TOXICOLOGICAL SCIENCES **53,** 224–236 (2000)
Copyright © 2000 by the Society of Toxicology

DNA-Damaging Effects of Genotoxins in Mixture: Modulation of Covalent Binding to DNA

Matthew K. Ross, Boctor Said, and Ronald C. Shank[1]

Environmental Toxicology Program, Department of Community and Environmental Medicine, University of California at Irvine, Irvine, California 92697–1820

Received April 6, 1999; accepted September 27, 1999

Modulation of DNA adduct formation by pre-existing adducts was examined in synthetic oligonucleotides and genomic DNA (calf thymus); genotoxins studied were *N*-acetoxy-acetylaminofluorene (N-AcO-AAF), aminofluorene (AF), aflatoxin B_1-8,9-epoxide (AFB_1-8,9-epoxide), and dimethylsulfate (DMS). Oligodeoxynucleotides containing either guanine-C8-AAF (Gua-C8-AAF) or Gua-C8-AF adducts and a neighboring unadducted guanine (G) (target G), located 1, 2, or 4 nucleotides from the adduct, were reacted, as single- (ss) or double-stranded (ds) substrates, with dimethylsulfate (DMS) or AFB_1-8,9-epoxide. A modified Maxam-Gilbert technique showed that the presence of the AAF adduct lowered the extent to which AFB_1-8,9-epoxide, but not DMS, reacted with target G. Binding of AFB_1-8,9-epoxide to the target G was attenuated (≥5-fold) when the target was located immediately adjacent to an AAF, but not AF, adduct in ds-DNA. Reaction with AFB_1-8,9-epoxide increased when the target G was located 2 or 4 nucleotides from the AAF adduct. Pretreatment of calf thymus DNA with AAF (0–1.8% nucleotides modified) reduced levels of Gua-N7-AFB_1 adducts formed after subsequent treatment with AFB_1-8,9-epoxide. Pretreatment of calf thymus DNA with AFB_1 did not alter levels of adducts formed after subsequent treatment with *N*-AcO-AAF. The supposition that aflatoxin B_1-binding to DNA may be altered by conformational changes in the helix, due to the presence of a pre-existing AAF adduct, is supported by the absence of an effect by AF and confirmation of local denaturation of the oligomer helix by use of chemical probes hydroxylamine and diethylpyrocarbonate. Nonetheless, the importance of changes in the nucleophilicity of neighboring nucleotides and local steric effects cannot be ruled out.

Key Words: **guanine adducts; aflatoxin B_1 (AFB_1); AFB_1-8,9-epoxide; *N*-acetoxy-acetylaminofluorine (N-AcO-AAF); dimethylsulfate (DMS); mixtures.**

A little explored area of DNA adduct research is the study of mechanisms by which genotoxins may interact to modulate adduct formation and subsequent mutagenesis. The toxicology of chemical mixtures has important public health implications, since humans are exposed to multiple chemicals in occupational settings, as a result of lifestyle choices and environmental exposures. Cancer and developmental toxicity risks for genotoxins in mixture are generally estimated by assuming additivity of the components, yet two or more genotoxins acting sequentially or simultaneously may present a greater or lesser risk than could be predicted by assuming additivity. Indeed, nonadditive mutagenic effects have been described for combinations of *N*-acetoxy-acetylaminofluorene (*N*-AcO-AAF) and aflatoxin B_1-8,9-epoxide (AFB_1-8,9-epoxide) in the *Salmonella* reversion assay (Said *et al.*, 1999).

There is considerable evidence that adduct formation is non-random and occurs with considerable specificity (Hemminki, 1993; Warpehoski and Hurley, 1988). DNA sequence-context is important in influencing the specificity of adduct formation, and neighboring nucleotides can significantly influence the reactivity of guanine toward genotoxins. GC-rich regions and runs of contiguous guanines in the genome have a higher probability of being targeted by genotoxins (Mattes *et al*., 1986; 1988; Said and Shank, 1991). Additionally, 5-methylcytosine in CpG sites can modulate the distribution of guanine adduct formation by N-methyl-N-nitrosourea (MNU; Mathison *et al*., 1993; Ross *et al*., 1999), benzo(a)pyrene diolepoxide (BPDE; Denissenko *et al*., 1997), *N*-AcO-AAF and AFB_1-8,9-epoxide (Chen *et al*., 1998). *In vitro* experiments have described enhanced binding of BPDE to guanines in *H-ras* codons 12 and 13 (Dittrich and Krugh, 1991) as well as to guanines in *p53* codons 157, 248, and 273 (Denissenko *et al*., 1996) and preferential binding of AFB_1-8,9-epoxide to guanines in the *p53* codon 249 (Puisieux *et al*., 1991). Mutational hot spots found in the p53 gene in human lung (Denissenko *et al*., 1996) and liver (Hsu *et al*., 1991) tumors correlated with the sequence-specific reactivity of the etiologically linked chemical carcinogens. Therefore, genotoxins (singly or in combination) may modify DNA bases in close proximity to one another and this may have important physiological effects, e.g., in DNA damage recognition and repair mechanisms (Bridges and Brown, 1992; Jones *et al*., 1998).

Drugs (Bailly *et al*., 1996; Chaires, 1985; Walker *et al*., 1985a,b) and genotoxins such as *N*-hydroxy-AAF and 4-nitroquinoline-1-oxide (Winkle and Krugh, 1981) and AFB_1 (Stone *et al*., 1988), non-covalently bind with a high degree of coop-

[1] To whom correspondence should be addressed. Fax: (949) 824-2793. E-mail: rcshank@uci.edu.

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

erativity to a small minority of high affinity sites on the DNA lattice. The precovalent binding affinity of these agents can dictate the ultimate specificity of covalent modification of the genome (Warpehoski and Hurley, 1988). "Clustering" of ligands within a localized region of the genome may occur and high affinity binding sites for different ligands could overlap one another. This overlapping could be biologically relevant, particularly since the cooperative phase of binding isotherms described for several drugs and carcinogens (Bailly *et al*., 1996; Chaires, 1985; Rosenberg *et al*., 1986; Stone *et al*, 1988; Walker *et al*, 1985a,b; Winkle and Krugh, 1981) saturate at low levels of bound ligand. This may apply to environmental exposures to genotoxins.

Previously, we have shown that prior treatment of DNA with both small (MNU) and bulky (AFB_1, AAF, benzo(a)pyrene) genotoxins inhibited the formation of DNA adducts after subsequent treatment of the adducted DNA with a second genotoxin of different chemical structure (Said *et al*., 1995; Said and Shank, 1991); this effect is not due to saturation of available guanines by the initial carcinogen treatment (Said *et al*., 1995). These binding alterations could be caused by (a) steric effects due to the initial DNA adduct, (b) conformational changes within the DNA polymer induced by the pre-existing adduct, and/or (c) decreases in the nucleophilicity of reactive centers in the DNA molecule (e.g., the *N7* position of guanine). These possible mechanisms are addressed in the current experiments that investigated the effect of pre-existing AAF adducts on the reactivity of neighboring guanine bases (target guanines) toward both small and bulky carcinogens.

In the present study, defined sequences of DNA containing a single adduct were used to determine what effect the adduct had on the reactivity of neighboring target guanines. This allowed control of adduct distribution, site-selectivity, and neighboring base content within the sequence of DNA such that modulations of target guanine reactivity could be directly attributed to the presence of initial adduct and not to nearest-neighbor effects, saturation of available sites by the initial carcinogen, or other uninterpretable effects.

MATERIALS AND METHODS

Chemicals and reagents. Calf thymus DNA (Type I), aflatoxin B_1 and piperidine were obtained from Sigma (St. Louis, Mo). The DNA was mechanically sheared and contaminating RNA and proteins were removed with RNase A and phenol extraction. The following oligodeoxynucleotides were obtained from Genosys Biotechnologies (The Lakes, TX): 5′-TTTTG$_1$G$_2$TTTT-3′ (10m0T), 5′-TTTTG$_1$TG$_2$TTT-3′ (10m1T), 5′-TTTTG$_1$TTTG$_2$T-3′ (10m3T), 5′-CCCCG$_1$G$_2$CCCC-3′ (10m0C), 5′-CCCCG$_1$CCCG$_2$C-3′ (10m3C), and their complementary strands. The shorthand notation for these oligomers refers to their size (e.g., 10m = 10-mer) as well as the number of intervening nucleotides between the two guanine residues (e.g., 0T, 1T, and 3T = the number of thymidines interspersed between the guanines). Thymidine or cytosine nucleotides were interspersed between guanines to control for nearest-neighbor effects on guanine reactivity. Oligomers were purified by HPLC when necessary. Dimethylsulfate (DMS) was obtained from Eastman Kodak Chemicals (Rochester, NY). *N*-acetoxy-2-acetylaminofluorene (*N*-AcO-AAF) was obtained from Chemysn (Lenexa, KS). Aflatoxin B_1-8,9-epoxide (AFB_1-8,9-epoxide) was synthesized using the method of Iyer and Harris (1993); the final product was dissolved in acetone and stored at –30°C. The stability of the epoxide was repeatedly checked by determining its ability to react with 10m0T (control reaction as described below). DMS solutions were prepared fresh before each experiment by mixing appropriate amounts in ethanol. Authentic standards of dG-*C8*-AAF and dG-*C8*-AF were prepared as described by Kriek and coworkers (1967). Gua-*N7*-AFB_1 was prepared as described by Essigmann and coworkers (1977). [γ-^{32}P]ATP (4500Ci/mmol) was obtained from ICN Chemicals (Costa Mesa, CA). T4 DNA kinase was from GIBCO-BRL (Bethesda, MD). All other chemicals and reagents not explicitly stated were reagent grade or better.

Preparation of DNA oligomers containing site-specific Gua-C8-AAF-and AF-adducts. Single and double stranded oligomers (~50–100 μg of 10m0T, 10m1T, 10m3T, 10m0C, and 10m3C) were treated with a molar excess of *N*-AcO-AAF in 10 mM sodium citrate buffer, pH 7.2 (total volume of 200 μl), incubated up to 12 h at 37°C, recovered free of unreacted carcinogen and purified by reversed-phase HPLC (described below). Aliquots were taken to determine oligomer yields and for 5′-end labeling (~250–500 ng). AAF adducts were converted to AF adducts by treating oligomers (10m0C.1AAF or 10m0C.2AAF) with basic mercaptoethanol (Zhou and Romano, 1993).

Characterization of site-specific AAF adducts in DNA oligomers. Oligonucleotide constructs (Fig. 1) were characterized by 3 different methods. First, oligomers containing AAF adducts were initially characterized by showing that their UV spectra agreed with literature reports (Koehl *et al*., 1989; Kriek *et al*., 1967). Second, approximately 500 ng of each purified oligomer was 5′ end-labeled with [γ-^{32}P]ATP and T4 kinase and analyzed by a modification of the Maxam-Gilbert (1977) piperidine method to determine adduct position. Third, selected oligonucleotides containing site-specific AAF or AF adducts and standards of dG-*C8*-AAF or dG-*C8*-AF were hydrolyzed in either anhydrous trifluoroacetic acid or 0.1 M HCl to liberate free Gua-*C8*-AAF or Gua-*C8*-AF adducts. Hydrolysates were analyzed by reversed-phase HPLC and ultraviolet detection. Identification was made by co-elution with authentic standards of Gua-*C8*-AAF or Gua-*C8*-AF.

Duplex DNA formation. Complementary strands of oligomers were annealed by mixing each in a 1:3 molar ratio (10mxT: 10mxA;$\times$ = 0, 1, or 3) in a total volume of 20 μl of annealing buffer (40mM Tris–HCl, pH 7.5; 20mM $MgCl_2$; 50mM NaCl). Samples were heated at 37°C for 15 min to completely denature the two strands, followed by a 15°C incubation for 30 min for annealing to take place, then cooling on ice for >1 h. Double-stranded 10m0C and 10m3C were prepared similarly by heating complementary oligonucleotides at 70°C (2 min) and slowly cooling over 1 h to ~35°C; the annealed DNA was then placed on ice (0°C) for ~1 h. In all cases, subsequent manipulations of double-stranded oligomers were done at 4°C to prevent strand dissociation. Duplex DNA formation was confirmed by electrophoretic separation of double- and single-stranded DNA on a 20% non-denaturing polyacrylamide gel (run at 250 V for 24 h at 4°C), as described previously (Suh *et al*., 1995), and comparing their different mobilities.

Reactions of oligomers with chemical carcinogens. End-labeled oligonucleotides (single- or double-stranded) containing site-specific adducts (~60 ng) were reacted with DMS (final conc. 10 or 53 mM) or AFB_1-8,9-epoxide (10 or 100 μM) in 10 mM sodium citrate buffer, pH 7.2 (final volume of 200 μl per reaction) and the final solvent concentration was ≤10% (v/v). Single-stranded DNA was reacted at 37°C for 30 min and double-stranded DNA at 4°C for 60 min. Reactions were stopped by either ethanol precipitation or by organic extraction. Positions of adducts in the oligonucleotides were determined by Maxam-Gilbert analysis using 1 M piperidine (90°C) for 30 min or 8 h. The extent of reaction at the target guanine (G_1 or G_2) was determined by densitometry using the freeware program NIH Image version 1.60.

HPLC analysis of products derived from reaction of ds10m0T, ds10m0T.1AAF, and ds10m0T.2AAF with AFB_1-8,9-epoxide. Non-radiolabeled oligonucleotides 10m0T, 10m0T.1AAF, and 10m0T.2AAF (1 μg of each) were individually mixed with a 3-fold molar excess of its complementary

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

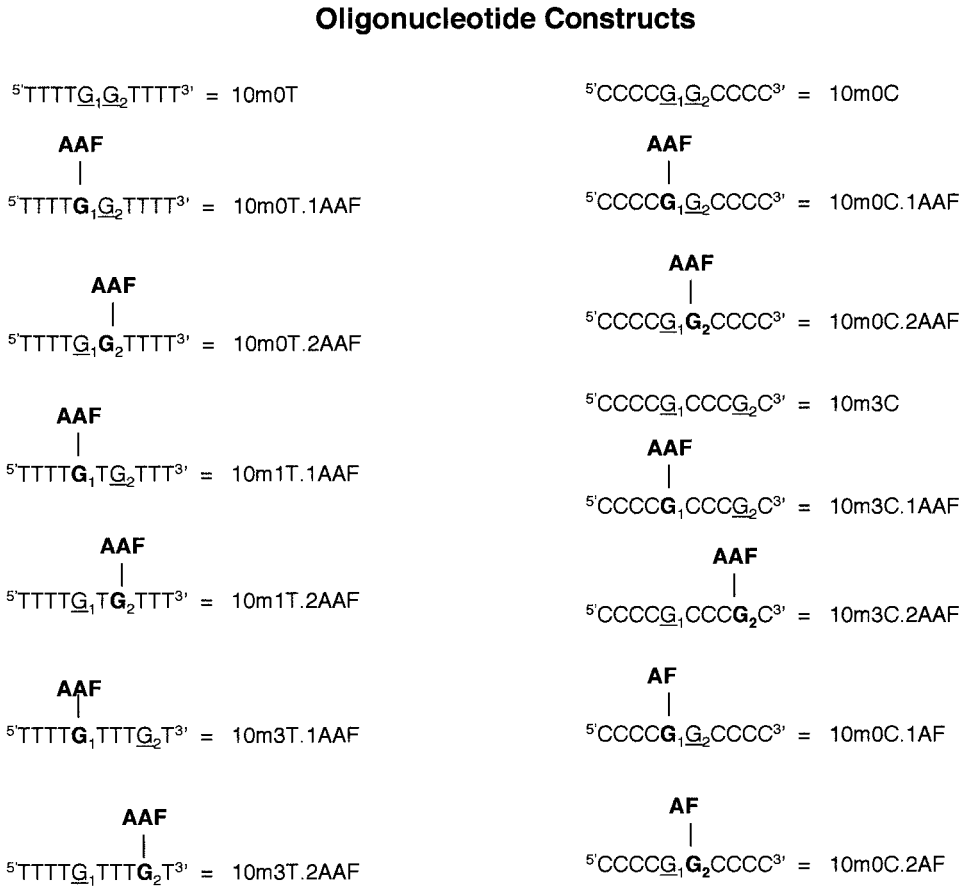

FIG. 1. Synthetic oligonucleotide constructs containing site-specific adducts used in study.

strand, and annealed. The duplex DNA was reacted with AFB_1-8,9-epoxide exactly as described above for the radiolabeled oligomers (100 μM final concentration). Following extraction of the reaction mix with ethyl acetate, the aqueous layers were analyzed by reversed-phase HPLC to determine the extent of reaction at the target guanine by the epoxide. Eluted oligomers were detected by UV absorption at 260 nm.

Chemical probe analysis of DNA conformation. Conformation of duplex oligonucleotides, 10m0C:*10m0G and 10m0C.1AAF:*10m0G (*denotes 5′-^{32}P labeled oligonucleotide) was analyzed by the chemical probe method of Johnston and Rich (1985) using hydroxylamine and diethylpyrocarbonate.

In vitro treatment of calf thymus DNA with combinations of N-AcO-AAF and AFB_1-8,9-epoxide. Pretreatment of DNA with N-AcO-AAF was followed by reaction with AFB1-8,9-epoxide. Calf thymus DNA (0.9 mg/ml) in 10 mM sodium citrate buffer, pH 7.2 (total volume 200 μl) was reacted (in triplicate) in the presence of variable amounts of *N*-AcO-AAF (250–500 μM) or vehicle at 37°C for various times up to 21 h to create increasing levels of AAF adducted DNA. Following initial treatment with *N*-AcO-AAF, each sample was extracted with ethyl acetate 3–4 times and the DNA was precipitated with ethanol. Following centrifugation, the DNA pellet was washed with 100% ethanol and dried. After suspension in 200 μl 10mM sodium citrate buffer, pH 7.2, each sample was pre-incubated at 37°C for 10 min followed by treatment with a final concentration of 100 μM AFB_1-8,9-epoxide. Reaction mixtures were incubated for 30 min at 37°C followed by organic solvent extraction and ethanol precipitation as above. After washing with ethanol and drying the pellets for 5–10 min in a Speed-Vac centrifuge, the DNA was resuspended in 180 μl of water and heated for 5–10 min at 37°C to ensure complete dissolution. Samples were subjected to mild acid hydrolysis (0.1 N HCl, 70°C, 45 min) to liberate both Gua-*C8*-AAF and Gua-*N7*-AFB_1 adducts from the DNA. Hydrolysates were cooled, filtered (0.45 μm) and analyzed by reversed-phase HPLC (Supelco LC-18, 5 μm, 250 × 4.6 mm, i.d., column; Supelco, Bellefonte, PA). Purines were eluted with 5% methanol in 0.01 M potassium phosphate, pH 7.0 for 5 min, followed by a linear gradient of 5–80% (v/v) methanol (in 0.01 M potassium phosphate buffer, pH 7.0) over 30 min and then holding at 80% methanol for 10 min; the flow rate was 1.0 ml/min. Eluted compounds were detected by ultraviolet absorbance (260 nm).

RESULTS

Preparation of DNA oligomers containing Gua-C8-AAF adducts at G_1 or G_2 positions. The modified 10-mers (Fig. 1), prepared by reacting *N*-AcO-AAF with unmodified oligomers, were isolated by HPLC; separation of the oligomer products is shown in Figure 2A. The major fractions were characterized as follows: (1) UV spectra of the adducted 10-mers (Fig. 2B) were similar to literature spectra of oligonucleotides containing *C8*-AAF guanine adducts (Essigmann *et al*., 1977), (2) a modified Maxam-Gilbert sequencing technique that enabled location of the site-specific adduct to be determined (Fig. 2C), and (3) acid hydrolysis of selected adducted 10-mers and identification of released modified guanine bases by HPLC (data not shown).

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

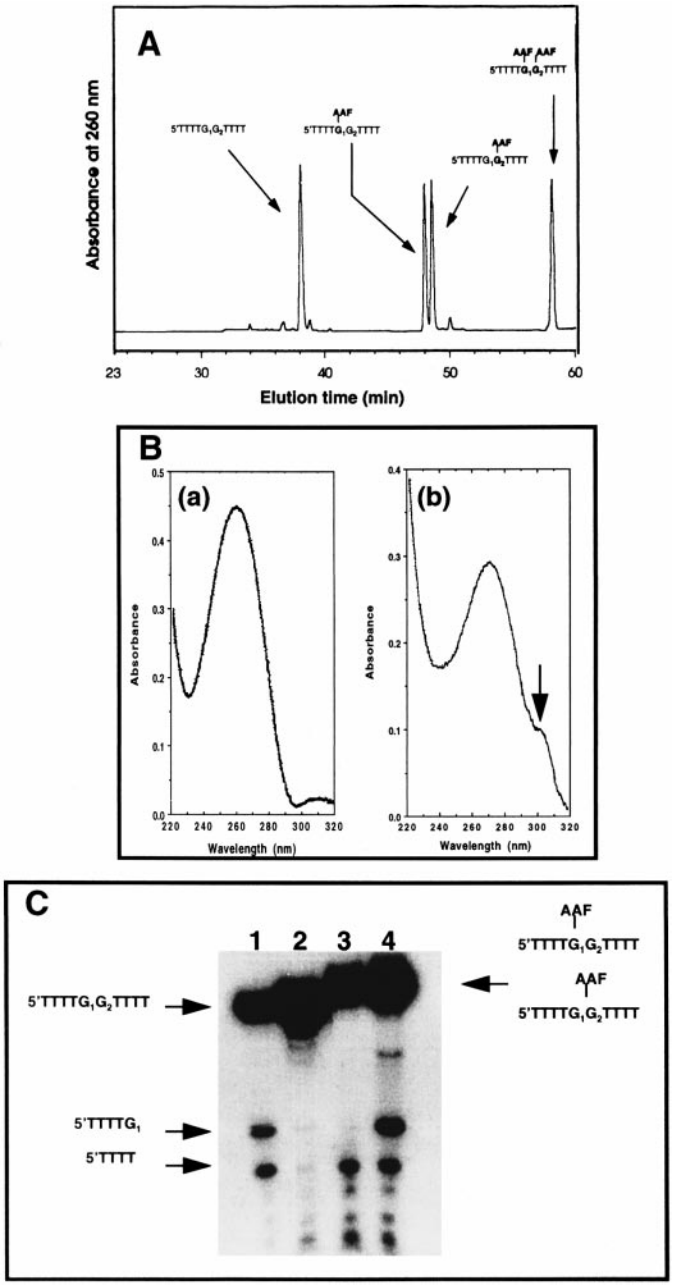

FIG. 2. Purification and analysis of unadducted 10m0T and AAF-adducted 10m0T isomers (as defined in Fig. 1). **(A)** HPLC fractionation of the aqueous phase following reaction with N-AcO-AAF and 10m0T. Mobile-phase: elution gradient of 5–50% (v/v) methanol (in 0.05 M triethylammonium phosphate, pH 7.0) over 60 min with a flow rate of 1.0 ml/min. **(B)** UV spectra of unadducted 10m0T (panel a) and adducted 10m0T.1AAF (panel b). The arrow denotes a shoulder at 305 nm that is characteristic of the presence of an AAF moiety within the oligonucleotide. **(C):** The designated fractions in **A** above were isolated and 5′ end-labeled with [γ-^{32}P]ATP and fraction identities confirmed by 1 M piperidine cleavage and subsequent 20% denaturing polyacrylamide gel electrophoresis. Lane 1: unmodified 10m0T, DMS treatment followed by 1 M piperidine; Lane 2: unmodified 10m0T, treated with 1 M piperidine only; Lane 3: 10m0T.1AAF, treated with 1 M piperidine; Lane 4: mixture of 10m0T.1AAF and 10m0T.2AAF that resulted from incomplete fractionation of the two products, treated with 1 M piperidine.

Reaction of adducted DNA oligomers with a second carcinogen. Modified 10-mers containing a single AAF or AF adduct were reacted with AFB_1-8,9-epoxide or dimethylsulfate to determine the effect of the first adduct on the formation Gua-N7-AFB_1 or Gua-N7-Me adducts.

Gua-C8-AAF influences alkylation by AFB_1-8,9-epoxide. Reaction of AFB_1-8,9-epoxide (10 and 100 μM) with the target guanine in 10m0T.1AAF or 10m0T.2AAF was inhibited in both double- and single-stranded DNA (Fig. 3; data not shown for single-stranded DNA). The inhibition was significantly more pronounced in double-stranded DNA than single-stranded. Figure 3A shows results obtained when piperidine incubation lasted for 30 min instead of 8 h. In this instance, cleavage of DNA at sites modified with AAF adducts (lanes 4–9) was barely detectable since AAF adducts do not destabilize guanine to the same degree as AFB_1 adducts (Johnson, *et al.*, 1987); however, cleavage of DNA modified with AFB_1 did occur (lanes 1–3). Attenuation of AFB_1 epoxide binding due to the presence of an AAF adduct is clearly evident (lanes 4–9). Densitometry of autoradiograms of polyacrylamide gels showed that reactivity of the target guanine in 10mOT.1AAF (i.e., G_2) towards 100 μM AFB_1-8,9-epoxide was reduced ~4-fold when compared with the same guanine in the unadducted control oligomer (10m0T) (Fig. 3B, compare lanes 5 and 3; inset) following 8 h piperidine incubation. Similarly, the reactivity of the target guanine in 10m0T.2AAF (i.e., G_1) towards AFB_1-8,9-epoxide (at the same concentration) was decreased about 19-fold when compared to its control guanine (Fig. 3B, inset).

Similar results that confirmed the above binding modulations were obtained when unlabeled double-stranded oligonucleotides, either unmodified or modified with AAF adducts (ds10m0T, ds10m0T.1AAF, and ds10m0T.2AAF), were reacted with AFB_1-8,9-epoxide (100 μM) and the resulting adducted oligonucleotides separated by HPLC instead of being subjected to piperidine treatment (Fig. 4).

Site-specific Gua-C8-AAF adducts do not mask reactivity of target guanine. dG-*C8*-AAF adducts are less sensitive to piperidine cleavage than are *N7*-guanine adducts, and chemical cleavage of DNA containing AAF adducts at the *C8* position of guanine is not quantitative. The presence of an AAF-adduct located on the 5′ guanine does not mask the reactivity of the 3′ guanine. As evidence of this, Figure 5 shows the piperidine cleavage products of 10m1T.1AAF, 10m1T.2AAF, and 10m1T.DA (5′-TTTTG_1(AAF)TG_2(AAF)TTT) after a 30-min incubation at 90°C. The intensity of the 2 bands derived from AAF adducts on the 10m1T.DA oligomer (Lane 3) were approximately equal (G_1: 9.1% of normalized radioactivity; G_2: 8.8% of normalized radioactivity); this is evidence that a G_1 adduct does not alter the ability to detect the G_2 adduct by the modified Maxam-Gilbert method used in these studies. A recent study (Raney *et al.*, 1990) reported that cleavage of

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

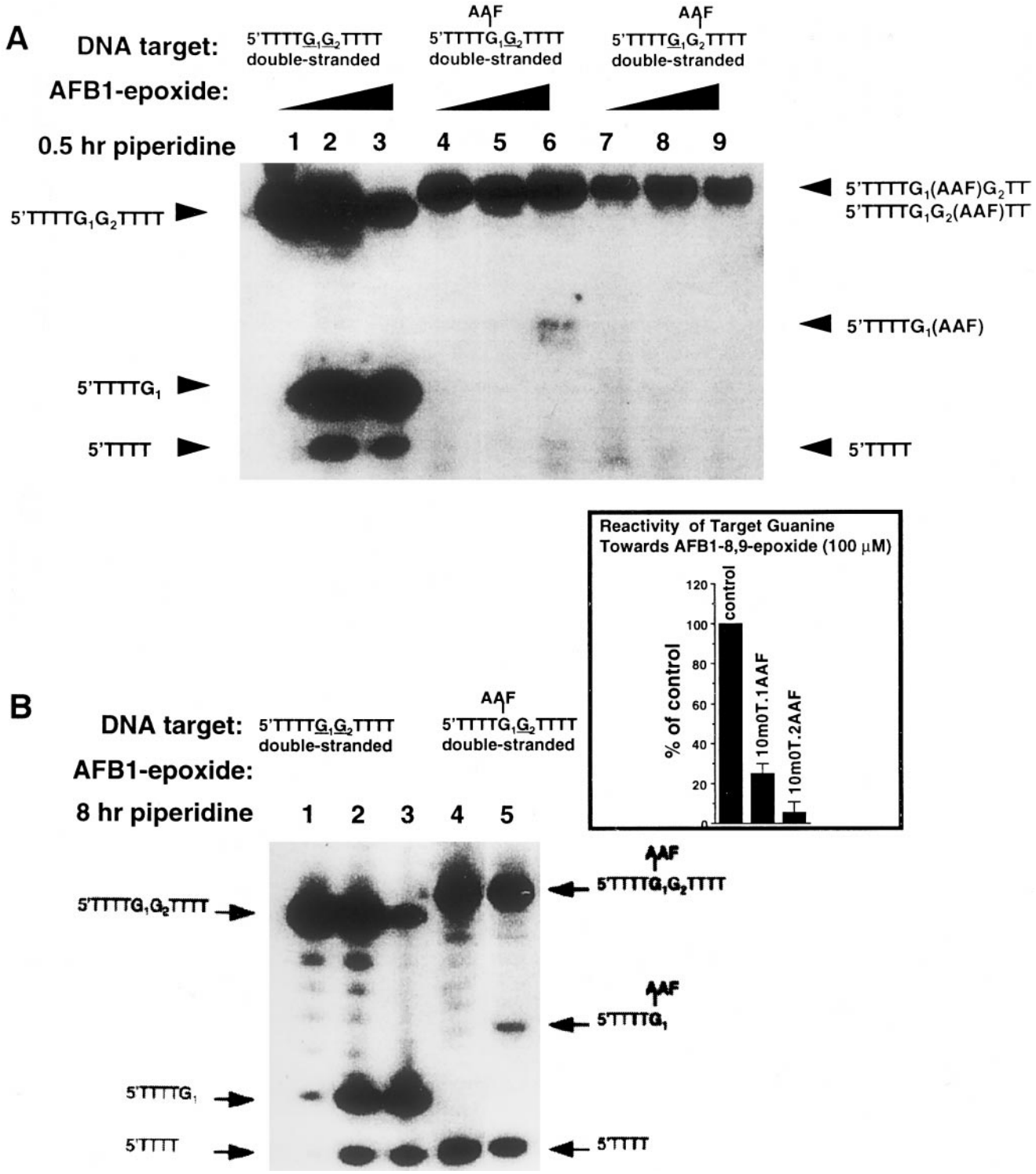

FIG. 3. Effect of N-AcO-AAF adduct on AFB_1-8,9-epoxide formation in 10-mer oligonulcetides. Autoradiogram of a denaturing 20% PAGE of cleavage products following exposure of double-stranded 5′-end-labeled 10m0T, 10m0T.1AAF, and 10m0T.2AAF to AFB_1-8,9-epoxide and subsequent 1 M piperidine incubation for 30 min (Panel A) and 8 h (Panel B). Details of reaction conditions are given in Materials and Methods. **ds10m0T** Lane 1: control (no carcinogen treatment); Lane 2: AFB_1-8,9-epoxide (10 μM); Lane 3: AFB_1-8,9-epoxide (100 μM). **ds10m0T.1AAF** Lane 4: control (no carcinogen treatment); Lane 5: AFB_1-8,9-epoxide (10 μM, Panel A; 100 μM, Panel B); Lane 6: AFB_1-8,9-epoxide (100 μM). **ds10m0T.2AAF** Lane 7: control (no carcinogen treatment); Lane 8: AFB_1-8,9-epoxide (10 μM); Lane 9: AFB_1-8,9-epoxide (100 μM). **Panel B Inset:** Relative amounts of Gua-N7-AFB_1 formed on target guanine, expressed as percent of control reaction [i.e., reaction of AFB_1-8,9-epoxide with unmodified 10m0T]. The column denoted by 10m0T.1AAF represents the relative band intensity of 5′$TTTTG_1$(AAF) (lane 5) divided by the band intensity of 5′-$TTTTG_1$ (lane 3) multiplied by 100. Background band intensities (determined from

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

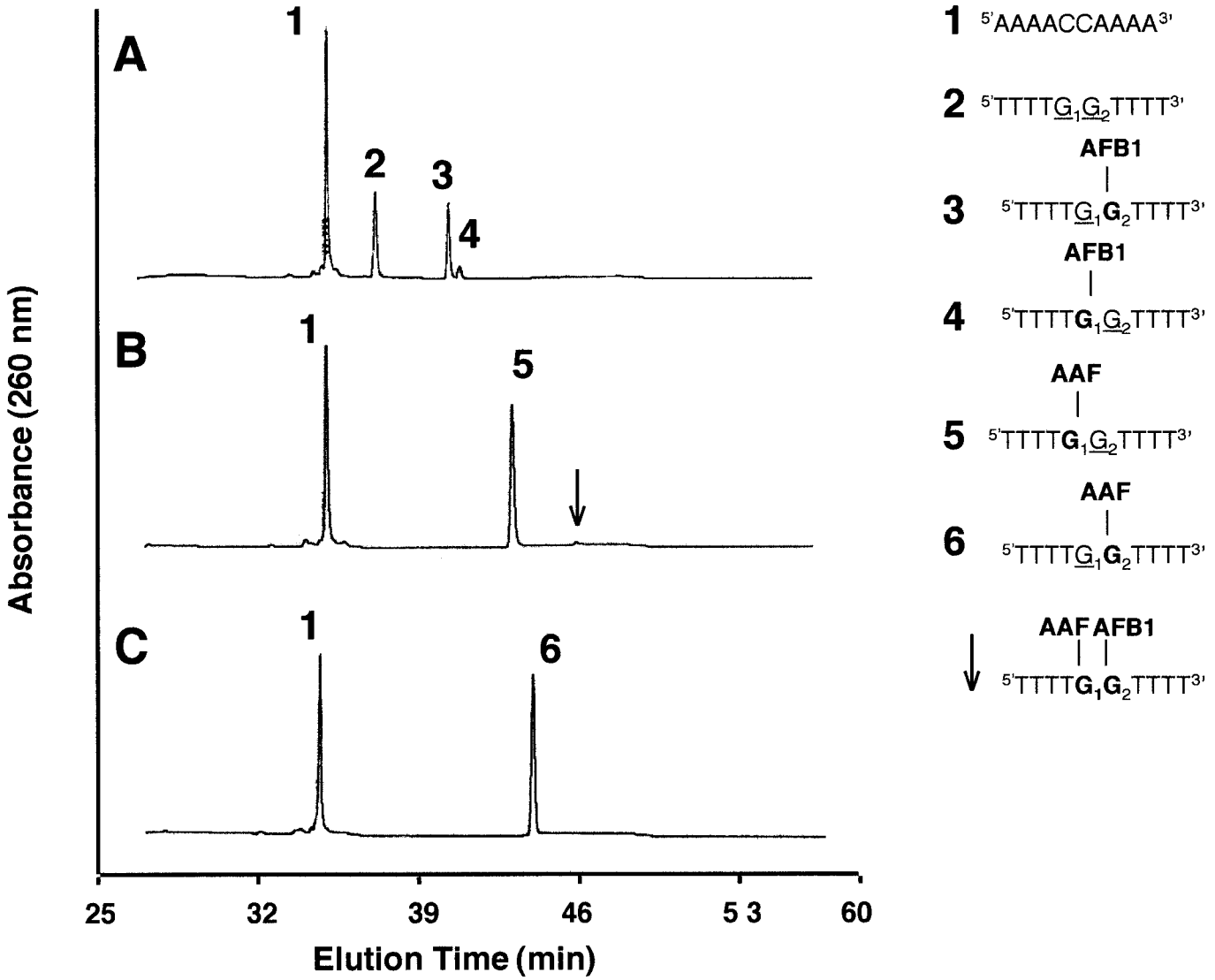

FIG. 4. HPLC chromatograms of duplex d(TTTTG$_1$G$_2$TTTT)•d(AAAACCAAAA) **(A)**, duplex d(TTTT**G$_1$**(**AAF**)G$_2$TTTT)•d(AAAACCAAAA) **(B)**, and d(TTTTG$_1$**G$_2$**(**AAF**)TTTT)•d(AAAACCAAAA) **(C)** following reaction with 100 μM AFB$_1$-8,9-epoxide. Peak numbers in each chromatogram are identified to the right. Details of reaction are given in Materials and Methods. HPLC conditions are the same as described in Fig. 3. Only the region on the chromatogram between 25 and 60 min is shown. The arrow (↓) designates the elution time of dual adducted oligomer, d(TTTT**G$_1$**(AAF)**G$_2$**(AFB$_1$)TTTT). To quantitate the amount of dual adducted oligomer (↓) formed in chromatogram **B**, the normalized % of UV absorbing material eluted as the dual adduct (3.4%), was divided by the normalized % of UV absorbing material eluted as peak 3 (43.5%) (which represents the control level of modification of G$_2$ in the non-AAF adducted oligomer) in chromatogram **A**. The level of dG-N7-AFB$_1$ modification found at G$_2$, in an oligomer that contained a site-specific AAF adduct, was ~8% of the AFB$_1$-derived modification level found for control G$_2$. Note that in **C**, no dual adducted oligomer was detected.

dG-*N7*-AFB$_1$ adducts by piperidine at 90°C after 30 min was quantitative.

When both 10m0T and 10m0T.1AAF were treated with 100 μM AFB$_1$-8,9-epoxide and incubated with piperidine for 30 min, the intensity of the band on the gel due to dG-*N7*-AFB$_1$ adduct at G$_2$ was attenuated in 10m0T.1AAF as compared to control 10m0T (Fig. 3A). Cleavage due to the AAF adduct is barely detectable in this set of reactions, but the presence of the AAF adduct in the oligomer is confirmed by the observation that uncleaved oligomer, with AAF adduct, migrates more

control reactions; lanes 1 and 4) were subtracted out. The relative amount of Gua-N7-AFB$_1$ formed on target guanine (G$_1$) in 10m0T.2AAF is also shown. Reactivity of target guanines (i.e., band intensities at either G$_1$ or G$_2$) in control oligonucleotides were set at 100%. Column denoted as control is shown for comparison purposes. (Error bars represent the SD of 3 separate reactions).

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

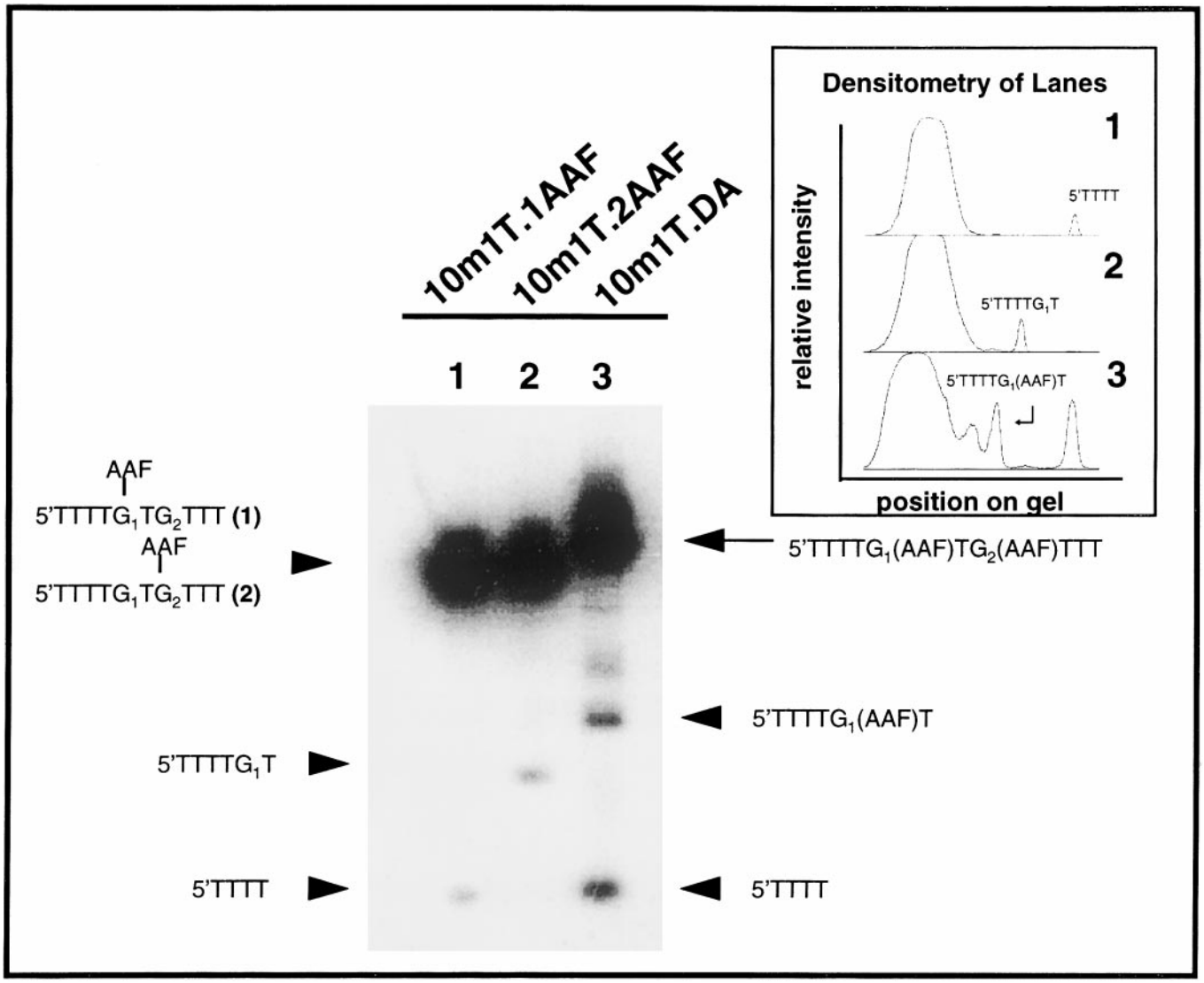

FIG. 5. The presence of two AAF adducts in a single oligonucleotide visualized by piperidine treatment of 5′ end-labeled DNA. Autoradiogram of a denaturing 20% PAGE of cleavage products from piperidine treatment of single-stranded end-labeled, **10m1T.1AAF** (Lane 1), **10m1T.2AAF** (Lane 2), and **10m1T.DA** (dual adducted oligomer) (Lane 3) is shown. **Inset:** Graphical representation of densitometry of lanes in autoradiogram.

slowly in the 20% polyacrylamide gel than in the oligomer without AAF adduct. This is consistent with the higher molecular weight of the AAF adducted oligomer as compared to unmodified oligomer, and confirms that the decrease in band intensity on the autoradiograms at G_2, after treatment with AFB_1-epoxide, is due to modulation of G_2 reactivity by the presence of an AAF adduct found at G_1.

Increasing distance between Gua-C8-AAF adduct and target guanine. Double-stranded oligonucleotides, containing AAF adducts at G_1 or G_2 with intervening nucleotides (either 1 or 3) between the site-specific adduct and the target guanine, were also reacted with AFB_1-8,9-epoxide. The effect of the AAF adduct on the formation of the AFB_1 adduct decreased with increasing separation of the target guanine (Fig. 6). The reactivity of the target guanine towards AFB_1-8,9-epoxide (100 μM final concentration) was moderately attenuated, ~3-fold in 10m1T (both isomers gave similar results; gel data not shown) and ~1.5-fold in 10m3T (both isomers gave similar results; gel data not shown), when compared to the strong attenuation that occurs when the AAF adduct was located immediately adjacent to the target guanine (~4-fold for 10m0T.1AAF and ~19-fold for 10m0T.2AAF). Because of the strong negative influence of the 5′ and 3′ nearest neighboring thymidines surrounding the target guanine in 10m3T oligos and because of unexplained high background that was consistently found after piperidine treatment of control 10m3T, another double-stranded oligonucleotide was constructed (10m3C; see Fig. 1). This oligonucleotide contained cytosines as the intervening nucleotides

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

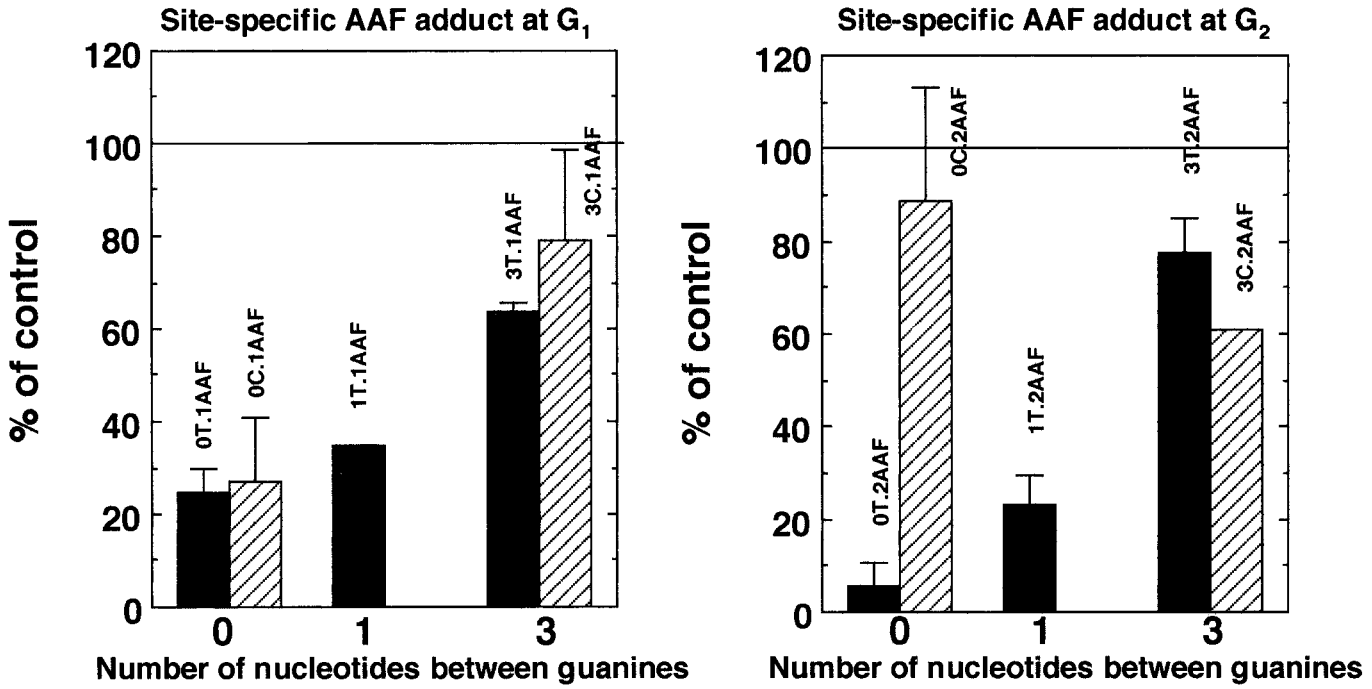

FIG. 6. Reactivity of target guanine towards AFB_1-8,9-epoxide (100 μM) as a function of increasing distance between AAF adduct and target guanine. A 10-mer oligomer containing one AAF adduct and one target guanine separated from the adducted guanine by 0, 1, or 3 pyrimidine oligonucleotides was reacted with 100 μM AFB_1-8,9-epoxide. The extent of interaction between the epoxide and the target guanine was determined by modified Maxam-Gilbert sequencing, PAGE, and densitometry. Dotted lines indicated 100% of control, i.e., the level of AFB_1 adduct formation achieved in the absence of a pre-existing AAF adduct.

between the site-specific AAF adduct and the target guanine. When this oligomer was annealed with its complement and reacted with AFB_1-8,9-epoxide, results similar to the inhibition seen with AAF-modified 10m3T oligomers were observed (an ~1.5-fold decrease) (Fig. 6). It therefore appeared that the reactivity of the target guanine towards AFB_1-8,9-epoxide increased as a function of the number of intervening nucleotides between the target guanine and the AAF adduct.

Comparison of AAF adduct with AF adduct on target guanine reactivity. DNA conformation may play a primary role in aflatoxin B_1 binding alterations. To investigate this, 10m0C oligomers containing an aminofluorene (AF) adduct instead of an acetylaminofluorene (AAF) adduct were constructed (Fig. 1). Gua-*C8*-AF adducts do not disrupt normal Watson-Crick base pairing between G:C base pairs within the DNA helix as opposed to Gua-*C8*-AAF adducts, which are inserted into the helix and the guanine moiety displaced out such that typical base-pairing is disrupted (Iyer *et al*., 1994; Pullman and Pullman, 1981; Raney *et al*., 1993). Therefore, AF and AAF adducts induce different conformational states within DNA. When oligomers 10m0C.1AF and 10m0C.2AF were reacted with AFB_1-8,9-epoxide, the target guanine was more reactive than the same guanine in oligonucleotides containing AAF adducts; particularly the target guanine in 10m0C.1AF (Fig. 7; compare lanes 6 and 12; compare lanes 9 and 16). Additionally, results from the 10m0C.1AAF treatment with AFB_1-8,9-epoxide confirmed the inhibition of aflatoxin B_1 binding seen when 10m0T.1AAF was similarly treated (compare Fig. 7, lane 6 with Fig. 3B, lane 5). The target guanine (G_2) of 10m0T.1AAF was slightly more reactive than that of 10m0C.1AAF. However, the reactivity of the target guanine in 10m0C.2AAF was significantly greater than the same guanine in 10m0T.2AAF. This may be due to the difference in nucleotide type that neighbors the target guanine on the 5′ side.

Distortion of local DNA conformation caused by the presence of a site-specific AAF adduct. Chemical probes were used to confirm that the AAF adduct caused local distortions in the helix. When unlabeled 10m0C.1AAF was mixed with its 5′ ^{32}P-end-labeled complement (10m0G) and treated with the probes diethylpyrocarbonate or hydroxylamine, there was significant hyperreactivity of both cytosines in the complementary strand towards hydroxylamine, with the cytosine opposite the unmodified guanine demonstrating a greater degree of enhanced reaction (Fig. 8). The band intensities due to hyperreactivity of cytosines towards hydroxylamine were approximately 2-fold greater than the control cytosines found in the oligonucleotide not containing an AAF adduct, as determined by densitometry. In addition, there appeared a slight enhancement in the reaction of guanines in the complementary strand toward the probe diethylpyrocarbonate. These results are consistent with the observations of Belguise-Valladier and coworkers (1991), who described a site of local denaturation around an AAF adduct found in a 162-bp fragment of DNA.

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

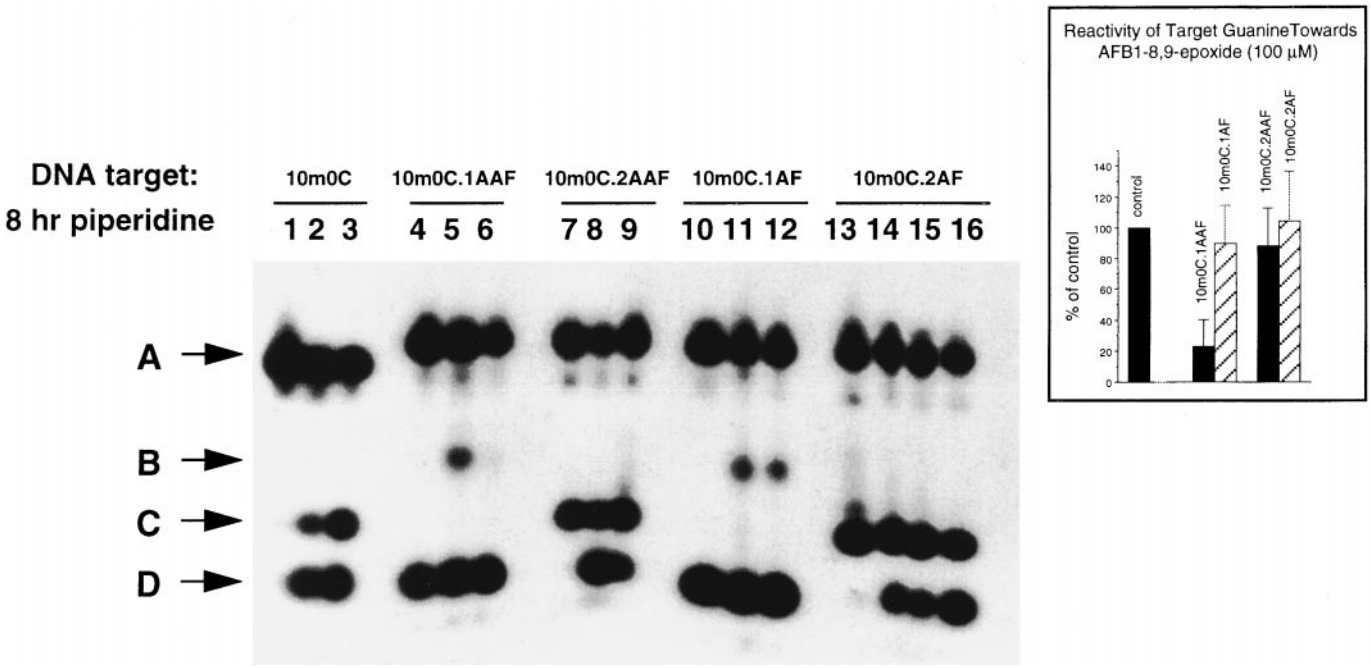

FIG. 7. Autoradiogram of a denaturing 20% PAGE of cleavage products following exposure of double-stranded end-labeled 10m0C, 10m0C.1AAF, 10m0C.2AAF, 10m0C.1AF, and 10m0C.2AF (as indicated) to either DMS or AFB_1-8,9-epoxide and subsequent 1 M piperidine incubation (8-h incubation). Details of reaction conditions are given in text. Lanes 1, 4, 7, 10, and 13: control (no carcinogen treatment); Lanes 2, 5, 8, 11, and 14: DMS (50 mM); Lanes 3, 6, 9, 12, and 16: AFB_1-8,9-epoxide (100 μM). (Lane 15 is a repeat load of the sample in Lane 14. The arrow denoted by **A** marks the location of full-length oligonucleotide (i.e., material resistant to piperidine-mediated cleavage) (all lanes); arrow **B** marks the location of 5′-$CCCCG_1$ (AAF) [lane 5 (indicates reaction of DMS at G2)] or 5′-$CCCCG_1$ (AF) [lanes 11, 12 (indicates reaction of DMS or AFB_1-epoxide at G2)]; arrow C marks the location of 5′-$CCCCG_1$ [lanes 2, 3 (indicates reaction of DMS or AFB_1-epoxide at G2), lanes 7–9 (due to presence of AAF adduct at G2), lanes 13–16 (due to presence of AF adduct at G2)]; arrow D marks the location of 5′-CCCC [lanes 2, 3 (indicates reaction of DMS or AFB_1-epoxide at G1), lanes 4–6 (due to presence of AAF adduct at G1), lanes 8, 9 (indicates reaction of DMS or AFB1-eposide at G1), lanes 10–12 (due to presence of AF adduct at G1), lanes 14–16 (indicates reaction of DMS or AFB_1-epoxide at G1)]. **Inset:** Relative amounts of Gua-N7-AFB_1 formed on the target guanine (G1 or G2) in the respective oligomers, expressed as the percent of control reaction [i.e., reaction of AFB_1-8,9-epoxide (100 μM final concentration) with unmodified 10m0TC]. The reaction of DMS in each case was ~100% of the control reaction (densitometry data not shown). Quantitation is as described in the legend to Figure 3.

Both chemical probes are used to sense the degree of denaturation or single-strandedness of DNA bases.

Inhibition of aflatoxin B_1 adduct formation in calf thymus DNA by pre-existing AAF adducts. To determine the applicability of the oligomer studies to genomic DNA, calf thymus DNA, treated with 100 μM AFB_1-8,9-epoxide only, contained 63 pmol Gua-*N*7-AFB_1/nmol total guanine. When calf thymus DNA was pretreated with *N*-AcO-AAF, so that between 0–7% of the total guanine nucleotides were adducted with AAF and then treated with AFB_1-8,9-epoxide (100 μM), the extent of adduction by the epoxide was inversely correlated with the amount of initial modification by AAF (Fig. 9A). The regression was significant ($t = 3.024$, $p \leq 0.05$) when analyzed by ANOVA. Additionally, when each data point was considered as an individual treatment group and compared to the control treatment group by Dunnett's test, two points (marked with ¶ symbol in Fig. 9A) were significantly different ($p \leq 0.05$). When the level of AAF modification was ~7% of the available guanines, the extent of dG-*N7*-AFB_1 adduction had decreased to 28 pmol/nmol total guanine, which is just less than one-half the initial level of modification by AFB_1 in the absence of AAF adducts. When the order of carcinogens was reversed, no effect was observed. (Fig. 9B).

DISCUSSION

Only a few investigations have examined the effects of mixtures of genotoxins or drugs at the level of DNA binding. Results similar to those reported here have been obtained for combinations of antitumor drugs and DNA binding. Tullius and Lippard (1982) demonstrated that pretreatment of a 165-bp restriction fragment derived from pBR322 with ethidium bromide switched *cis*-diaminedichloroplatinum (II) (*cis*-platin) binding sites; sites that were usually preferential for *cis*-platin binding were blocked or attenuated and new sites, particularly a d(GGGGGGCGG) sequence, were dominant.

Bailly and coworkers (1997) found in three different restriction fragments that prior treatment of DNA with actinomycin caused alterations in the sequence specificity of bleomycin●Fe-mediated cleavages, including the disappearance of cleavage sites and appearance of new sites. The authors argued that the

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

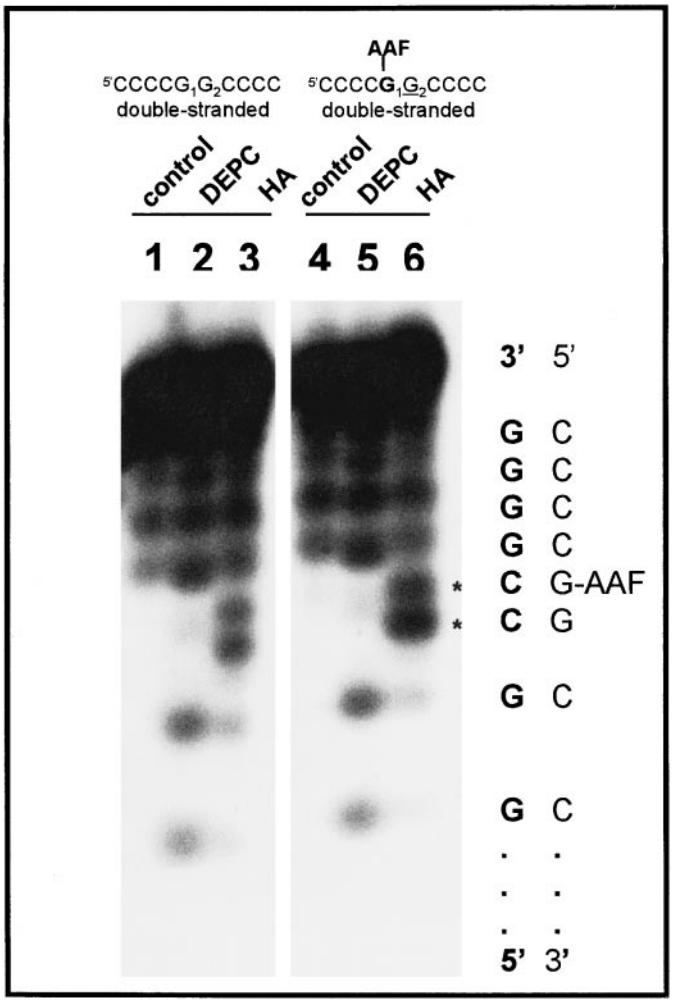

FIG. 8. Chemical probes used to determine DNA conformation. Autoradiogram of a denaturing 20% PAGE of cleavage products following exposure of double-stranded 10m0C and 10m0C.1AAF to either diethylpyrocarbonate (DEPC) or hydroxylamine (HA) and subsequent 1 M piperidine incubation (30-min incubation) is shown. The complementary strand was 5′ end-labeled (as designated by the bold type face of the sequence on the right side of the autoradiogram). Details of reaction conditions are given in Materials and Methods. **ds10m0C** Lane 1: control (no probe); Lane 2: DEPC; Lane 3: HA; **ds10m0C.1AAF** Lane 4: control (no probe); Lane 5: DEPC; Lane 6: HA. The asterisks denote cytosines in the complementary strand that exhibited marked hyper-reactivity towards the probe HA.

results provided evidence of "mutual interference" between two individual small molecules binding with DNA.

Misra and co-workers (1983) used combinations of intercalative agents and AFB_1 to study the mechanisms of sequence-specific binding to DNA by AFB_1-8,9-epoxide; simultaneous treatment of a 174-bp restriction fragment derived from pBR322 with [^{3}H]-AFB_1 (activated by chloroperoxybenzoic acid) and either various non-reactive AFB_1 structural analogues or ethidium bromide led to a decreased level of AFB_1 covalently bound to DNA, and a reduced sequence-specificity of AFB_1 reactivity. These observations were thought to be due to competitive non-covalent binding by the non-reactive ligands and/or to alterations in helix conformation due to the binding of intercalative agents.

The findings reported here describing attenuation or blockage of reaction by AFB_1 epoxide on guanine when located immediately adjacent to a dG-*C8*-AAF adduct, on the same sequence of DNA, are similar to those of Kobertz and coworkers (1997), who showed that the presence of an intercalation inhibitor (a thymidine-benzofuran derivative) on the non-target strand, at an intercalation site immediately 5′ to a guanine on the target strand, protected the guanine from reaction with AFB_1-epoxide (due to prevention of epoxide intercalation). The reactivity of the epoxide shifted to another intercalation site and reacted with the *N7* atom of guanine in a stereospecific manner.

An initial AAF adduct, but not an AF adduct, inhibits Gua-N7-AFB_1 formation at the target guanine in oligonucleotides. The efficient alkylation by AFB_1-8,9-epoxide of G_2 in ds10m0T oligomers (which did not contain AAF adducts) concurs with the reported sequence-specificity of aflatoxin adduction (Benasutti *et al*., 1988). 5′-GG*T closely follows 5′-GG*G as the most reactive sequence in DNA towards AFB_1-8,9-epoxide (G* = targeted guanine). The nearest neighboring bases to a target guanine (5′-XG*Z-3′) that best predict reactivity towards AFB_1-8,9-epoxide are described as follows, 5′-side (i.e, X-position): G > C > A > T and 3′-side (i.e., Z-position): G > T > C > A (Benasutti, 1988).

Steric influences and conformational alterations in the DNA polymer, mediated by the site-specific AAF adduct, were initially thought to be probable causes of the described AFB_1 adduction alterations. The bulky *C8*-acetylaminofluorene adduct destabilizes the normal *anti* conformation of guanine in DNA in favor of the *syn* conformation, whereas, *C8*-aminofluorene adducts do not (Evans *et al*., 1980). Hydrogen bonding of Gua and Cyt is disrupted because the guanine moiety is displaced out of the double helix, into the major groove, while the acetylaminofluorene moiety is inserted into the helix with some evidence of base-fluorene stacking interactions, which can contribute to structural stabilization (O'Handley *et al*., 1993). The initial intercalation of AFB_1-epoxide may be reduced by either (or a combination of) (*i)* local denaturation of DNA, caused by the AAF adduct, or (*ii)* steric interference which physically blocks the AFB_1-epoxide from effectively intercalating. The fact that alkylation of the target guanine by the epoxide increases as it is placed 2 and 4 nucleotides away from the site-specific AAF adduct supports both possibilities. The distortion of local DNA conformation in ds10m0C.1AAF, caused by the presence of the AAF adduct, was confirmed with the chemical probes, hydroxylamine and diethylpyrocarbonate, and is consistent with reports describing AAF-induced perturbations of DNA conformation cited above. It was also observed that the reactivities of single-stranded AAF-adducted 10m1T and 10m3T towards AFB_1 epoxide were not attenuated

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

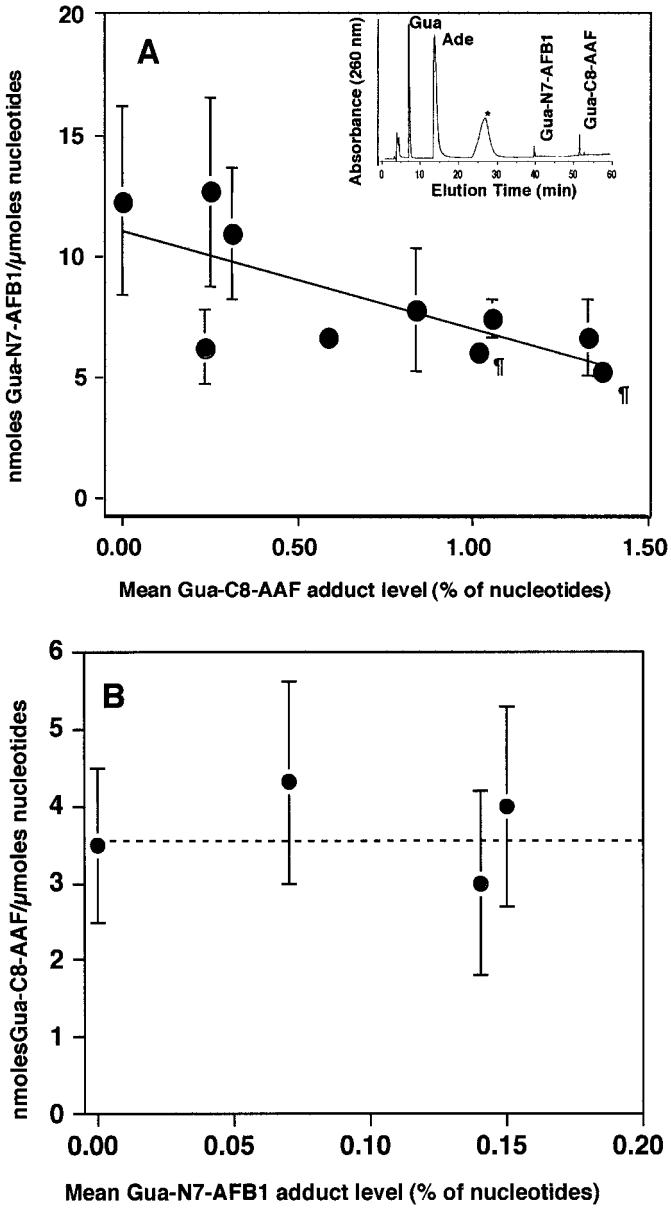

FIG. 9. **(A)** Extent of AFB_1-8,9-epoxide adduct formed in calf thymus DNA pretreated with N-AcO-AAF. The abscissa represents initial mean levels of DNA modification by AAF expressed as % of total nucleotides (mean ± SD of 3 separate Gua-C8-AAF determinations: 0.234 ± 0.117, 0.253 ± 0.039, 0.312 ± 0.039, 0.585 ± 0.059, 0.838 ± 0.019, 1.014 ± 0.039, 1.053 ± 0.059, 1.326 ± 0.370, 1.365 ± 0.273). The ordinate represents the subsequent level of DNA modification by AFB_1. The solid line through the data points was determined by the method of least squares: $y = 11.0 - 4.08x$; $R^2 = 0.535$. Error bars represent the SD of 3 separate Gua-N7-AFB_1 determinations. Data points designated by the ¶ were significantly different from the control group when ANOVA and Dunnett's tests were performed ($p \leq 0.05$). The inset is a representative HPLC chromatogram for the fractionation of an acid hydrolysate of dual treated DNA. The broad peak indicated by an asterisk was found after both control and carcinogen treatments of DNA; it consisted of pyrimidine oligonucleotides as determined from absorbance spectra measured at 3 different pH values (1, 7, 13). Continued acid hydrolysis of samples did not increase the relative amounts of eluted material indicated by the asterisk. **(B)** Extent of N-AcO-AAF adduct formed in calf thymus DNA pretreated with AFB_1. The abscissa represents initial mean levels of DNA modification by AFB_1 expressed as % of total nucleotides (mean ± SD of 3 separate Gua-N7-AFB_1 determinations: 0.07 ± 0.01, 0.14 ± 0.02, 0.15 ± 0.02). The ordinate represents the subsequent level of DNA modification by AAF (nmol Gua-C8-AAF/total nmol nucleotides) (±SD). No significant differences in Gua-C8-AAF levels were observed when the amounts of Gua-N7-AFB_1 adducts in DNA increased ($p \leq 0.05$).

to the same degree as their corresponding double stranded oligonucleotides (data not shown). This would also support a conformational rationale for binding alterations. Additionally, the observation that a bulky AF adduct does not lead to significant changes in AFB_1-*8,9*-epoxide binding to a neighboring guanine, while an AAF adduct does, suggests that conformation may be a more important parameter than steric hindrance.

There are two possible reasons for the apparent contradiction in data that describes a block in the reaction of AFB_1-epoxide with guanine in the sequence context 5′-T$\underline{G}_1G_2$(AAF)-3′, while there appears no block in the context 5′-C$\underline{G}_1G_2$(AAF)3′. First, the difference may be related to the enhanced neighboring effect C has, relative to T, when found 5′ to a target G. The sequence-context 5′-C$\underline{G}$G is ~4-fold more reactive than 5′-T$\underline{G}$G towards AFB_1-epoxide (Benasutti *et al.*, 1988). The positive influence of the 5′ C neighboring the target guanine may override the negative influence of the 3′ G(AAF) adduct. Second, the difference may lie in the thermal stability of the 2 oligonucleotides. Double-stranded 10m0C.2AAF is GC-rich and presumably has a higher melting temperature than 10m0T.2AAF. The decreased stability of the 10m0T.2AAF oligomer could lead to increased single-strandedness with a subsequent reduction in the concentration of intercalation sites 5′ to the target guanine and subsequent reduced covalent adduct formation. Therefore, perturbations in DNA conformation, induced by an AAF adduct, may be dependent upon the sequence-context in which it is found.

It is therefore a reasonable assumption, with the model oligonucleotides used in the present study, that the efficiency of initial non-covalent binding of AFB_1-8,9-epoxide with DNA substrate will determine the rate of Gua-*N7*-AFB_1 formation. This non-covalent binding serves to concentrate the reactive epoxide from the bulk solution by intercalation of the planar portion of the aflatoxin moiety above the 5′ face of the target guanine (Raney *et al.*, 1990; 1993). The molecule is therefore oriented for efficient backside attack by *N7*-guanine on the *exo*-stereoisomer of the epoxide (via an S_N2-type mechanism) to form the covalently bonded adduct (Iyer *et al.*, 1994). Therefore, the inhibition of *N7*-AFB_1 formation on the target guanine in oligonucleotides containing site-specific AAF adducts may result, firstly, from a reduction in the rate of non-covalent association, thereby decreasing the effective concentration of intercalated non-covalent AFB_1-epoxide complex. It then follows that less covalently bound AFB_1 would be formed at the target guanine. Secondly, the attenuated adduction by

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

AFB_1-epoxide is probably not due to perturbations in the "molecular electrostatic potential" (i.e., nucleophilicity) (Pullman and Pullman, 1981) of the target *N7* guanine atom, because there were no observable changes in the reactivity of the S_N2-type carcinogen DMS with AAF-adducted oligomers as compared to control oligomers. DMS is a small methylating agent that was used to probe the nucleophilicity of the target *N7*-guanine.

HPLC analysis of calf thymus DNA treated sequentially with carcinogens. In order to confirm the observations in the oligomer model in which the presence of an AAF adduct contributed to formation of a subsequent adduct by AFB_1, calf thymus DNA was treated with the 2 carcinogens in each order of sequence. Prior modification of calf thymus DNA with *N-AcO*-AAF caused a reduction in the number of AFB_1 epoxide-derived Gua-*N7*-AFB_1 adducts that was dependent upon the initial levels of AAF adducts. However, when the order of carcinogens was reversed, no effect was observed.

It is unlikely that the decrease in the number of AFB_1 adducts was due to saturation of available binding sites by AAF molecules, as the maximum level of AAF adduction involved $<7\%$ of the total guanine bases. Favored binding sites along the DNA lattice for both AAF and AFB_1 with a potential for overlap (or close interaction) of these binding sites is possible. The presence of an AAF adduct at its high affinity site could effectively occupy the high affinity site for AFB_1-epoxide, although global levels of AAF adducts may be relatively low. However, the molecular details of such an interaction are not apparent and were investigated further by using oligonucleotides containing site-specific adducts.

In conclusion, non-additivity between 2 chemical carcinogens at the level of DNA adduct formation has been demonstrated and may help understand similar interactions demonstrated in an *in vivo* system in which pretreatment of *Salmonella typhimurium* with AAF, induced alone (Said *et al*, 1999). These results should be useful for development of risk assessment models, in consideration of mixtures to chemical carcinogens.

ACKNOWLEDGMENT

This work was supported by the U.S. Environmental Protection Agency Grant No. R825809.

REFERENCES

Bailly, C., Hamy, F., and Waring, M. J. (1996). Cooperativity in the binding of echinomycin to DNA fragments containing closely spaced CpG sites. *Biochemistry* **35**, 1150–1161.

Bailly, C., Kenani, A., and Waring, M. J. (1997). Altered cleavage of DNA sequences by bleomycin and its deglycosylated derivative in the presence of actinomycin. *Nucleic Acids Res.* **25**, 1516–1522.

Bailly, C., Suh, D., Waring, M. J., and Chaires, J. B. (1998). Binding of daunomycin to diamminopurine- and/or inosine-substituted DNA. *Biochemistry* **37**, 1033–1045.

Belguise-Valladier, P., and Fuchs, R. P. P. (1991). Strong sequence-dependent polymorphism in adduct-induced DNA structure: Analysis of single N-2-acetylaminofluorene residues bound within the NarI mutation hot spot. *Biochemistry* **30**, 10091–10100.

Benasutti, M., Ejadi, S., Whitlow, M. D., and Loechler, E. L. (1988). Mapping the binding site of aflatoxin B_1 in DNA: Systematic analysis of the reactivity of aflatoxin B_1 with guanines in different DNA sequences. *Biochemistry* **27**, 472–481.

Bridges, B. A., and Brown, G. M. (1992). Mutagenic DNA repair in *Escherichia coli* XXI. A stable SOS-inducing signal persisting after excision repair of ultraviolet damage. *Mutat. Res.* **270**, 135–44.

Chaires, J. B. (1985). Long-range allosteric effects on the B to Z equilibrium by daunomycin. *Biochemistry* **24**, 7479–7486.

Chen, J. X., Zheng, Y., West, M., and Tang, M. S. (1998). Carcinogens preferentially bind at methylated CpG in the p53 mutational hot spots. *Cancer Res.* **58**, 2070–2075.

Denissenko, M. F., Chen, J. X., Tang, M. S., and Pfeifer, G. P. (1997). Cytosine methylation determines hot spots of DNA damage in the human p53 gene. *Proc. Natl. Acad. Sci. USA* **94**, 3893–3898.

Denissenko, M. F., Pao, A., Tang, M. S., and Pfeifer, G. P. (1996). Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hot spots in p53. *Science* **274**, 430–432.

Dittrich, K. A. and Krugh, T. R. (1991). Mapping of (+/−)-anti-benz[a]pyrene diol epoxide adducts to human c-Ha-ras1 proto-oncogene. *Chem. Res. Toxicol.* **4**, 277–281.

Essigmann, J. M., Croy, R. G., Nadzan, A. M., Busby, Jr., Reinhold, V.N., Buchi, G., and Wogan, G. N. (1977). Structural identification of the major DNA adduct formed by aflatoxin B_1 *in vitro*. *Proc. Natl. Acad. Sci. USA* **74**, 1870–1874.

Evans, F. E., Miller, D. W., and Beland, F. A. (1980). Sensitivity of the conformation of deoxyguanosine to binding at the C-8 position by N-acetylated and unacetylated 2-aminofluorene. *Carcinogenesis* **1**, 955–959.

Hemminki, K. (1993). DNA adducts, mutations, and cancer. *Carcinogenesis* **14**, 2007–2012.

Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. (1991). Mutational hot spot in the p53 gene in human hepatocellular carcinomas. *Nature* **350**, 427–428.

Iyer, R., and Harris, T. M. (1993). Preparation of aflatoxin B_1 8,9-epoxide using m-chloroperbenzoic acid. *Chem. Res. Toxicol.* **6**, 313–316.

Iyer, R. S., Coles, B. F., Raney, K. D., Thier, R., Guengerich, F. P., and Harris, T. M. (1994). DNA adduction by the potent carcinogen aflatoxin B_1: Mechanistic studies. *J. Am. Chem. Soc.* **116**, 1603–1609.

Johnson, D. L., Reid, T. M., Lee, M. S., King, C. M., and Romano, L. J. (1987). Chemical stability of oligonucleotides containing the acetylated and deacetylated adducts of the carcinogen N-2-acetylaminofluorene. *Carcinogenesis* **8**, 619–623.

Johnston, B. H., and Rich, A. (1985). Chemical probes of DNA conformation: Detection of Z-DNA at nucleotide resolution. *Cell* **42**, 713–724.

Jones, W. R., Johnston, D. S., and Stone, M. P. (1998). Refined structure of the doubly intercalated d[TAT(AFB1)GCATA]2 aflatoxin B1 adduct. *Chem. Res. Toxicol.* **11**, 873–881.

Kobertz,W. R., Wang, D., Wogan, G. N., and Essigmann, J. M. (1997). An intercalation inhibitor altering the target selectivity of DNA damaging agents: Synthesis of site-specific aflatoxin B1 adducts in a p53 mutational hot spot. *Proc. Natl. Acad. Sci. USA* **94**, 9579–9584.

Koehl, P., Burnouf, D., and Fuchs, R. P. P. (1989). Construction of plasmids containing a unique acetylaminofluorene adduct located within a mutation hot spot. *J. Mol. Biol.* **207**, 355–364.

Kriek, E., Miller, J. A., Juhl, U., and Miller, E. C. (1967). 8-(N2-Fluorenylacetamido)guanosine, an arylamidation reaction product of guanosine and

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017

the carcinogen N-acetoxy-N-2-fluorenylacetamide in neutral solution. *Biochemistry* **6**, 177–182.

Mathison, B. H., Said, B., and Shank, R. C. (1993). Effect of 5-methylcytosine as a neighboring base on methylation of DNA guanine by N-methyl-N-nitrosourea. *Carcinogenesis* **14**, 323–327.

Mattes, W. B., Hartley, J. A., and Kohn, K. W. (1986). DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards. *Nucleic Acids Res*. **14**, 2971–2987.

Mattes, W. B., Hartley, J. A., Kohn, K. W., and Matheson, D. W. (1988). GC-rich regions in genomes as targets for DNA alkylation. *Carcinogenesis* **9**, 2065–2072.

Maxam, A. M., and Gilbert, W. (1977). Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol*. **65**, 499–560.

Misra, R. P., Muench, K. F., and Humayun, M. Z. (1983). Covalent and noncovalent interactions of aflatoxin with defined deoxyribonucleic acid sequences. *Biochemistry* **22**, 3351–3359.

O'Handley, S. F., Sanford, D. G., Xu, R., Lester, C. C., Hingerty, B. E., Broyde, S., and Krugh, T. R. (1993). Structural characterization of an N-acetyl-2-aminofluorene (AAF) modified DNA oligomer by NMR, energy minimization, and molecular dynamics. *Biochemistry* **32**, 2481–2497.

Puisieux, A., Lim, S., Groopman, J., and Ozturk, M. (1991). Selective targeting of p53 gene mutational hot spots in human cancers by etiologically defined carcinogens. *Cancer Res*. **51**, 6185–6189.

Pullman, A., and Pullman, B. (1981). Molecular electrostatic potential of the nucleic acids. *Q. Rev. Biophys*. **14**, 289–380.

Raney, K. D., Gopalakrishnan, S., Byrd, S., Stone, M. P., and Harris, T. M. (1990). Alteration of the aflatoxin cyclopentenone ring to a δ-lactone reduces intercalation with DNA and decreases formation of guanine N7 adducts by aflatoxin epoxides. *Chem. Res. Toxicol*. **3**, 254–261.

Raney, V. M., Harris, T. M., and Stone, M. P. (1993). DNA conformation mediates aflatoxin B_1-DNA binding and the formation of guanine N7 adducts by aflatoxin B_1 8,9-exo-epoxide. *Chem. Res. Toxicol*. **6**, 64–68.

Rosenberg, L. S., Carvlin, M. J., and Krugh, T. R. (1986). The antitumor agent mitoxantrone binds cooperatively to DNA: Evidence for heterogeneity in DNA conformation. *Biochemistry* **25**, 1002–1008.

Ross, M. K., Mathison, B. H., Said, B., and Shank, R. C. (1999). 5-Methylcytosine in CpG sites and the reactivity of nearest neighboring guanines toward the carcinogen aflatoxin B_1-8,9-epoxide. *Biochem. Biophys. Res. Commun*. **254**, 114–119.

Said, B., Ross, M. K., Hamade, A. K., Matsumoto, D. C., and Shank, R. C. (1999). DNA-damaging effects of genotoxins in mixture: 1. Non-additive effects of aflatoxin B_1 and N-acetylaminofluorene on their mutagenicity in Salmonella typhimurium. *Toxicol. Sci*. **52,** 226–231.

Said, B., Ross, M. K., Salib, T., and Shank, R. C. (1995). Modulation of DNA adduct formation by successive exposures of DNA to small and bulky chemical carcinogens. *Carcinogenesis* **16**, 3057–3062.

Said, B., and Shank, R. C. (1991). Nearest-neighbor effects on carcinogen binding to guanine runs in DNA. *Nucleic Acids Res*. **19**, 1311–1316.

Stone, M. P., Gopalakrishnan, S., Harris, T. M., and Graves, D. E. (1988). Carcinogen-nucleic acid interactions: Equilibrium binding studies of aflatoxins B1 and B2 with DNA and the oligodeoxynucleotide d(ATGCAT)2. *J. Biomol. Struct. Dyn*. **5**, 1025–1041.

Suh, M., Ariese, F., Small, G. J., Jackowiak, R., Liu, T. M., and Geacintov, N. E. (1995). Conformational studies of the (+)-trans, (−)-trans, (+)-cis, and (−)-cis adducts of anti-benzo[a]pyrene diol epoxide to N2-dG in duplex oligonucleotides using polyacrylamide gel electrophoresis and low-temperature fluorescence spectroscopy. *Biophys. Chem*. **56**, 281–296.

Tullius, T. D., and Lippard, S. J. (1982). Ethidium bromide changes the nuclease-sensitive DNA binding sites of the antitumor drug cis-diamminedichloroplatinum (II). *Proc. Natl. Acad. Sci. USA* **79**, 3489–3492.

Walker, G. T., Stone, M. P., and Krugh, T. R. (1985a). Ethidium binding to left-handed (Z) DNAs results in regions of right-handed DNA at the intercalation site. *Biochemistry* **24**, 7462–7471.

Walker, G. T., Stone, M. P., and Krugh, T. R. (1985b). Interaction of drugs with Z-DNA: Cooperative binding of actinomycin D or actinomine to the left-handed forms of poly(dG-dC), poly(dG-dC), and poly(dG-m5dC).Poly(dG-m5dC) reverses the conformation of the helix. *Biochemistry* **24**, 7471–7479.

Warpehoski, M. A., and Hurley, L. H. (1988). Sequence selectivity of DNA covalent modification. *Chem. Res. Toxicol*. **1**, 315–333.

Winkle, S. A., and Krugh, T. R. (1981). Equilibrium binding of carcinogens and antitumor antibiotics to DNA: Site-selectivity, cooperativity, allosterism. *Nucleic Acids Res*. **9**, 3175–3186.

Zhou, Y., and Romano, L. J. (1993). Solid-phase synthesis of oligonucleotides containing site-specific N-(2′-deoxyguanosin-8-yl)-2-(acetylamino)fluorene adducts using 9-flourenylmethoxycarbonyl as the base-protecting group. *Biochemistry* **32**, 14043–14052.

Downloaded from http://toxsci.oxfordjournals.org/ at University of North Texas on January 21, 2017