Radiolytic signature of Z-DNA

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ABSTRACT
Ionizing radiations induce various damages in DNA via the hydroxyl radical OH' generated by the radiolysis of water. We compare here the radiosensitivity of B- and Z-DNA, by using a Z-prone stretch included in a plasmid. In the supercoiled plasmid, the stretch is in the Z-form, whereas it is in the B-form when the plasmid is relaxed. Frank strand breaks (FSB) and alkali-revealed breaks (ARB) were located and quantified using sequencing gel electrophoresis. We show that B- and Z-DNA have the same mean sensitivity towards radiolytic attack, for both FSB and ARB. Nevertheless, the guanine sites are more sensitive, and the cytosine sites less sensitive in Z- than in B-DNA, leading to a characteristic signature of the Z-form. The comparison of experiments with the outcome of a Monte Carlo simulation of OH' radical attack suggests that transfer of initial damage from a guanine base to its attached sugar or the adjacent 3' cytosine is more important in Z-DNA than in B-DNA.

INTRODUCTION
Ionizing radiations lead to cell death, mutagenesis, carcinogenesis and chromosomal aberrations, and DNA is considered to be the critical target of these damaging agents. They induce strand breaks, abasic sites and modifications of bases (1). In aqueous aerated solution, DNA damage is mainly due to attacks on sugars and bases by hydroxyl radicals (OH') emanating from the radiolysis of water (2). OH' radicals abstract a hydrogen atom from sugars and bases, or get added to the bases, thus generating sugar and base radicals. These last radicals engender breaks of the phosphodiester backbone, either directly at neutral pH (frank strand breaks, FSB) or after alkaline treatment (alkali-revealed breaks, ARB). A radical transfer process corresponding to hydrogen abstraction from the sugar by the base—OH' radical and leading also to an FSB was proposed for γ- and UV-irradiated DNA (3–5).

The extensive work performed until now on the radiolysis of DNA considers DNA as a homogeneous polymer from the structural point of view. Only few studies reported a dependence of radiosensitivity on the sequence of nucleotides and related it to some variations in the B-structure, e.g. size of grooves (6–9). Apart from a study comparing single- and double-stranded DNA (9), the radiosensitivity of different forms of DNA (Z-, H-, and branched forms), has not been investigated until now.

In the present work, we compare the radiosensitivity of B- and Z-DNA. The different exposure to the solvent of bases and sugars in the two forms (10) (and consequently access of OH' radicals) entitle us to expect a difference in radiosensitivity. We performed this comparison by observing radiation-induced FSB and ARB in a Z-prone stretch included in a plasmid. This system presents a very important advantage. The torsional constraint due to supercoiling of the native plasmid forces the stretch to be in the Z-form, and relaxation of the plasmid triggers the Z—B transition of the stretch (11). Therefore, irradiating the relaxed or the supercoiled plasmid allows to observe the same stretch in the B- or in the Z-form, respectively. No additives (such as metal ions or polyamines) are necessary to trigger the B—Z transition, as for a linear DNA (12). Such additives might interfere with radiolysis by protecting or sensitizing effects.

We have used for this purpose sequencing gel electrophoresis to locate and quantify the breaks in the DNA. The experimental data were compared to the outcome of a Monte Carlo simulation of OH' radical attack.

MATERIAL AND METHODS
Plasmid pCG40 and the Z-prone sequence (CG)₉AATT(CG)₉
The plasmid pCG40 is derived from plasmid pGEM-4Z (Promega). At the BamHI site of the poly linker was inserted the sequence (CG)₉AATT(CG)₉, which can transit into the Z-form when the plasmid is negatively supercoiled, i.e. in its natural supercoiled form (13). A fragment of 92 bp (H/S fragment) containing this sequence can be obtained after digestion by the restriction enzymes HindIII and SacI at their unique sites on the plasmid.

The plasmid was prepared from a transformed E. coli strain (DH5) by the method of alkaline lysis (14). The negatively supercoiled form of the plasmid was purified on a CsCl gradient in the presence of ethidium bromide (BET) (14). After removing

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CsCl and BET, fractions of 100 μg of the plasmid were stored at -20°C at a concentration of 500 μg/ml in 10 mM Tris–HCl, 1 mM EDTA, pH 7.5. The final solution of plasmid contained 97% supercoiled form, as judged by the quantitative analysis of an agarose gel after electrophoresis and BET staining.

Irradiations

γ-Photon irradiation was performed with a 90Co teletherapy unit (Centre Hospitalier Régional d’Orléans). The dose mean lineal energy was 1.9 keV/μm, and the dose rate was 9 Gy/min.

Samples of several μl were irradiated in polypropylene tubes, immersed in an ice bath 1 cm below the surface of the water. For all irradiations, DNA was dissolved in 10 mM potassium phosphate buffer, pH 7.25.

Determination of the number of single-strand breaks per plasmid

Supercoiled plasmid (10 μl at 75 μg/ml in 10 mM potassium phosphate pH 7.25) was submitted to irradiation, and then electrophoresed on agarose gel (1.2% in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, pH 8) for 5 h at 450 V/m. After BET staining, the fractions of supercoiled, relaxed and linear forms were assayed by fluorescence scanning (Camag TLC scanner II), and the number of single-strand breaks (SSB) was determined as previously described (15).

Post-irradiation labelling of the H/S fragment

The irradiated plasmid was linearized with HindIII, treated with alkaline phosphatase (Boehringer), and labelled at the 5'-termini by T4 polynucleotide kinase (BRL) with [γ-32P]ATP (specific activity >185 TBq; Amersham), according to Sambrook et al. (14). The labelled linear plasmid was digested with SacI, leading to the 92 bp H/S fragment containing (CG)9AATT(CG)9, and a 2680 bp fragment. Both fragments were separated by electrophoresis on an 8% polyacrylamide gel (acrylamide:bisacrylamide ratio of 20:1) in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8). The position of the H/S fragment was determined as previously described (15).

Location of the radiation-induced breaks

The labelled fragments were dissolved in a loading buffer (98% deionized formamide, 10 mM EDTA, 7 M urea and 0.025% bromophenol blue), heated at 100°C for 3 min and rapidly cooled to 0°C for 5 min. Then DNA was layered on a sequencing gel (12% acrylamide (acrylamide:bisacrylamide ratio of 19:1), 7 M urea, 30% formamide in TBE buffer). The gel was run for 200 min at 42 W, fixed for 30 min in a bath containing 10% acetic acid and 10% ethanol, and dried. The radioactivity in the bands was assayed using a PhosphorImager and the ImageQuant software (Molecular Dynamics).

When required, piperidine treatment of DNA, identical to that used in the Maxam–Gilbert sequencing procedure (14), was performed before dissolving in the loading buffer.

Unirradiated H/S fragment was submitted to the Maxam–Gilbert sequencing procedure (14) of purines and pyrimidines, to provide an identification of the bands on the sequencing gels.

Despite the presence of two denaturing agents (7 M urea and 30% formamide) in the gels, all the bands were not clearly resolved, due to the presence of the large self-complementary sequence 5'-(CG)9AATT(CG)9-3'. When more than the first half part of this sequence is present in a single-stranded oligonucleotide, hairpin formation compacts the electrophoretic pattern, and only the bands corresponding to the first (CG)9 track can be correctly resolved. Our analysis is thus limited to this part.

Diethylpyrocarbonate (DEPC) treatment

5 μg of plasmid were mixed with 3 μl of DEPC in 100 μl of TBE buffer and incubated under mixing for 30 min at 20°C. Excess of reactant was eliminated by ether, and the labelling and the isolation of the fragment were done as described above. The N7 sites of purines are carboxyethylated, and revealed as strand breaks by piperidine treatment before loading on sequencing gels (16,17).

Modelling of OH· radical attack on DNA

To simulate OH· radical attack on DNA in dilute aqueous solution, we have used a model described in detail elsewhere (18). The model assumes that OH· radicals are distributed homogeneously in the solution. The three-dimensional atomic structure of DNA is generated from Arnott’s data (19,20), and enveloped by a virtual cylinder 4 nm in diameter representing the volume of interest. OH· radicals start their diffusive motion in a random direction from the cylinder coat. Their diffusion in the volume of interest is simulated by the Monte Carlo method. At each diffusive jump, the OH· radical is displaced in space by 1 Å. According to the model of Smoluchowski (21), the OH· radicals which diffuse in a sphere centred at the reaction site of a given moiety, and with a radius equal to the reaction radius of this moiety, will react with a probability of 1. The considered

![Figure 1. Relaxation of the plasmid upon γ-irradiation. (Top) kinetics of SSB induction in the plasmid pGEM 4Z. The Gray (Gy, SI unit of absorbed dose), corresponds to an absorbed energy of 1 J/kg of matter. (Bottom) Electrophoretic patterns of the DEPC-treated Z-prone sequence after 2.5 and 50 Gy irradiation of the plasmid. Notice the different scales for the two doses.](5566 Nucleic Acids Research, 1994, Vol. 22, No. 25)
reaction sites in DNA are C(6) and C(5) in pyrimidines, C(4), C(5) and C(8) in purines, and all carbon positions in the sugar moiety (1,22). The reaction radii calculated from the rate constant for OH\(^-\) reaction with the DNA constituents (23) are: 1.2 Å for deoxyribose, 2.9 Å for adenine and cytosine, 3.0 Å for thymine and 4.3 Å for guanine. If an OH\(^-\) radical diffuses out of the volume of interest, the simulation is terminated, and a new starting OH\(^-\) radical is generated.

To compare calculations with experiments, we had to generate in a computer a DNA structure in which the first part is in the Z form, and the second part in the B form. Since we were not able to simulate the region of the junction between the two forms, we have separately generated two duplex oligonucleotides, (3'-GC-5')\(_6\) in the Z-form and 3'-CTAGGAGATCTC-5' in the B-form, coupled them and used them together for the simulations of OH\(^-\) attack. The sequence of the generated piece of DNA is the upstream sequence of the part of the H/S fragment which has been correctly resolved in the experiments. The consequence of this simplification is that the calculated values for nucleotides at the coupled ends cannot be used for comparison. Similarly, to avoid an influence of end effects, the GC at the beginning and the C at the end of the generated piece of DNA were not used for the comparison. On average, at least 20 000 reacting OH\(^-\) were generated per nucleotide, which allowed us to keep the statistical error of the results presented well below 5%.

**RESULTS**

**Determination of the yield of single-strand breaks (G)**

The supercoiled plasmid was irradiated with \(\gamma\)-rays in the dose range 0-20 Gy. The number of SSB was determined and plotted as a function of dose (Fig. 1, top). Linear regression over the experimental data leads to a G value of 0.045 SSB/plasmid/Gy.

After 2.5 Gy irradiation, 10.5% of the plasmids are relaxed. This corresponds to the induction of 0.11 SSB/plasmid on average. According to Poisson's law, 95% of the relaxed plasmids contain only one SSB, the others containing two or more breaks. Therefore we can reasonably assume that a break observed in the Z-prone sequence is the first and unique break induced by the radiation in the majority of the plasmids. Thus this break occurred in the supercoiled plasmid, when the Z-prone sequence was in the Z-form. For 50 Gy, the plasmids contain in average 2.25 SSB/plasmid. Since the Z-prone region represents 3% of the plasmid, 97% of the first breaks that relax the plasmids are located outside the Z-prone sequence. When the second break arises, the plasmid is already relaxed, and therefore this break occurs in the B-form.

An extensively used chemical probe of Z-DNA, DEPC, reacts at the N7 position of purines. When the purines are in syn conformation in the Z-form, N7 is more accessible to carboxyethylation than in the B-form, and the reaction is considerably enhanced. We have used this probe to check the structure of DNA. Indeed, the alternating CG region was still in Z-form after 2.5 Gy and in B-form after 50 Gy irradiation (Fig. 1, bottom).

**Post-irradiation labelling of the H/S fragment**

Since the H/S fragment must be irradiated in the intact supercoiled plasmid, labelling must be performed after irradiation. Thus we had to ensure that the phosphatase – kinase enzymatic system did not process the radiation-induced breaks. Supercoiled plasmids were either linearized by HindIII or irradiated at 20 Gy to induce ~1 break/plasmid on average. Both samples were labelled at 5'-termini, and the supercoiled and circular relaxed forms were separated by agarose gel electrophoresis (the dose is too low to induce any detectable linear molecules). The amount of DNA in each band, and consequently the number of breaks, was determined by measuring the fluorescence after BET staining, and the radioactivity was assayed by scintillation counting of the pieces of gel. As the labelling of the HindIII-linearized fragment was complete, the ratio [radioactivity/strand breaks] was taken as the reference. The corresponding ratios for the supercoiled and the circular relaxed form were found to be 0 and 3% of the reference value, respectively, showing that the 5'-ends generated by radioisotope are not labelled by our procedure.

**Radiolysis of plasmids**

Plasmids were irradiated with either 2.5 or 50 Gy of \(\gamma\)-rays. After post-irradiation labelling, the H/S fragments were layered on sequencing gels to identify the FSB produced upon irradiation. Piperidine treatment of the samples before gel electrophoresis allows detection of the sum of FSB and ARB. To compare calculations with experiments, we had to generate the corresponding ratios for the supercoiled and the circular relaxed form were found to be 0 and 3% of the reference value, respectively, showing that the 5'-ends generated by radioisotope are not labelled by our procedure.

After 50 Gy irradiation, the probability of FSB occurrence does not strongly depend on the sequence. However, FSB + ARB show a more pronounced dependence on the sequence. The reactivity at the guanine sites is systematically greater than the average reactivity.

After 2.5 Gy irradiation, the part of the studied sequence outside the Z-prone region behaves to a large extent as after irradiation at 50 Gy. In the (CG)\(_b\) track however, the yield of FSB as well as of FSB + ARB at guanine sites is significantly enhanced as compared to 50 Gy irradiation. At cytosine sites,
the yield of FSB is considerably smaller, and that of FSB + ARB is similar to that measured after 50 Gy irradiation. FSB production is particularly high at a thymine site located 10 bases away from the end of the alternated (CG)₉ track.

Modelling of the chemical attack of DNA

Figure 3 shows the calculated patterns of OH· radical attack on deoxyribose and on nucleosides. In the Z-form of DNA, the calculated level of attack is higher for G sites than for C sites by a factor of 2 for sugars and by a factor of 13 for nucleosides. For B-DNA, there is practically a random attack on deoxyribose, but for nucleosides, one can observe base and sequence dependence of the attack.

In the same figure, the calculated values are compared to the experimental ones. The attack on sugar is compared with the measured yield of FSB, and the attack on nucleosides is compared to the measured yield of cleavage of piperidine-treated DNA (FSB + ARB). There is a good qualitative and quantitative agreement between both sets of data for regions in the B-form. The agreement is, however, only qualitative for the part in the Z-form.

DISCUSSION

It has already been shown that alternating (CG)₉ sequences included in negatively supercoiled DNA adopt the Z-form, without any additive (Mg²⁺, spermidine, etc) known to induce the B–Z transition in relaxed DNA (11). We can thus expect that in the native supercoiled plasmid the Z-prone sequence is in the Z-form, whereas it switches to the B-form when the plasmid is relaxed by the first radiation-induced strand break, i.e. when the torsional stress disappears. We have used the chemical probe DEPC to check whether, under our experimental conditions, the (CG)₉ track was indeed in the Z-form at 2.5 Gy, and in the B-form at 50 Gy. DEPC has been widely used as a specific reagent.
for purines in Z-form DNA. After irradiation at 2.5 Gy, the strong reaction of DEPC with purines proves that the Z-prone sequence was effectively in the Z-form, and consequently that the damage observed in this sequence were produced in Z-DNA. In contrast, the very weak reaction after 50 Gy irradiation shows that in this case the DNA was in the B-form.

For the FSB observed in the regions of DNA in B-form (Fig. 2), the dependence on the nature and on the sequence of the bases was weak, as already reported (7, 24). In the region of DNA in the Z-form, the probability of breakage at G sites was twice as high, and that at C sites even more than twice as low as the mean breakage probability in B-DNA. This leads to a "splitting" in the pattern of breakage probabilities in the Z-part: the G sites appear more radiosensitive and the C sites more radioprotected compared to the random sequences of B-DNA (Fig. 4). The comparison between the Z- and B-forms reveals a particular signature of the Z-form after analysis of the radiation-induced FSB. Nevertheless, the mean probability of breakage in the Z-form averaged over all nucleotides of the Z-prone stretch is equal to that in the B-form averaged over all nucleotides of the B-part of the studied sequence. The exceptional radiosensitivity of the thymine site situated in the B-DNA 10 bases away from the end of the (CG)₉ track has no straightforward explanation.

The analysis of the sum of FSB and ARB (after piperidine treatment) shows, that for both B- and Z-forms, guanine sites are more sensitive than the other sites. In the Z-prone region, the difference between both forms is not as pronounced as for FSB, and therefore, the signature of the Z-form is less marked for FSB + ARB than for FSB alone.

It is generally considered that FSB are mainly induced by a direct OH radical attack on the sugar, whereas ARB are due to OH attack on bases, leading to abasic sites and modified bases that induce strands breaks upon alkaline treatment (1). The calculation of the probability of attack by Monte Carlo simulation of OH radical diffusion and its reaction with DNA has been compared to the experimental results. The patterns of FSB were compared with the patterns of attack on the sugar alone, and the patterns of FSB + ARB were compared with the patterns of attack on the whole nucleoside (base + sugar).

For both DNA forms, measured and calculated patterns agree well from the quantitative point of view. The quantitative agreement is, however, good for B-DNA only. In this case, the good concordance between experimental data obtained under neutral conditions (FSB) and modelling of OH attack on sugars (Fig. 3, top) confirms that OH-induce sugar radicals are mainly responsible for FSB and that OH-induced base radicals contribute only slightly to the formation of this damage in B-DNA. The agreement between the experimental data obtained after alkaline treatment (FSB + ARB) and the simulation of the attack at nucleosides in B-DNA (Fig. 3, bottom) corroborates the idea that the base radicals contribute significantly to the formation of ARB in this form. Thus, the major determinants of the measured variability in the radiosensitivity of nucleotides seems to be the character of the chemical reaction of the OH radical with sugars and bases, together with their specific position in the sequence. The same was concluded from our previous study on longer oligonucleotides in B-form (18).

We observed, however, that in the alternating (CG)₉ sequences, the calculated values for guanosines are always greater than the measured FSB + ARB. These differences are small in the case of the B-form of the (CG)₉ sequence (data not shown). For Z-DNA, there are large differences between the calculated values and the measured FSB at the G sites. A radical transfer from guanine, the most reactive moiety towards the OH radical, to its sugar and/or to an adjacent base can account for these discrepancies. There is evidence that the formation of a strand break can occur via the transfer of OH damage from base to sugar (5). The mechanism of the damage transfer could be an intra-DNA H atom abstraction, suggested as a pathway for strand break formation by pyrimidine radicals (3,4).

In Z-DNA, the guanosine nucleotides are in the syn position, i.e. the base is located over the sugar, and is firmly stacked with the cytosine in the 3' position, but not at all with the 5' one (10). These structural features of Z-DNA may promote a transfer of initial damage from guanine to its own sugar or to its 3'-neighbouring cytosine.

To obtain a good fit between calculation and experiment for FSB at guanine sites and for FSB + ARB at cytosine sites, we have to assume a radical transfer of 30% from guanine to the associated deoxyribose, and of 10% to the 3' cytosine, respectively. The values calculated in taking into account this transfer are compared to the experimental values in Fig. 5.

In conclusion, the present work shows that: (i) B- and Z-DNA have the same mean sensitivity (averaged over all nucleotides) towards radiolytic attack, concerning both FSB and ARB; (ii) in Z-DNA, the guanine sites are more sensitive, and the cytosine sites less sensitive than in the same sequence in the B-form, leading to a characteristic signature of the Z-form; (iii) the transfer of initial damage from guanine base to its sugar or an adjacent base is probably more important in Z-DNA than in B-DNA; (iv) stochastic simulation of OH radical attack on DNA can predict qualitative patterns of strand breakage.

The response to radiation injury of a DNA presenting a sequence-dependent structural polymorphism is not uniform along the molecule, e.g. in Z- and B-form regions. This may be important, since Z-regions present in vivo (25—27) seem to be involved in biological processes such as gene regulation and recombination (28).

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