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Polymorphism in *N*-2-acetylaminofluorene induced DNA structure as revealed by DNase I footprinting

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ABSTRACT

In this paper, we have constructed double stranded helices (60-mers) containing a single N-2-acetylaminofluorene (-AAF) adduct covalently bound to one of the three guanine residues of the *NarI* site ($G^1G^2CG^3CC$). This sequence was identified as a strong frameshift mutation hot spot for many carcinogens that bind to the C8 position of guanine. Using DNase I as a probe for DNA conformation we show i) that the average size of the helix deformation extends over 3 to 5 base pairs in both directions from the adduct site, and ii) that there is a strong polymorphism in the adduct induced DNA conformation. The present study supports the idea that adducts induce specific sequence dependent local conformational changes in DNA that are differentially recognized and processed by the enzymatic machineries that lead to repair or mutagenesis.

INTRODUCTION

The covalent modification of DNA by agents such as UV light, active oxygen derivatives or carcinogens yields DNA lesions or adducts. The biological functioning of DNA is hindered by these adducts, and therefore sophisticated mechanisms have evolved in order to repair these lesions in an error-free manner. However, some adducts escape repair and may be converted into mutations when, for instance, replication proceeds through a template containing a lesion. Recently, it has become apparent that both repair and mutagenesis mechanisms exhibit a strong DNA sequence dependence (1, 2). It has been shown that both phenomena (i.e. repair and mutagenesis) are not linked by an inverse relationship. Indeed, a poorly repaired adduct does not necessarily yield high levels of mutation and conversely an adduct that gives rise to a mutation hot spot is not inevitably an adduct that is refractory to error-free repair (1).

Our laboratory has been involved in the study of the mechanism of action of a chemical carcinogen, N-2-acetylaminofluorene (AAF). This chemical is representative of a large family of carcinogens and mutagens that have in common the ability to bind covalently to the C8 position of guanine. This family includes the aromatic amines, the nitroarenes, nitroheterocyclic compounds, food mutagens and active oxygen species that give rise to 8-oxo-guanine.

N-Acetoxy-N-2-acetylaminofluorene (N-Aco-AAF), the reactive form of AAF, has been shown to induce mainly frameshift mutations in bacteria (3). These mutations mostly occur within two types of sequences : runs of guanines, and *NarI*-sequences ($G^1G^2CG^3CC$). Although, the guanine residues of the *NarI* site are about equally reactive (4), 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 *NarI* site leads to high mutation frequencies (2). Similarly, UvrABC mediated incision studies using double stranded oligomers bearing single AAF adducts within the *NarI* 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 (1). Taken together, these observations suggest that the specificity of repair and mutagenesis is related to the adduct induced DNA structure which in turn depends both on the chemical structure of the adduct and on the local DNA sequence.

Many studies have used randomly modified double stranded DNA or synthetic polynucleotides to investigate the structural alterations that covalently bound AAF residues impose upon DNA. Two major modes of binding have been described.

1) In a random sequence of DNA, AAF induces a local denaturation of B-DNA with the fluorene ring being inserted between the base-pairs and the guanine being rotated outside the double helix. This has been termed the Insertion-Denaturation (5, 6) , or base displacement model (7).

2) In poly d(CpG)n sequences, AAF promotes a B→Z conversion of the helix (8, 9, 10). When bound to left handed Z-DNA, AAF residues are located outside of the helix and the base-pairing is maintained.

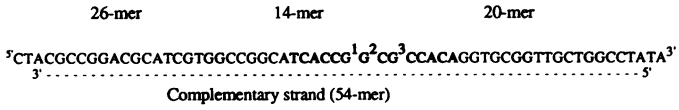
It should be stressed that both models were deduced from low resolution structural data and must therefore be considered as average modes of binding. The observations that single AAF adducts exhibit large differences in mutagenicity and repair depending on their position within the DNA sequence suggests that there might be large differences in the respective adduct induced DNA structures. In order to obtain further insight into these structures at the nucleotide level, we have used DNase I as an enzymatic probe to visualize the extent of the structural deformation of double stranded oligomers bearing single adducts.

In this paper, we show : i) the usefulness of DNase I in measuring the size of the DNA structure alteration induced by a single adduct; and ii) a polymorphism in the adduct induced DNA structure as a consequence of the position of the AAF adduct within a mutation hot spot.

MATERIAL 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 adduct was achieved by ligation of chemically synthesized oligonucleotides as previously described (1) with minor modifications. One of these oligonucleotides (a 14-mer) contains the AAF adducted guanine. The synthesis, purification, and characterization of the modified oligonucleotides has been described previously (11). Briefly, for each construction the corresponding 5'-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 an AAF modified 60-mer as shown in scheme I. After a heating-reanealing cycle, the oligonucleotides were ligated with T4 DNA ligase.



Scheme 1.

The AAF-modified 60-mers were labeled at either the 3' or 5'-ends with ^{32}P. For the construction of the 5'-end labeled AAF-containing fragment, the 26-mer was treated with polynucleotide kinase and [$\gamma^{32}P$]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% sequencing 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 strand not containing the AAF lesion, the complementary 60-mer was labeled either on the 5'- or the 3'-end. 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.

DNase I digestion, densitometry and data processing

Digestions were carried out for 10 min at 37°C in 10μl buffer containing 70mM Hepes pH8, 15mM MgCl2, 1.5mM CaCl2, 0.03mM DTT, 50mM NaCl, 30μg/ml BSA and 10 ng/ml DNase I (Boehringer). Reaction was stopped by addition of 5mM EGTA. DNA was precipitated with ethanol acetate and loaded onto 20% denaturing polyacrylamide gel. Autoradiograms of the gels were scanned on a CS-9000 Shimadzu scanning densitometer.

RESULTS AND DISCUSSION

Size of the adduct induced DNA conformational change

The substrates used in the present study were double stranded oligomers (60-mers) containing a single *NarI* site located approximately in the middle of the DNA fragment. Single AAF adducts covalently bound to one of the three guanine residues of the *NarI* sequence $G^1G^2CG^3CC$ were constructed as described in Material and Methods. All three AAF modified oligomers and the unmodified control DNA were ^{32}P-end labeled at the 3' or 5' extremities of either the adduct containing strand or the complementary strand. These substrates were subjected to a mild digestion with bovine pancreatic deoxyribonuclease I (DNase I) and the hydrolysis products analysed by electrophoresis on sequencing gels. DNase I has been widely used to study sequence-dependent structural variations of DNA (12, 13, 14, 15) and to monitor the interaction of drugs that bind noncovalently (16) or covalently (17, 18) to DNA. DNase I interacts with DNA and cuts each strand independently.

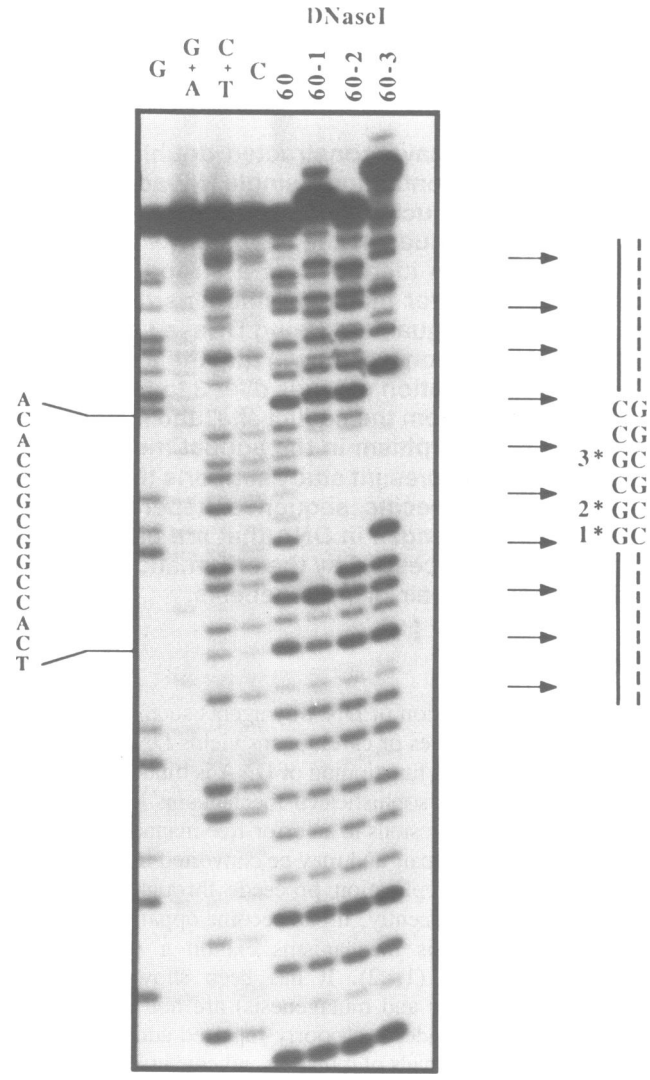


Figure 1. DNase I footprinting of AAF containing strand. Lanes G,G+A,C+T,C are the Maxam & Gilbert sequencing reactions. Lane 60 refers to the non modified 60-mer; 60−1, 60−2, 60−3 indicate the position of AAF adduct. The DNA was 5'-end labeled. When DNase I cleavage occurs 3' to the site of AAF binding, there is a retardation in migration on the sequencing gel due to the presence of the fluorene residues. This gives rise to some difficulty in the attribution of bands when the adduct containing strand is labeled at the 5' end. The correctness of the assignments in this region was therefore confirmed by analysis of 3' end labelled fragments (data not shown). In the scheme on the right hand side, the heavy line represents labeled modified strand and the dotted line represents the unlabeled complementary strand. Numbers indicate the modified guanine residues.

Crytallographic structure of DNA-DNase I complex has shown that an exposed loop of the protein binds in the minor groove of B-DNA and that interactions occur with the backbone of both strands (19, 20). Global geometric parameters important for efficient cutting by the nuclease are the width of the minor groove and the flexibility or bendability of DNA.

In the unmodified control fragment, DNase I was found to cut at every internucleotidic bond with varying efficiencies, reflecting local differences in the geometry of the double helix (fig 1 and 2). When single AAF bearing fragments were digested with DNase I, a very distinct print of the adduct was seen (fig 1 and 2). This print shifted by one or two nucleotides according to the position of the adduct and was essentially characterized by the total lack of DNase I cutting over several base pairs around the position of the adduct in both strands. Moreover, in the complementary strand of the duplexes with the AAF adduct at position G^3 and G^1 the print contained one and two specific hypersensitive bonds, respectively.

In order to visualize the intrinsic print of the adducts, the intensities of all the cleavage products were measured by scanning densitometry. Due to slight differences in intensities between samples, we normalized the different tracks by taking into account the intensities of ten bands in a region not affected by the AAF adducts. For each modified substrate, the normalized intensity of a given band ($I_{mod.}$) was divided by the normalized intensity of the corresponding band in the unmodified substrate ($I_{unmod.}$). The ratios $I_{mod.}/I_{unmod.}$ were plotted for all three substrates over 25 base pairs centered around the position of the adduct (fig 3). When the activity of DNase I is not influenced by the AAF induced structural alteration, the ratio $I_{mod.}/I_{unmod.}$ is close to 1. Within the region where the structural alteration influences the activity of DNase I the ratios $I_{mod.}/I_{unmod.}$ range from values close to zero (internucleotidic bonds that are protected from cutting) to values greater than 5 and up to ≈ 50 reflecting bonds that become hypersensitive to DNase I cutting. On the average, the size of the structural alteration is sensed on both strands and extends over 6 to 10 base pairs in the vicinity of the adduct. When the chemical nuclease orthophenanthroline-copper (OP_2Cu) was used to probe the same substrates, a distinct print was seen over a 4 to 6 base pairs long region centered around the adduct (21). The slightly larger size of the AAF induced structural alteration sensed by DNase I as compared to the corresponding deformation sensed by OP_2Cu might be related to the difference in size of the two probes itself (22). In agreement with the present measurements, Fuchs and Daune (23) have estimated the average

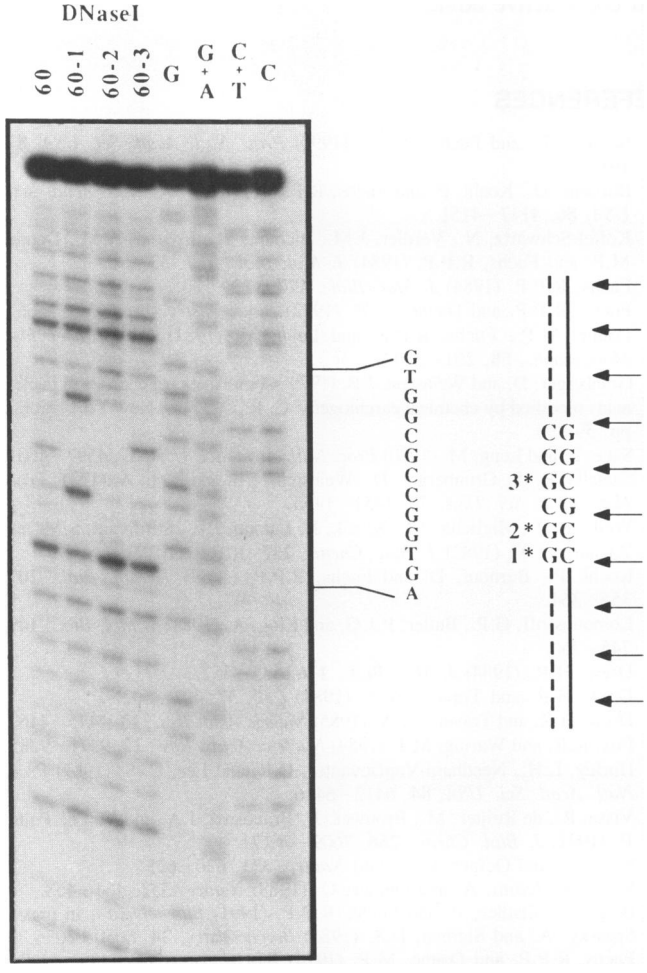


Figure 2. DNase I footprinting of the 3'-end labeled complementary strand. Lanes G,G+A,C+T,C correspond to the Maxam & Gilbert sequencing reactions. Sites of DNase I cutting correspond directly to the DNA sequence ladder. In the scheme on the right hand side, the heavy line represents labeled modified strand and the dotted line represents the unlabeled complementary strand. Numbers indicate the modified guanine residues.

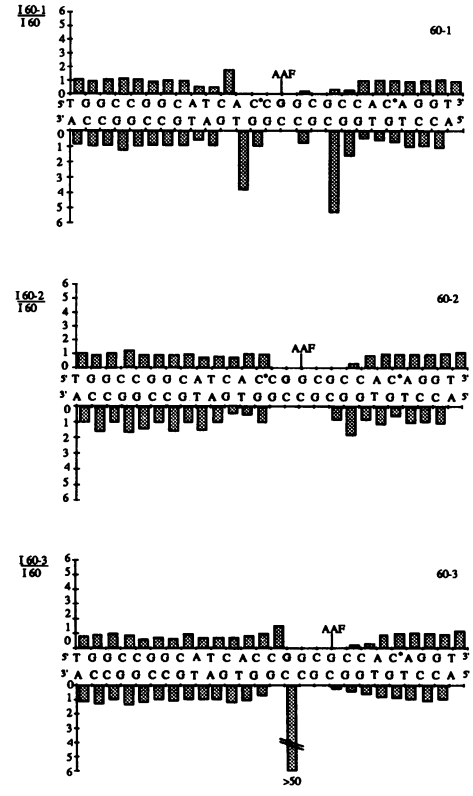


Figure 3. Quantitative representation of DNase I cleavage for the three single modified 60-mers. The autoradiograms were scanned and the bands intensity normalized as described in the text. The sensitivity of any given internucleotidic bond to DNase I was determined by dividing the normalized intensity of the band in the AAF modified substrate by the normalized intensity of the corresponding band in the unmodified substrate. These ratios are represented by bars. DNase I cleavage of the AAF modified strand is shown above the sequence, and cleavage of the complementary strand is shown below the sequence.

size of the AAF-induced local denaturation zone to be equal to ≈7−8 bp by means of formaldehyde unwinding kinetics of DNA samples which had been randomly modified with AAF.

Polymorphism in adduct induced DNA structure

For all three AAF containing substrates, DNase I reveals a clear difference in its cutting pattern in the vicinity of the adduct indicating a polymorphism in the adduct-induced DNA structure. Although it is not possible from these kind of data to draw precise conclusions about the actual structure of the individual distortions that AAF adducts impose upon the DNA helix, these data provide 'visual' evidence of the polymorphism in adduct induced DNA structure at nucleotide level resolution. The G^2 modified oligomer presents a total protection towards DNase I cutting extending two and three bases to the 5′ and 3′ sides of the adduct, respectively. Total protection is also achieved in the complementary strand over a total of 4 base pairs centered around the cytosine complementary to the modified guanine. The print is essentially restricted to this area and no hypersensitive bond is seen in contrast to the oligomers modified at G^1 and G^3 where a more extended print is seen in both strands and hypersensitive bonds appear in the complementary strand (fig 3). For both the G^1 and G^3 adducts, the DNase I scission patterns show similarities in the AAF bearing strand. Total inhibition of cleavage occurs on the three phosphodiester bonds located to the 5′ side and only one to the 3′ side of adduct. A region of reduced cleavage extended an additional four and two internucleolitic bonds for G^1 and G^3 adducts· respectively. In the complementary strand, of both the G^3 and G^1 modified oligomers, the internucleotidic bond between the second and third base 3′ to the cytosine complementary to the modified guanine was hypersensitive to DNase I cutting. These DNase I hypersensitive bonds suggest strong modification of DNA backbone geometry

On the basis of circular dichroïsm experiments, it has been suggested that upon binding of AAF to G^3 there is a local induction of a Z-like DNA region (24). For the G^3 modified oligomer, the modified guanine is part of a dinucleotide ^{5}'CpG^3' unit that can undergo a B→Z transition. In agreement with this hypothesis is the recent finding that diethylpyrocarbonate (DEPC) reacts with the guanine 3′ to the C paired with G^3 suggesting that this guanine is indeed in the *syn* conformation as expected in Z-DNA (21). The strong DNase I hypersensitive bond might therefore be located at one of the B/Z junctions that are formed. A similar observation has been made recently by Ramesh and Brahmachari (25) showing a DNase I hypersensitive bond at the B/Z junction in a plasmid containing a (CpG)16 insert that is in the Z conformation.

Two sites of enhanced cleavage can be observed for the G^1 modified oligomer but are of lower intensity than that observed for the G^3 modified oligomer. In this case, although G^1 is also part of a CpG dinucleotide, no local Z-like structure has been suggested by CD data, in spite of the transient *syn* conformation of the guanine on the complementary strand suggested by DEPC reactivity (21). The difference between G^1 and G^3 is most likely related to the differences in ability of the flanking base pairs to accomodate the B/Z junctions.

CONCLUSION

The biological importance of the adduct induced structural polymorphism of DNA is exemplified by recent data involving both excision repair and mutagenesis (1,2). Indeed, within the same *NarI* sequence context as studied here, a five to ten fold reduction in incision efficiency between AAF adducts located at position G^1 and G^2 respectively, was observed (1). Even more importantly, in the context of mutagenesis where the *NarI* site was discovered as being an extremely strong hot spot for −2 frameshifts induced by many mutagens binding to C(8) of guanine (26, 27) , it was found that adducts at G^3 induced mutagenesis over 100 fold more efficiently than adducts at positions G^1 or G^2 (2, Veaute and Fuchs, unpublished results) . The present study supports the idea that adducts induce specific sequence dependent local conformational changes in DNA that are differentially recognized and processed by the enzymatic machineries that lead to repair or mutagenesis.

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