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The ultimate carcinogen of 4-nitroquinoline 1-oxide does not react with Z-DNA and hyperreacts with B–Z junctions

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ABSTRACT

DNA secondary and tertiary structures are known to affect the reaction between the double helix and several damaging agents. We have previously shown that the tertiary structure of DNA influences the reactivity of 4-acetoxyaminoquinoline 1-oxide (Ac-4-HAQO), the ultimate carcinogen of 4-nitroquinoline 1-oxide (4-NQO), being more reactive with naturally supercoiled DNA than with relaxed DNA. The relative proportion of the three main stable adducts and of an unstable adduct, that resulted in strand scission and/or AP sites, was also affected by the degree of supercoiling of plasmid DNA. In this study we examined the influence of Z-DNA structure on the reactivity of Ac-4-HAQO by mapping the distribution of the two main Ac-4-HAQO adducts, C8-guanine and N2-guanine, along a $(dC\text{-}dG)_{16}$ sequence inserted at the BamHI site of pBR322 plasmid DNA. This insert adopted the left-handed Z and right-handed B structure depending on the superhelical density of the plasmid. Sites of C8-guanine adduct formation were determined by hot piperidine cleavage of Ac-4-HAQO modified DNA, while N2-guanine adducts were mapped by the arrest of the 3′-5′ exonuclease activity of T4 DNA polymerase. The results showed that Ac-4-HAQO did not react with guanine residues when the $(dC\text{-}dG)_{16}$ sequence was in Z conformation, while hyperreactivity at the B–Z junction was observed. These results indicate that Ac-4-HAQO can probe the polymorphism of DNA at the nucleotide level.

INTRODUCTION

4-Nitroquinoline 1-oxide (4-NQO*) is a potent mutagen and carcinogen, both in prokaryotes and eukaryotes (1). 4-NQO can be converted chemically to 4-acetoxyaminoquinoline 1-oxide (Ac-4-HAQO), which represents a good model for the ultimate carcinogen of 4-NQO, since it reacts *in vitro* with DNA producing the same adducts as those formed *in vivo* by 4-NQO (2). The three major stable DNA adducts formed by 4-NQO *in vivo* and by Ac-4-HAQO *in vitro* are: 3-(deoxyguanosin-N2-yl)-4-aminoquinoline 1-oxide (dGuo-N2-AQO) and N-(deoxyguanosin-C8-yl)-4-aminoquinoline 1-oxide (dGuo-C8-AQO) on guanine, and 3-(deoxyadenosin-N6-yl)-4-aminoquinoline 1-oxide (dAdo-N6-AQO) on adenine (3; 4). The relative proportion of the stable adducts is influenced by the primary, secondary and tertiary structure of target DNA. Analysis at the sequence level has shown that some sequences are more reactive than others towards this compound, although the rules governing this specificity appear complex (5). In the reaction with duplex DNA, N2-guanine, C8-guanine, and N6-adenine adducts are in the proportion of 5:3:2, respectively, while with single-stranded DNA the ratio is 1:7:2. In negatively supercoiled topoisomers ($\Delta Lk \approx -8$), the relative proportion of the three main adducts changes to 8:1.5:0.5. Furthermore, we found that naturally supercoiled DNA is more reactive than single-stranded DNA and even more reactive than relaxed DNA with this compound. We have also shown that single-strand breaks of unidentified origin (SSBs) and/or apurinic/apyrimidinic (AP) sites, that are likely to correspond to the alkali labile lesions induced by 4-NQO *in vivo* (6), are among the Ac-4-HAQO-induced lesions. The yield of these sites is also influenced by the tertiary structure of DNA, exhibiting a 2.5 fold increase in relaxed in comparison with naturally supercoiled plasmids (7). Thus, the dynamic properties of the double helix seem to be important factors in determining the reactivity of Ac-4-HAQO.

Negative supercoiling is one of the most important among the 'driving forces' that can modulate the conformation of DNA structure by promoting the transition of particular sequences to alternative secondary structures, such as Z-DNA (8; 9; 10). Z-DNA is a left-handed double helix that can be adopted by alternating purine-pyrimidine sequences. The most relevant feature of this conformation is that the repetitive unit is no longer a single base pair but a dinucleotide, in which purines adopt the *syn* while pyrimidines the *anti* conformation. The result of this new architecture is that stacking of the bases and exposure of their nucleophylic centres, the targets for several damaging agents, are widely different from those of B-DNA (11). Such structural changes have been shown to influence the reactivity of Z-DNA towards small chemicals that modify specific bases (12). Among them are the crosslinking agent 4,5′,8 trimethylpsoralen and the well known carcinogen N-hydroxy-2-aminofluorene (N-OH-AF), which have been shown to react with B-DNA but not with Z-DNA (13; 14). Evidence

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also exists in which N-acetoxy-AAF-modified poly(dC-dG)-poly(dC-dG) can assume the Z-DNA conformation more readily (15). In the case of Ac-4-HAQO, the two major adducts, dGuo-C8-AQO and dGuo-N2-AQO, have different influences on the conformation of poly(dC-dG)-poly(dC-dG). The N2-adduct inhibits the ethanol-induced B-Z transition of poly(dC-dG)-poly(dC-dG), while the C8-adduct does not affect this transition (16).

In order to test whether alternative DNA secondary structures could influence the reactivity of Ac-4-HAQO, we have studied the reaction of Ac-4-HAQO with a $(dC\text{-}dG)_{16}$ sequence inserted at the BamHI site of pBR322; this sequence adopts a normal B conformation when plasmid DNA is relaxed, while it adopts a Z conformation when DNA is naturally supercoiled. We report here that Ac-4-HAQO reacts with the $(dC\text{-}dG)_{16}$ insert only when in the B-form; when this sequence is in the Z-form the only observed reactivity is at the guanines of the B−Z junction at the 3′ side of the insert.

MATERIALS AND METHODS

Enzymes and chemicals

T4 DNA polymerase, *Nae*I and *Eco*RI restriction endonucleases, calf thymus intestinal alkaline phosphatase, ethidium bromide and Molecular Weight Marker V (MWM V) were purchased from Boehringer-Manheim, Germany. T4 polynucleotide kinase was from New England Bio Labs, USA. DNA topoisomerase I and apurinic/apyrimidinic (AP) endonuclease were purified from HeLa cells as described (17; 18). (γ-^{32}P)ATP (specific activity 3000 Ci/mmol) was obtained from Amersham, UK. Maxam and Gilbert sequencing kit was from Merck, Germany. Diacetyl-4-hydroxyaminoquinoline 1-oxide (di-Ac-4-HAQO) was prepared as previously described (2), and stored in a desiccator over P_2O_5 in the dark at −20°C. NACS-37 ion-exchange chromatography resin was obtained from BRL, USA.

Preparation of substrates

Plasmid pLP32 DNA (4398 bp) (8) was purified from HB101 *E. coli* cells by chloramphenicol amplification and alkaline-lysis method, as described (19); separation of the negatively supercoiled form (RFI) from the nicked form (RFII) was obtained by elution with a linear gradient of 0.3 to 0.7 M NaCl in 10 mM Tris−HCl, 1 mM Na_2-EDTA (pH 7.2) on a NACS-37 column (1 cm×2.5 cm) at 4°C. Fractions containing the RFI form were collected and plasmid DNA was recovered by ethanol precipitation; DNA was stored at −20°C in H_2O at a concentration of 400 μg/ml.

Relaxed (RFIV) pLP32 DNA was prepared by treating 50 μg of pLP32 RFI DNA with 200 U of DNA topoisomerase I (1 unit of enzyme is the amount of enzyme required to relax 0.5 μg of plasmid DNA in 30 min. at 30°C) in a final volume of 250 μl containing 50 mM Tris−HCl, pH 7.2, 120 mM KCl, 10 mM $MgCl_2$, 0.1 mM Na_2-EDTA and 0.1 mM dithiothreitol (DTT). Incubation was carried out for 30 min. at room temperature. The reaction was terminated by addition of distilled, neutralized phenol; the DNA was ethanol precipitated and resuspended in 2 mM Na-citrate pH 7.0 to a final concentration of 250 μg/ml.

The *Nae*I-*Eco*RI 440 bp DNA fragment was obtained as follows: 3 μg of pLP32 DNA were digested with 9 units of *Nae*I restriction endonuclease; the linearized plasmid was 5′-end labelled with ^{32}P by the phosphatase-polynucleotide kinase method (19). A second digestion with 15 units of *Eco*RI endonuclease was performed and, after separation of the products on a 5% polyacrylamide gel, the ^{32}P-end labelled *Nae*I-*Eco*RI fragment was located as a band on the gel following autoradiography. The band was excised and DNA recovered by electroelution (19). After ethanol precipitation, DNA was resuspended in 50 μl of H_2O.

DNA modification with Ac-4-HAQO

Plasmid pLP32 DNAs were modified with Ac-4-HAQO in 2 mM Na-citrate, pH 7.0 at the indicated molar ratio 'R' (Ac-4-HAQO to DNA mononucleotides). Ac-4-HAQO was prepared by treating di-Ac-4-HAQO compound with DTT in DMSO solution as previously described (2). Reaction mixtures were incubated in the dark for 15 min at room temperature. Chemically modified plasmid DNAs were purified from unreacted carcinogen by ethanol precipitation as previously described (2) and resuspended in H_2O to a final concentration of 100 μg/ml.

Quantitation of single strand breaks

The number of SSBs formed in pLP32 DNA was calculated by measuring the conversion of covalently closed DNA circles (RFI and RFIV) into circles containing single-strand scissions (RFII). Quantitative analysis of the closed and nicked forms, separated by electrophoresis on 1% agarose gel in the presence of 1 μg/ml ethidium bromide, was performed by scanning the negatives of gel photographs with a Beckman DU8 gel scanner (20). The average number of SSBs (μ) was calculated assuming a Poisson distribution as $\mu = -\ln x$, where x is the fraction of plasmid DNA remaining in the RFI form.

The number of AP sites was calculated by the same methodology, as the number of sites sensitive to AP endonuclease. Modified pLP32 DNA (0.4 μg) was incubated for 30 min at 37°C with excess AP endonuclease in a final volume of 20 μl containing 100 mM Tris−HCl, pH 7.4, 120 mM KCl, 10 mM $MgCl_2$, 0.1 mM Na_2-EDTA and 0.1 mM DTT. The reaction was stopped by addition of the electrophoresis dye mix, and reaction products were analysed on agarose gel, as above.

Piperidine digestion of Ac-4-HAQO-modified 440-bp DNAs

DNA samples (10 μl, 20000 cpm) were treated with 1 M piperidine (100 μl) at 90°C for 10 min (2; 5). After the reaction, piperidine was removed in a Speed-Vac concentrator (Savant Instruments, Inc., Farmingdale, NY). Reaction products were dissolved in 5 μl of formamide plus dyes, heated to 90°C for 2 min and quickly chilled in an ice bath. The samples were analysed on a denaturing 8% polyacrylamide/7M urea sequencing gel, alongside with the Maxam and Gilbert sequence ladder of the same fragment. After electrophoresis, at 70 W for 1.5 h, gels were autoradiographed by using two intensifying screens at −70°C.

Digestion of UV-irradiated and Ac-4-HAQO-modified 440-bp DNAs with T4 DNA polymerase (3′-5′) exonuclease

DNA concentration was determined by electrophoresis on a 5% polyacrylamide gel the ^{32}P-labelled fragments in parallel with a known concentration of MWM V. After ethidium bromide staining, the intensity of the labelled bands was compared with that of the 458-bp band of MWM V, by scanning the negative of the gel photograph. Unmodified ^{32}P-end labelled 440-bp fragment was UV irradiated on ice under a 254 nm germicidal lamp at a dose of 1230 J/m^2. At this fluency an average of 6 cyclobutane pyrimidine dimers and 1.6 (6-4) photoproducts per

fragment were produced. Unmodified, UV-irradiated and Ac-4-HAQO modified samples were digested for 30′ at 37°C with 1 unit of T4 DNA polymerase per 2.5 ng of DNA in a final volume of 80 μl containing 33 mM Tris-acetate, pH 7.8, 10 mM $Mg(OAc)_2$, 66 mM KOAc, 0.5 mM DTT and 0.1 mg/ml BSA. The reaction was stopped by addition of 1 μg of carrier DNA. After extraction with buffer saturated phenol, and ethanol precipitation, DNA fragments were separated on a denaturing 8% polyacrylamide/7M urea sequencing gel.

RESULTS

Before starting the analysis of the reactivity of the $(dC\text{-}dG)_{16}$ insert of pLP32 DNA in the Z-form with Ac-4-HAQO, we first established if the insert adopted the Z conformation when the plasmid was naturally supercoiled. By two dimensional agarose gel electrophoresis, we showed that the B to Z transition took place in correspondence of topoisomer with $\Delta Lk = -17$ (data not shown); therefore, naturally supercoiled pLP32 DNA ($\Delta Lk = -27$) was suitable for our study. In addition, we have controlled the integrity of the modified DNA. In fact, we have shown in a previous work that, in addition to the three major stable adducts (dGuo-N2-AQO, dGuo-C8-AQO, dAdo-N6-AQO), the reaction between Ac-4-HAQO and DNA led to the appearance of SSBs, after agarose gel electrophoresis, and to sites sensitive to AP endonuclease (7). Since we knew about the fragile nature of AP sites in our agarose gel system, we were not able to establish whether SSBs were formed during the modification treatment or resulted from phosphodiester bond disruption of unbroken AP sites during agarose gel electrophoresis. In the event of strand breaks beng formed during the damaging treatment, relaxation of negatively supercoiled DNA molecules with consequent loss of Z-DNA structure would have taken place. Therefore, we have estimated the maximum amount of molecules that might have lost the Z-form during the modification treatment, by calculating the fraction of molecules containing SSBs present at the end of the damaging treatment as a function of the Ac-4-HAQO/DNA ratio (R). Fig. 1A shows the electrophoretic pattern of relaxed (RFIV) and negatively supercoiled (RFI) pLP32 DNA samples, immediately after

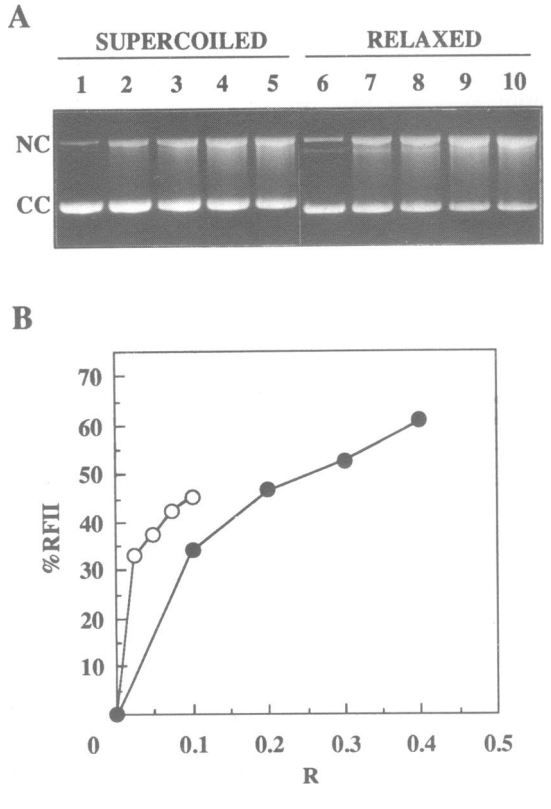

Figure 1. Analysis of the amount of broken molecules immediately after treatment of DNA with Ac-4-HAQO. Supercoiled (RFI) and relaxed (RFIV) closed circular DNAs (CC) were separated from RFII nicked circular DNAs (NC) by agarose gel electrophoresis in the presence of 1 μg/ml ethidium bromide. The fraction of nicked molecules were related to the Ac-4-HAQO dose (R=Ac-4-HAQO/DNA) used in the modification treatment. **A.** Lanes 1 and 6, no modification; lanes 2–5, RFI DNA modified with Ac-4-HAQO at a molecular ratio to DNA (R) of 0.025, 0.050, 0.075, 0.1; lanes 7–10, RFIV DNA modified with R of 0.100, 0.200, 0.300, 0.400. **B** shows the relationship between the fraction of molecules in the NC form and the dose (R) of Ac-4-HAQO; (○) modified RFI; (●) modified RFIV.

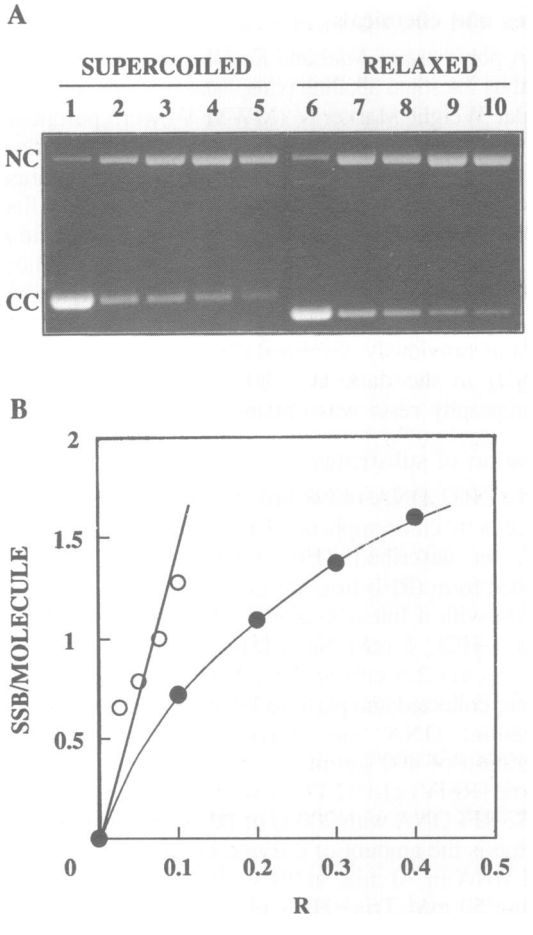

Figure 2. Quantitation of adducts per molecule formed after modification of DNAs with Ac-4-HAQO. **A.** After treatment of Ac-4-HAQO modified DNAs with AP endonuclease, CC and NC DNA forms were separated as in Fig. 1A and the number of total SSBs per molecule calculated. Lanes 1 and 6, no modification; lanes 2–5, RFI DNA modified with an R of 0.025, 0.050, 0.075, 0.1; lanes 7–10, RFIV DNA modified with an R of 0.100, 0.200, 0.300, 0.400. **B.** The values obtained were used to determine the number of stable adducts per molecule for RFI (○) and RFIV (●) forms according to the relationship stable adducts/SSB of 40 for supercoiled DNA and 17 for relaxed DNA (7). The data represent the average of two independent experiments.

Table 1. Average number of dGuo-C8-AQO and dGuo-N2-AQO adducts and SSBs expected to be formed in the 440 bp NaeI/EcoRI single-stranded fragment, as inferred from the average number of SSBs measured after AP endonuclease digestion of naturally supercoiled and relaxed Ac-4-HAQO-modified pLP32 DNA (Fig.2).

DNA form	R	dGuo-C8-AQO	dGuo-N2-AQO	SSBs
RFI pLP32	0.025	0.39	0.65	0.032
	0.050	0.47	0.79	0.039
	0.075	0.60	0.99	0.050
	0.100	0.77	1.28	0.064
RFIV pLP32	0.100	0.18	0.31	0.036
	0.200	0.28	0.46	0.054
	0.300	0.35	0.58	0.069
	0.400	0.41	0.68	0.080

treatment with Ac-4-HAQO. In agreement with our published results (7), the damaging treatment caused modifications that resulted in strand scissions upon agarose gel electrophoresis (Fig. 1). This initial breakage represented about 50% of the total number of breaks measured after AP endonuclease digestion (Fig. 2 and data not shown). However, the presence of a smear of ethidium bromide stainable material between the closed circular and nicked circular DNAs indicated that breakage of at least some AP sites took place during agarose gel electrophoresis. Therefore, the amount of broken molecules measured at the end of the damaging treatment was an overestimation of the number of molecules that, following breakage of phosphodiester bonds, might have lost the Z-DNA conformation during the 15 minutes of the modification treatment.

We have also previously established a relationship between the total number of SSBs measured after AP endonuclease digestion of Ac-4-HAQO modified DNAs, and the number of stable adducts formed per molecule. In relaxed DNA, the ratio was one chain break every 17 stable adducts while in negatively supercoiled DNA this value was of one break every 40 stable adducts (7). We used these ratios to calculate the approximate number of dGuo-C8-AQO, dGuo-N2-AQO and strand breaks formed per pLP32 molecule (4398 bp) (Fig. 2). The relative number of adducts in the 440 bp fragment, containing the insert $(dC\text{-}dG)_{16}$, was then calculated (Table I). For our study, the levels of modification chosen were such that in supercoiled DNA the maximum amount of nicked molecules which might have been present at the end of the damaging treatment was lower than 45%. Since naturally supercoiled DNA reacts more efficiently than relaxed DNA, we have compared samples which have been damaged with Rs expected to introduce a comparable level of modification in the two topologically different substrates. In order to determine whether the $(dC\text{-}dG)_{16}$ insert in the Z-form reacted with Ac-4-HAQO, we mapped, at sequence level, the distribution of C8 and N2 guanine adducts along the insert either in the B or Z-DNA conformation. After modification, supercoiled and relaxed pLP32 DNAs were digested with the restriction endonuclease *Nae*I and the fragments 5′-end labelled with ^{32}P using the phosphatase-kinase procedure. The fragment, carrying the *Eco*RI site, was then digested with *Eco*RI endonuclease and the resulting (440 bp) *Nae*I-*Eco*RI fragment containing the $(dC\text{-}dG)_{16}$ insert was purified by electrophoresis on polyacrylamide gel.

The dGuo-C8-AQO adducts were mapped by treatment with hot piperidine, that has been shown to break DNA at these modified residues (2; 5). The resulting digests were analysed on sequencing gels along with the corresponding Maxam and Gilbert sequence ladders. When the $(dC\text{-}dG)_{16}$ insert was in the B-DNA conformation (Fig. 3, lanes 6−9), bands corresponding to all guanines residues could be seen on the autoradiogram. This result indicated that all guanines could give rise to dGuo-C8-AQO adducts, confirming what has been previously observed by Panigrahi and Walkers (5). However, when the insert was in the Z-DNA conformation (Fig. 3, lanes 2−5), only the guanines of the B−Z junction at the 3′ side of the insert were modified (because of the 'compression' it cannot be determined whether only one or both guanine residues are modified by Ac-4-HAQO). As judged by the relative intensity of the bands, the guanines at the 3′ side are about 20 times more reactive than the most reactive guanines on each side of the insert in B conformation.

Sites of dGuo-N2-AQO adduct formation were obtained by utilising the ability of these lesions to halt the progression of the 3′-5′ exonuclease activity of T_4 DNA polymerase (5; 21). In agreement with Haseltine and coworkers (22), who reported that this enzyme shows pause sites at G-C rich sequences, we found that the guanines of the $(dC\text{-}dG)_{16}$ insert were very strong points of arrest for enzyme's progression, those at the 3′ side being more efficient than those at the 5′ side. In preliminary experiments the complete 3′-5′ exonuclease digestion of the undamaged fragment could be achieved only with a high concentration of enzyme. Unfortunately, in these conditions the dGuo-N2-AQO adducts were not an absolute block for T4 polymerase-associated 3′-5′ exonuclease as indicated by the fact that the intensity of the bands in the sequences flanking the insert was lower in the T4 DNA polymerase than in the piperidine digest of the same fragment (data not shown). This is in contrast with the expected 2:1 ratio of the N2 and C8 guanine adducts (see Table I). Furthermore, the amount of ethanol precipitable material decreased with increasing enzyme concentrations up to complete digestion of the fragment. Fig. 4 shows the pattern of T4 DNA polymerase digestion of *Nae*I-*Eco*RI fragments modified with Ac-4-HAQO, when the $(dC\text{-}dG)_{16}$ insert was in the B (lane 4) and Z-form (lane 3). In order to discriminate whether the bands of arrest at the $(dC\text{-}dG)_{16}$ sequence, observed in Ac-4-HAQO modified DNAs, were due to dGuo-N2-AQO adducts or to increased pausing of the enzyme at the (dC-dG)16 sequence as a result of the presence of adducts in other parts of the *Nae*I/*Eco*RI fragment, we used the same UV-irradiated fragment as control (lane 2). UV lesions constitute points of arrest to T4 DNA polymerase digestion and are not formed in the (dC-dG)16 sequence. Therefore, the appearance of bands in this region cannot be due to UV damage but to a reduced rate of digestion of the damaged fragment. As it can be seen, in the lane containing DNA modified in the B-form (lane 4), band distribution qualitatively followed the chemical cleavage patterns observed after piperidine treatment of the same DNA (Fig. 3, lanes 6−9).

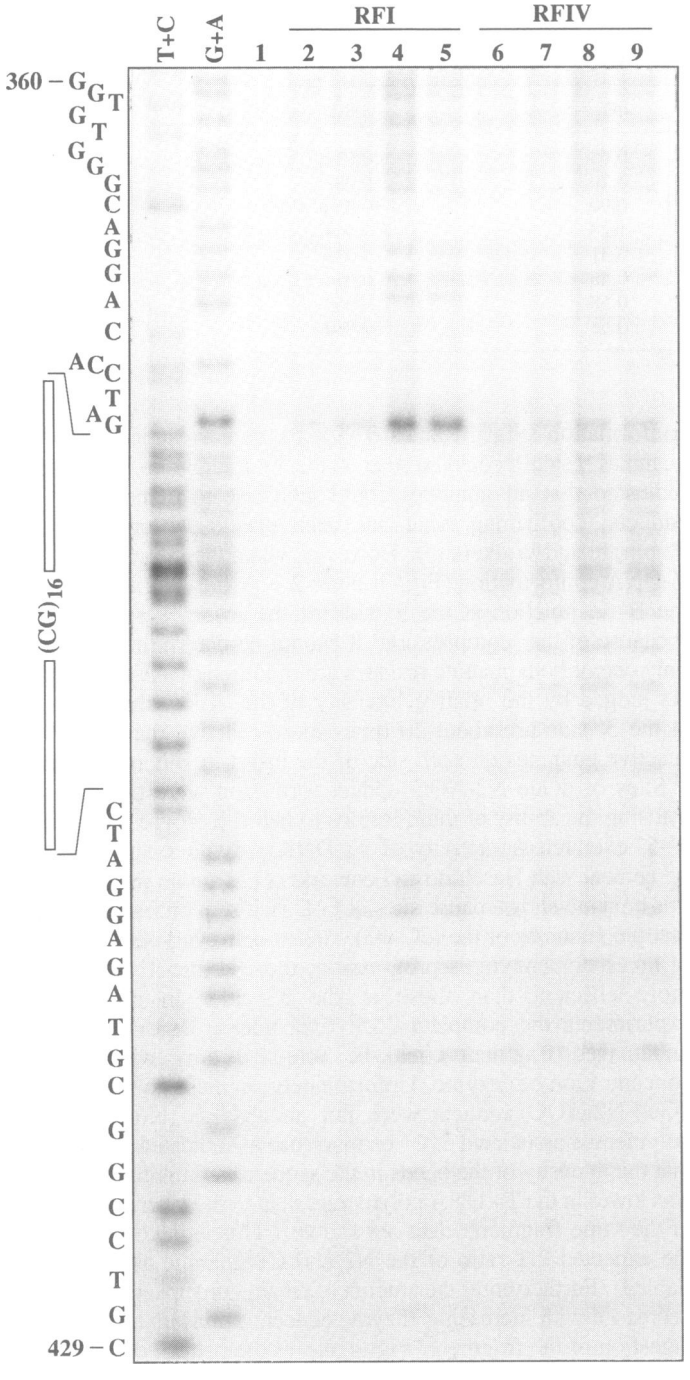

Figure 3. dGuo-C8-AQO adducts formation within the *Nae*I-*Eco*RI fragment containing the $(dC\text{-}dG)_{16}$ insert. Supercoiled (RFI) and relaxed (RFIV) pLP32 DNAs, modified with increasing amount of Ac-4-HAQO (R), were digested with *Nae*I and *Eco*RI. The *Nae*I-*Eco*RI fragments 5′ end-labelled at the *Nae*I terminus were treated with 1 M piperidine to obtain dGuo-C8-AQO-specific reaction. The positions of the damaged-bases were identified by comparison with Maxam and Gilbert sequencing reactions of T+C and G+A. The $(dC\text{-}dG)_{16}$ insert in the *Bam*HI site is indicated as a box. Lane 1, no modification; lanes 2–5, RFI DNAs modified with R of 0.025, 0.050, 0.075, 0.100; lanes 6–9, RFIV DNAs modified with Rs of 0.100, 0.200, 0.300, 0.400.

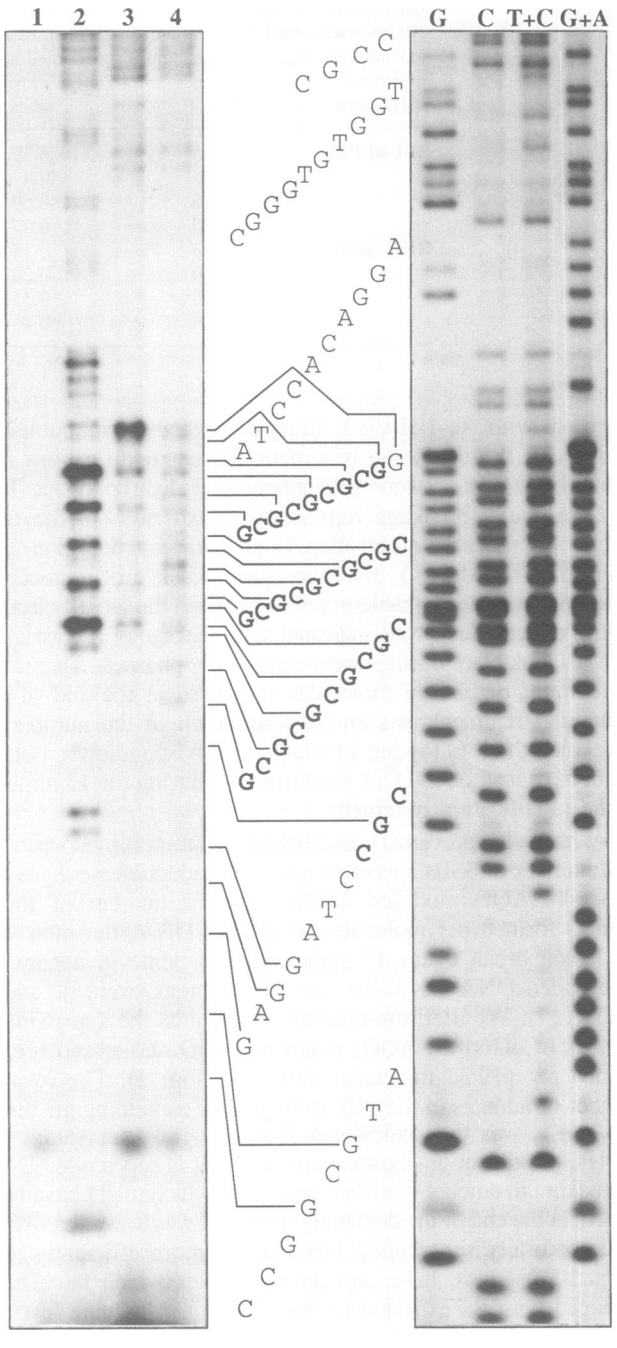

Figure 4. dGuo-N2-AQO adducts formation within the *Nae*I-*Eco*RI fragment containing the $(dC\text{-}dG)_{16}$ insert. Supercoiled (RFI) and relaxed (RFIV), modified with an R of 0.1 (lane 3) and 0.4 (lane 4), respectively. The *Nae*I-*Eco*RI fragments 5′ end-labelled at the *Nae*I terminus were digested with T4 DNA polymerase (1 unit every 2.5 ng of DNA) in parallel with the same undamaged (lane 1) and UV-irradiated (lane 2) fragments. The positions of the damaged-bases were identified by comparison with Maxam and Gilbert sequencing reactions of the same fragment.

These data suggest that in B-DNA, N2-adducts can be formed at all G-residues. There is a constant shift of about three to four nucleotides towards the higher molecular weights in the enzyme-generated fragments with respect to the chemically generated fragments. This shift can be accounted for in the following way: 1) one nucleotide shift is due to the destruction of the guanosine residues by the Maxam and Gilbert reaction; 2) for polynucleotides containing 30 or 30 to 50 nucleotides, 1 or 0.5 nucleotide shift is due to the 3′-OH fragment generated by the enzyme that migrated 1 or 0.5 nucleotide slower, respectively, than the corresponding 3′-phosphate generated by the chemical

cleavage reaction; 3) there is an additional nucleotide shift of two nucleotides due to the arrest of enzyme digestion before two nucleotides on the 3'-side of the dGuo-N2-AQO adduct. These data are consistent with published work showing that, on Ac-4-HAQO modified linear fragments, the T4 DNA polymerase arrests 3−4 nucleotides prior to the damaged bases (5). When the insert adopted the Z-form (Fig. 4, lane 3), we see bands analogous to those observed in the UV-irradiated fragment indicating that the presence of damage in the sequence outside the insert caused the enzyme to pause at every other guanine of the insert. We also see a very intense band corresponding to the two guanines of the B−Z junction at the 3' side of the insert. Since in the UV-irradiated control these guanines are only week pausing points, it is likely that this band is due to the presence of dGuo-N2-AQO adducts. These data suggest that also N2-guanine adducts are preferentially formed only at the 3' side B−Z junction but not in Z-DNA.

DISCUSSION

The results reported in this paper demonstrate that guanine residues, when located in an alternating purine−pyrimidine tract in Z-DNA conformation, do not react with Ac-4-HAQO; on the contrary, guanine residues, when located in sequences at the interface between a section of left-handed Z-DNA and right-handed B-DNA, are hyperreactive. These observations indicate that DNA polymorphism can play an important role in determining the reactivity of the C8 and N2 positions of guanine with Ac-4-HAQO. These results parallel much of what has been previously observed by Rio and Leng (13) with N-OH-AF. With the same supercoiled plasmid (pLP32) we used, these authors have shown that N-OH-AF does not react with the C8 position of guanine in Z-DNA but it hyperreacts with the two guanines at the 3' side B−Z junction. In another example, Sinden and colleagues (14), have reported a very poor cross-linking activity of 4,5',8 trimethylpsoralen at AT sequences when in the Z-DNA conformation and a pronounced hyperreactivity of the same sequences when located at the B-Z junctions. The methylating agent MNU instead reacts with B- and Z-DNA with equal efficiency (23).

The structure of the regions at the interface between Z-DNA and B-DNA is unknown. However, numerous evidence exists which indicate a distinctive and unusual conformation of this site (24). Salient features of the B−Z junction are as follows: i) it is short, probably 3 base pairs, with only one base pair showing dramatically altered properties, ii) it is energetically costly to form, iii) it shows a reduced base stacking. This last property could offer some explanation for the observed reactivity since the base pairs at the junction can certainly rotate more easily, than if they are closely held by stacking interactions on both sides (25). In addition, reduced base stacking should facilitate intercalation because of the lower free energy barrier that must accompany the disruption of stacked base pairs at the intercalation sites during the course of the binding reaction. Evidence for a preferred binding of intercalators to B−Z junctions, with respect to B-DNA, comes from studies showing a higher binding affinity of ethidium bromide to a deoxyoligonucleotide containing a B−Z junction (26) over a standard B-form. A characteristic activation of DNA cleavage at a B−Z junction by dynemicin A has also been reported (27). Dynemicin A is an hybrid molecule with an anthraquinone core that is thought to intercalate. The increased photobinding of the 4,5',8 trimethylpsoralen to B−Z junctions has been attributed to its intercalative mode of interaction with DNA (14).

Based on the higher reactivity of negatively supercoiled DNA, with respect to relaxed DNA, with Ac-4-HAQO, we have previously proposed a non-covalent intercalative mode of binding followed by the covalent addition of the carcinogen to DNA (7). This mode of DNA-carcinogen interaction could shed light on the hyperreactivity of Ac-4-HAQO with the B-Z junction at the 3' side of the $(dC\text{-}dG)_{16}$ insert, and especially justify the increased formation of the dGuo-N2-AQO adduct. It is possible to postulate that the geometry of intercalation and the sterically permitted alignment between the reactive centres of the bases and of the damaging agent vary according to the helical twist and may be extremely appropriate at the B−Z junction. Once the dGuo-N2-AQO has been formed at the B−Z junction, it may reside in the groove in the Z direction, similarly to what Hingerty has suggested for the modification by N-acetoxy-2-acetyl-aminofluorene at the guanine amino group (28). However, an alternative explanation may exist especially for the formation of the dGuo-C8-AQO. Evidence of the reactivity to chemical and enzymatic probes specific for single-stranded DNA suggests that the junction regions between Z- and B-DNA may possess transient single-stranded character (29). Thus, we cannot exclude the possibility that the hyperreactivity of the B−Z junction with Ac-4-HAQO might depend on the unpairing of the bases. In fact, we have shown that single-stranded DNA is more reactive than duplex DNA with Ac-4-HAQO, and in single-stranded DNA, dGuo-C8-AQO represents 70% of all stable adducts while only 30% in duplex DNA (7).

The hyperreactivity of the structural transition point between the B and Z-DNA conformation to chemical carcinogens is of particular interest since Z-DNA has been detected in prokaryotes (30) and in mammalian cell nuclei (10), and supposedly plays a role in regulation of transcription and genome rearrangement. A recent search has revealed a non-random distribution of potential Z-forming sequences in human genes with a strong tendency to be located at transcription initiation sites in the gene (31). Furthermore, alternating purine-pyrimidine sequences have been described as being adjacent to the translocation breakpoints observed in lymphoid tumours (32) and found to be hot spot for recombination (33). Since sequences capable of forming the Z-DNA conformation exist *in vivo*, our results may be relevant in the processes of mutagenesis and chemical carcinogenesis especially in view of the possibility that adducts in non-B DNA may be poorly repaired (34; 35; 36).

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ABBREVIATIONS

4-NQO, 4-nitroquinoline 1-oxide; Ac-4-HAQO, Acetyl-4-hydroxyaminoquinoline 1-oxide; dGuo-N2-AQO, 3-(deoxy-guanosin-N2-yl)-4-aminoquinoline 1-oxide; dGuo-C8-AQO, N-(deoxyguanosin-C8-yl)-4-aminoquinoline 1-oxide; dAdo-N6-AQO, 3-(deoxyadenosin-N6-yl)-4-aminoquinoline 1-oxide; N-OH-AF, N-hydroxy-2-aminofluorene; RFI, negatively

supercoiled DNA form; RFIV, relaxed DNA form; RFII, nicked circular DNA form; AP, apurinic/apyrimidinic; SSBs, single-strand breaks; DMSO, dimethylsulphoxide; DTT, dithiothreitol.

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