 dinucleotide unit may accommodate the two B–Z junctions more favorably than the bases surrounding the potential Z-DNA unit in the G_1 (^{5}CpCpGpG^3) context. Because of the sequence context of G_1, the modified substrate might not easily form a stable G_3-like conformation and may instead adopt a more dynamic conformation.

Conclusion. The construction of DNA helices containing single adducts and the use of several chemical probes have allowed us to investigate the structural alteration that a carcinogenic adduct imposes upon the DNA structure. In previous studies we identified the *Nar*I site ($G_1G_2CG_3CC$) as a strong frameshift mutation hot spot for carcinogens that bind to the C8 position of guanine. We have compared the structural alterations induced by the covalent binding of a single AAF residue to the three guanines that belong to the *Nar*I site. By use of a probe (1,10-phenanthroline–copper) that is sensitive to the geometry of the DNA backbone, the deformation of the DNA structure was found to extend over a region of 4–6 bases centered around the adduct and sensed by the probe in both strands. However, it is possible that some DNA unwinding beyond the actual size of deformation could be induced by the OP_2Cu because this compound interacts with DNA through partial intercalation; therefore, the real size of the AAF-induced deformation could be shorter than 4–6 bases. The patterns of reactivity of the base-specific probes were found to be different for all three substrates, revealing a strong polymorphism of the adduct-induced DNA structure.

The observed polymorphism strongly influences the processing of the different adducts (repair, conversion into a mutation, etc.). Indeed, induction of −2 frameshift mutations within the *Nar*I mutation hot spot was found to be triggered solely by binding of AAF to G_3 (Burnouf et al., 1989). The work of Burnouf et al. (1989) and our present study suggest that the binding of AAF to G_3 might induce a local Z-DNA type of structure. We suggest that this unusual DNA structure is recognized and processed into a −2 frameshift mutation by an SOS-dependent pathway (Janel-Bintz et al., 1991; Koffel-Schwartz & Fuchs, 1989) that requires further characterization.

Excision repair mediated by the UvrABC excinuclease was also found to be strongly influenced by the position of the adduct within the *Nar*I site (Seeberg & Fuchs, 1990). Adducts at G_1 and G_3 were found to be incised more readily than the adduct at G_2. It can be suggested from the present work that parameters such as the position of the adduct (interior or exterior to the helix) or the mobility of the adduct are important for the excision repair efficiency. However, an understanding of the determinants that influence the cutting efficiency by the excision repair complex await a more detailed picture of the distorted DNA structure.

The fact that a given adduct (i.e., dG-C8-AAF) can induce various sequence-dependent DNA distortions that then lead to different biological processing implies that the ultimate understanding of repair and mutagenesis mechanisms will require knowledge of the various classes of adduct-induced DNA structures.

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