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Complexes of mismatched and complementary DNA with minor groove binders. Structures at nucleotide resolution via an improved hydroxyl radical cleavage methodology

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Abstract

Tumor cell lines can replicate faster than normal cells and many also have defective DNA repair pathways. This has led to the investigation of the inhibition of DNA repair proteins as a means of therapeutic intervention. An alternative approach is to hide or mask damaged DNA from the repair systems. We have developed a protocol to investigate the structures of the complexes of damaged DNA with drug like molecules. Nucleotide resolution structural information can be obtained using an improved hydroxyl radical cleavage protocol. The use of a dT_n tail increases the length of the smallest fragments of interest and allows efficient co-precipitation of the fragments with poly(A). The use of a fluorescent label, on the 5' end of the dT_n tail, in conjunction with modified cleavage reaction conditions, avoids the lifetime and other problems with ³²P labeling. The structures of duplex DNAs containing AC and CC mismatches in the presence and absence of minor groove binders have been investigated as have those of the fully complementary DNA. The results indicate that the structural perturbations of the mismatches are localized, are sequence dependent and that the presence of a mismatch can alter the binding of drug like molecules.

Keywords

DNA; minor groove; lesion; DNA repair; hydroxyl radical; fluorescence; mismatch

1. Introduction

There are many potential routes to therapeutic intervention in cancer and the leading targets include DNA replication and DNA repair. Since many tumor cell lines replicate more rapidly than normal cells replication has been a target for decades. Some inhibitors of replication that have been found useful are arabinosides, the platinum drugs, alkylating agents, nitrogen mustards, bleomycin and neocarzinostatin. Some of these molecules may work by other mechanisms. Replication inhibitors are also used as anti-virals.

DNA damage can lead to cell morbidity and mortality. Tumor cell lines can have defective DNA repair and the absence of nucleotide excision, mismatch or recombinational repair may lead to the presence of more damaged DNA than normal cells. DNA repair pathways have

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become one of the leading targets for drug discovery with many protein targets in many tumor cell lines [1–4]. For example inhibition of PARP-1, poly(ADP-ribose) polymerase, in cells with mutant forms of BRCA1 or BRCA2 can lead to double strand breaks and cell death without the introduction of DNA damaging agents [2–4]. Some of the other repair proteins being investigated are MSH2 and ERCC1 in small cell lung cancer [3] and RAD51 in glioma cancer [5]. There are many reviews of the use of single and multiple inhibitors of DNA repair [1, 3, 4, 6–13].

A potential alternative approach is to target the damaged DNA rather than the repair systems or the replication machinery. Complexes of damaged DNA with drug like molecules could inhibit the repair process by masking the damaged site or other mechanism. It may be the case that the targeting of specific types of DNA damage can be made tumor cell line specific by exploiting the competence of the cells in a specific nucleotide excision, base excision, mismatch or recombination pathway. A first step in this approach is to find molecules that selectively bind to a type of damaged DNA and then investigate whether the resulting complex inhibits repair and/or replication specifically in tumor cells. Molecules that target of specific types of damaged DNA may be also be useful as research tools to identify the presence of specific types of damaged DNA in complex systems including cells.

There is a clear need for an approach that can offer structural information about the interactions of a wide range of drug like molecules with any type of DNA lesion site under physiological conditions. The methodology needs to be able to obtain results that can compare the lesion site with both the undamaged DNA and the lesion site in a complex with the drug like molecule of interest. Similarly, the methodology needs to be able to be applicable to the complexes of the drug like molecules with undamaged DNA and DNA containing the lesion sites of interest. A variety of methods have been used to examine the structures of DNA lesion sites. NMR and x-ray offer the potential to obtain atomic resolution information. There are structures of many complexes of damaged DNAs with repair enzymes [14] and relatively few for the free DNAs [15]. The thermodynamics of many lesion sites has also been investigated [16–18].

Footprinting experiments, also known as chemical protection or structural interference, have been used in thousands of investigations. The hydroxyl radical cleavage reaction offers the most information of any chemical probe of nucleic acid structure, and the results have been shown to be particularly useful in studying the structures of RNA and RNA folding, ribosome assembly, to following the time course of RNA folding and other processes [19–27]. The hydroxyl radical cleavage reaction effectively reports on the accessibility of the sugar to a water molecule sized probe primarily in the minor groove [19, 20, 27]. Cleavage reactions have been used to monitor small molecule binding to RNAs [28]. That is, the extent of cleavage is proportional to the solvent accessible surface area of the sugar. The comparison of the information from hydroxyl radical cleavage reactions with subsequently determined crystal structures has shown that the hydroxyl radical results are very reliable [25]. Hydroxyl radical reactions do not require crystals, the high concentrations needed for NMR or even most optical experiments, can be carried out under a wide range of conditions allowing the effects of temperature, salt concentration and the concentration of ligands to be investigated [19, 27]. Hydroxyl radical cleavage reactions are now being used to monitor the structural variation in the human genome [29].

Hydroxyl radical reactions have *not* been used to examine the structural effects of lesions on modest length duplex DNAs even though this is potentially an excellent approach for this problem. There are good practical reasons for this. Short DNAs are not readily amenable to the usual hydroxyl radical cleavage protocol and the methods used to prepare many of the damaged sites of interest in DNA place a limitation of modest length on the DNA [30–35].

The practical problems to investigating cleavage reactions of modest length DNAs are interrelated and include separating the small fragments from the reaction reagents so that high quality gels can be obtained; precipitating the small fragments at high enough yield to allow detection and overcoming the length dependence of the ethanol precipitation used to separate the DNA fragments from the reaction reagents.

An examination of tandem AG mismatches used samples of about 60 nucleotides in length and the results showed that there was on the order of a hundred, or more, fold variation in band intensity from the larger to smaller fragments [36]. Examination of the same samples by nuclease cleavage gave fairly uniform band intensities as a function of length [36]. An examination of the binding of distamycin and netropsin to duplexes of over 120 nucleotides in length exhibited an enormous variation in intensity from the larger to smaller fragments giving rise to significant baseline variation as well as a strong background [37]. These factors make the quantification of the results challenging. There appears to have been only a couple of studies of the hydroxyl radical cleavage of modest length DNAs and these were apparently limited to the middle portion of the DNAs [19, 38].

We have found that the purification and length dependent yield problems can be largely solved through the use of dT_n tails and co-precipitation of the cleavage reaction products with poly(A). The DNA containing the lesion, or lesions, is prepared with a dT tail (Fig. 1A). This increases the lengths of the fragments as well as allowing for efficient and reproducible co-precipitation of even those fragments with a single nucleotide from the duplex region. This allows the use of DNA that are short enough to give high quality gel results that allow precise quantification of the band intensities and hence the reactivity of each site. This is similar in strategy to the well known use of poly(dT) to purify mRNAs that contain poly(A) tails.

An additional improvement comes from the use of fluorescent labeling, rather than ³²P end labeling, to allow the samples to be prepared once and used over long time periods. The usual hydroxyl radical cleavage reaction destroys fluorescent dyes, and this has apparently contributed to a lack of interest in this approach. As demonstrated below, the reaction conditions can be adjusted to obtain both sufficient cleavage and low levels of destruction of the dye. The reproducibility of the results is also enhanced by the use of low levels of cleavage.

Combining these innovations allows the cleavage reactions to examine damaged DNAs at high sensitivity and nucleotide resolution using the modest length DNAs. This generally useful methodology is demonstrated here by comparing the cleavage patterns of 29mer DNA with and without mismatches in the presence and absence of the minor groove binders [39] DAPI, 42,6-diamidino-2- phenylindole, and Hoechst 33258.

2. Materials and Methods

2.1 DNA samples

The DNA oligonucleotides were obtained from Integrated DNA Technologies in HPLC purified form. The 29mer DNAs were dissolved in PB3 buffer, 2.5 mM Na₂HPO₄, pH 7. These 100 µL samples were then precipitated with 10 µL of 3 M NaCl and 500 µL of chilled ethanol. After three hours of incubation on ice the samples were centrifuged at 13000 rpm and supernatant was removed. The pellet was dried using a speed-vac. The concentration of the samples was determined by absorption using a Nanodrop spectrometer.

2.2 Oregon Green labeling

The DNA samples with a dT tail containing the modified amine, 5AmMC6T, at the 5' end were dissolved in 100 μ L of autoclaved, doubly distilled H₂O, extracted one time with an equal volume of phenol, then twice with chloroform and ethanol precipitated as above. The DNA was then dissolved in 100 μ L of water at 100 μ M to which 250 μ g of Oregon Green 514 carboxylic acid succinimidyl ester, from Invitrogen, in 14 μ L DMSO and 75 μ L of 0.1 sodium tetraborate at pH 8.5 were added. The reaction mixture was incubated overnight on a shaker at low oscillation. The labeled DNA was purified by three ethanol precipitations to remove the free Oregon Green. The extent of labeling was estimated by comparing the absorption at 260 nm and that at 480 nm. The extent of labeling was found to be greater than 90% for all samples used in this study. The sequences of the labeled samples used here are given below.

CG complementary duplexes—T* indicates the position labeled with Oregon Green.

5'-T*TTTTTTTTTTAGTCACTATGAATCAACTACTTAGATGGT-3'

3'-TCAGTGATACTTAGTTGATGAATCTACCA-5'

5'-AGTCACTATGAATCAACTACTTAGATGGT-3'

3'-TCAGTGATACTTAGTTGATGAATCTACCATT*TTTTTTTTTT-5'

CC mismatch duplexes

5'-T*TTTTTTTTTTAGTCACTATGAATCAACTACTTAGATGGT-3'

3'-TCAGTGATACTTACTTGATGAATCTACCA-5'

5'-AGTCACTATGAATCAACTACTTAGATGGT-3'

3'-TCAGTGATACTTACTTGATGAATCTACCATT*TTTTTTTTTT-5'

AC mismatch duplexes

5'-T*TTTTTTTTTTAGTCACTATGAATCAACTACTTAGATGGT-3'

3'-TCAGTGATACTTAATTGATGAATCTACCA-5'

5'-AGTCACTATGAATCAACTACTTAGATGGT-3'

3'-TCAGTGATACTTAATTGATGAATCTACCATT*TTTTTTTTTT-5'

2.3 DMS footprinting

The reaction mixture consisted of 1 μ L of 0.1 M DMS added to 20 μ L of 2.5 μ M Oregon Green labeled DNA. After 10 min incubation at room temperature up to 5 μ L of 30 mM poly(A) was added to the sample and the DNA was precipitated with 100 μ L chilled ethanol. After three hours on ice the samples were centrifuged at 13,000 rpm, the supernatant was removed and the pellet was dried using a speed vac.

2.4 Pyrrolidine treatment

The DNA samples were dissolved in 20 μ L of 0.1 M pyrrolidine and heated at 363 K for 30 minutes. After cooling on ice the samples were dried using a speed-vac and lyophilized. The samples were dissolved in 30 μ L of autoclaved water and lyophilized a second time to remove any residual pyrrolidine.

2.5 Duplex sample preparation

The DNA duplexes were prepared by combining 2.5 μM labeled DNA with 3.75 μM of the complementary strand in 100 mM KCl, 2.5 mM Na_2HPO_4 , pH 7. The samples were heated at 363 K for 15 min and cooled gradually to room temperature. For the binding experiments duplex DNA was incubated with DAPI and Hoechst at final concentrations 5, 2.5 and 1.25 μM .

2.6 Hydroxyl radical cleavage reaction

The hydroxyl radical cleavage reaction was initiated by adding one-twentieth volume each of the 10 mM Fe(II)/20 mM EDTA solution, 100 mM sodium ascorbate and 0.04% H_2O_2 , 1:800 dilution of a 30% solution, to the DNA sample. After 1 min the reaction was quenched by one-fourth volume of 230 mM thiourea. For the “standard reaction” discussed below the H_2O_2 was a 1:100 dilution of a 30% solution. The sample was then ethanol precipitated after addition of one-fourth volume of 30 mM poly(A). The samples were subjected to the pyrrolidine treatment. The pyrrolidine treatment was found to regenerate most of the Oregon Green fluorescence lost by the hydroxyl radical reaction.

2.7 Gel electrophoresis and imaging

The DNA samples were dissolved in 5 μL of autoclaved water and 5 μL of 10 M urea. After heating to 90 $^\circ\text{C}$ for 4 minutes and cooling on ice the samples were loaded on a gel that had been pre-run at 45 W for 40 min. The gels used are 20 cm $\times$ 40 cm, 0.75 mm, 15% polyacrylamide denaturing gel in TBE buffer. TBE is 0.1 M Tris base, 0.1 M boric acid and 1 mM EDTA at pH 8. Electrophoresis was carried out at 45 W for 5 hours. The samples reacted with DMS, dimethyl sulfate, with electrophoresis at 60 W for 4 hours.

The gels were imaged using a Typhoon Trio with the fluorescence scanning at the Green-excited mode at 532 nm with the emission filter at 526 nm and the photomultiplier at 600 V. The intensities of all of the bands were within the dynamic range of the imager.

The gel image was analyzed using Semi-Automated Footprinting Analysis software SAFA v1.1. The results were exported to Excel. The sum of the intensities in each lane was normalized. The percentage change in the normalized intensities of each individual band was calculated to generate the histograms presented below. The results presented below used the average of three separate experiments.

To estimate the reproducibility of the procedure a set of experiments were run at least seven times. The analysis of the results indicated that the standard deviation in the intensity of any single band is on the order of 7% and the standard error about 2%. Details are presented in the supplementary data.

Some of the bands of the smaller fragments appear as doublets. Tullius and co-workers [19] have shown that hydroxyl radical cleavage can lead to more than one band due to the attack of the hydroxyl radical at different sugar positions. About 80% of the hydroxyl radical attacks occur in the minor groove at the 5'', 5' and 4' positions. This does not alter the comparison of the results between samples.

2.8 Solvent accessible surface area calculations

The crystal structures of complexes of DNA in complex with DAPI, [40], structure 3GJH, and Hoechst 33258 [41], structure 296D, were downloaded from the PDB website. Water molecules were deleted and hydrogen atoms added to the structure. The water accessible surface areas of the sugar hydrogen atoms were calculated using Chimera 1.5.3rc software. The Surface/Volume from Web tool was used and the surface computed using Molecular

Surface MSMS application with the surface probe size 0.14 nm. Values for each atom of the complex were transferred to Excel. To compare the solvent accessible area of duplex with and without a ligand, DAPI and Hoechst were deleted from the structures and calculations repeated. This allowed calculation of the percent changes in the water accessible surface area of all of the hydrogen of each sugar for each residue.

3. Results and Discussion

The overview of the protocol in Fig. 1A shows that the DNA of interest contains a dT_n tail on one of the duplex strands that has a 5' terminal, covalently attached fluorescent label. Oregon Green is used as the fluorescent dye since it has excellent optical properties, and we have been using it for other purposes [42]. When standard cleavage conditions were used the results were found to be essentially useless due to smeared bands, low intensity and other problems as indicated by the results in Fig. 1B. Investigation indicated that there were at least two independent problems to overcome.

The smearing of the gel bands is most likely due to the incomplete removal of the reactants and quenchers present in the hydroxyl radical cleavage reaction mixture. The use of repeated ethanol precipitation reduces the amount of these contaminants but each round of ethanol precipitation also reduces the amount of DNA. Furthermore, the efficiency of ethanol precipitation depends on the length of the fragment so the relative intensities of the bands were found to show considerable variation depending on the number of ethanol precipitations used, the temperature used for the precipitation and other conditions. A second problem is that the standard hydroxyl radical cleavage conditions led to destruction of the Oregon Green fluorescence and too much cleavage of the DNA.

Our approach was to first improve the purification step. To obtain higher yield and more reproducible purification of the DNA cleavage products a dT_n tail was added on the 5' end. The tail increases the length of the smallest fragment of interest. The dT_n tail was combined with the use of poly(A) as a co-precipitant and this combination is similar to the use of dT_n to purify poly(A) containing mRNAs. The effects of the dT_n tail and poly(A) co-precipitation were first examined using a DMS, dimethyl sulfate, cleavage reaction since the cleavage products are more readily isolated from DMS than from hydroxyl radical reactions. Those results indicated that 3 mM poly(A) is more than sufficient.

These methods were then applied using from 0 to 3 mM of poly(A) on the 5' labeled, single stranded DNA using the optimized hydroxyl radical reaction that is described below. The results in Fig. 1C show that poly(A) increases the yield of all of the fragments, and in particular enhances the yield of the smaller fragments as well as giving rise to fairly uniform band intensities as a function of fragment length. Poly(A) is cheap, stable, readily available and does not interfere with detection of the fluorescence or running of the gels. It is well known that adding any nucleic acid enhances ethanol precipitation yield. Since poly(A) works fine and it is as inexpensive and available as any other polynucleotide we did not examine alternatives. It was found that a tail length of 11 is sufficient for the smallest fragment of interest as well as good co-precipitation.

The use of poly(A) co-precipitation and the dT₁₁ tails largely solves the purification and fragment length problems. This allowed us to move on to the dye destruction and too much cleavage problems. The results for the optimization of the hydroxyl radical reaction shown in Fig. 1D were obtained using 3 mM poly(A) as co-precipitant. The results show that reducing the hydroxyl radical concentration, 1 being the standard, by 8 to 16 fold from the standard conditions allows high intensity bands to be obtained as well as sufficient cleavage.

Treatment of the fragments with pyrrolidine, as described in 2.4, regenerates the fluorescence of the Oregon Green.

The amount of DNA loaded on the gel was then adjusted so that the strongest bands would be about a quarter of the intensity needed to saturate the detector of the imager. The dynamic range of the GE Typhoon imager is 100,000 to 1 and the linearity of over this range has a standard deviation of 7.5%. The reproducibility of the gel band intensities obtained using these optimized cleavage reaction conditions was investigated by doing seven independent reactions for several different DNAs. It was found that the standard deviation for the band intensities is on the order of 7% with a standard error on the order of 2% as described in the supplementary data.

There have only been a couple of reports of hydroxyl radical cleavage reactions on DNAs of modest length.[19, 38] These studies reported on the cleavage of the central residues of the DNAs to examine the effects of deuteration on cleavage [19] and on the structure of Holliday junctions [38]. This small number of reports is an indication of the difficulties of using the standard hydroxyl radical protocol on DNAs of modest length.

This improved protocol allows, in principle, the labeling of both strands as long as the two dyes have sufficiently distinct emission and absorption profiles. However, labeling both strands requires having an exact stoichiometry as the single strands are more susceptible to cleavage than duplex DNA. In practice we have found that using an excess of the unlabeled strand gives the most reproducible results.

3.1 Comparison of cleavage of mismatch and complementary sites

The extent of hydroxyl radical cleavage of duplexes containing either a CC or an AC mismatch have been determined and compared with the results on the fully complementary duplex. The results in Fig. 2 are presented as histograms of the percent change in cleavage in the mismatched DNAs relative to the fully complementary duplex. The presence of an AC mismatch gives rise to decreased cleavage of the A and the C residues in the mismatch. The change at the A site is on the order of 25% (Fig. 2A) which is quite significant with about half as much change observed at the C residue (Fig. 2B). The structural perturbation due to the presence of the mismatch appears to be quite localized. The two bases adjacent to the A exhibit somewhat more cleavage in mismatched DNA than in the fully complementary DNA. The two bases adjacent to the C have very small changes in the cleavage relative to the complementary DNA.

The cleavage of a DNA containing a CC mismatch has also been examined. The cleavage at both C residues decreases relative to that in the complementary DNA. The cleavage at the two C residues is not the same. The C residue in the d(TTACTTG) region (Fig. 2D) exhibits less cleavage than the C in the d(CAACTAA) region (Fig. 2C). This indicates that the structure of the CC mismatch depends on the sequence context. For both the AC and CC mismatches there is more change in cleavage in the d(CAAXTAA) region, with X being C or A, than in the opposite strand. In the CC mismatch case the structural perturbation appears to be localized as was found for the AC mismatch case. The percent change in cleavage for CC is larger than for AC. This is in general agreement with studies on changes in thermal stability that indicated that the CC mismatch is more destabilizing than the AC mismatch [16–18]. These results are compared to those predicted from structural studies in 3.4.

3.2 Changes in cleavage due to minor groove binders

The effects of the minor groove binders DAPI and Hoechst 33258 on the extent of cleavage of complementary duplex DNA have been examined. The footprints of various minor

groove binders have been previously investigated using DNase I cleavage [21, 43, 44]. DNase I footprinting has been used to investigate the sequence preferences, of binding constants and kinetics of minor groove binders [21, 43, 44]. However, the resolution of the DNase I cleavage is many nucleotides. For example, the DNase I footprint of Hoechst 33258 appears to be about 8 nucleotides which is much larger than the binding site and as such does not give information about the protection of the individual nucleotides [21]. The protocol described here can offer additional resolution to aid the development of sequence specific binders.

The cleavage of a fully complementary DNA in the presence of DAPI and Hoechst 33258 has been investigated and compared to that of the free 29mer duplex DNA. The results in Fig. 3 show that DAPI has a footprint of three nucleotides on each strand with the largest protection being on the order of 30%. The magnitude of the protection is similar on the two strands. These results show that the hydroxyl radical cleavage method can be used to identify the binding site of drug like molecules at nucleotide resolution.

The extent of protection with Hoechst 33258 is on the order of 30% which is similar to that observed with DAPI as shown in Fig. 3. The footprint of Hoechst 33258 is larger and with more protection than that observed with DAPI. The footprint patterns of Hoechst 33258 and DAPI are consistent with minor groove binding and the crystal structures of complexes of the molecules with duplex DNA as discussed in 3.4.

3.3 Cleavage of mismatched sites in the presence of minor groove binders

The binding of DAPI to the DNA containing the CC mismatch has been investigated using the same reaction conditions as used with undamaged DNA. The results in Fig. 4 indicate that the presence of the mismatch alters the footprint of DAPI. There is significant protection of four residues, TCAA, with the C being involved in the mismatch, while only the CAA residues exhibited significant protection in the absence of the CC mismatch. In addition the extent of protection is larger in the presence than in the absence of the mismatch. On the other strand three residues, TGA, are protected with the T residue adjacent to the mismatch exhibiting more protection than in the DNA without the mismatch.

The percentage changes in cleavage shown are relative to the duplex DNA with a CC site. The binding of either DAPI or Hoechst 33258 leads to more protection of the residues in the CC mismatch than to the residues in the GC complementary pair. For Hoechst 33258 there is a significantly different profile with the mismatched DNA than the fully complementary DNA. The cleavage reactions of the CC mismatch and undamaged DNA with DAPI and with Hoechst 33258 were examined as a function of concentration over the range of 0.2 to 2 per DNA and the extent of protection was found to follow a typical binding curve.

3.4 Comparison of cleavage results with crystal structure predictions

Crystal structures of complexes of Hoechst 33258 [41], PDB 296D, and DAPI [40], PDB 3GJH, with DNA are available. The DNAs used for the crystal structures were relatively short palindromes of similar but not the same sequences as used for the cleavage experiments. The water accessible surface areas of the protons of the sugars have been calculated both for the complexes and for the DNA alone. The predicted changes in solvent accessible surface areas due to the presence of DAPI and Hoechst 33258 are shown in Fig. 5. The predictions are in quite good agreement with the observed cleavage results. The predicted Hoechst 33258 protection footprint is larger than for DAPI as is observed; the footprint pattern in the predicted protection of the two strands due to minor groove binding is similar to that observed; the percent predicted protection is a bit higher than observed but this may be due to less than saturation conditions in the experiments. These comparisons

indicate that the solution state cleavage results can be used to validate, at nucleotide resolution, crystal and modeling studies of the complexes of the drug like molecules with damaged and undamaged DNAs.

There are crystal structures for an AC mismatched sites in a palindromic decamer[45] and a dodecamer [46]. In both cases the crystal packing breaks the symmetry. The solvent accessibility has been calculated from the structure, PDB 1D99, of the dodecamer d(CGCAAATTCGCG). The predicted surface areas of the A4 residues of the two AC mismatches are similar and slightly higher than the average for this DNA. The predicted surface areas of T8, C9, G10 and C11 each differ by about 30% between the two strands making the interpretation of the predicted surface areas for the C9 in the AC mismatch challenging. An NMR structure investigation on an AC mismatch was carried out[47] but the coordinates have not been deposited.

There is no crystal structure of a CC mismatch but there is structural information on a bulky rhodium complex inserted at a CC mismatch but this does not seem relevant to the free mismatch.[48] The crystal structure of a 8-oxoG-A mismatch [49], PDB 3JR4, predicts that the solvent accessible surface area of the sugar hydrogen of the oxoG is about 30% smaller than the other residues while that of the A is about 25% more. Modeling of the CC and AC mismatched sites via molecular dynamics or other protocol has the potential to find those structures that are consistent with the cleavage results and are energetically reasonable.

3.5 Summary and future studies

These results show that the combination of a dTn tail, poly(A) co-precipitation and optimized reaction conditions allow the hydroxyl radical cleavage to be used to examine the structural effects of mismatches, the sites of ligand binding and the effects of a mismatch site on minor groove binding. The methods described here could also be used to examine the cleavage of DNA by antitumor agents such as bleomycin, neocarzinostatin and melphalin as well as by agents like metal-bipyridyl complexes. It appears that a CC mismatch has a significant effect on the binding of Hoechst 33258 and a much smaller effect on the binding of DAPI. These samples are not readily investigated by atomic resolution methods. The size of the DNAs used allows for high resolution gels to be obtained as well as for the preparation of samples with a wide range of lesions including those involving thymine glycol, oxidized bases and covalently attached groups and such studies are being undertaken. The methodology can also be applied to complexes of quadruplex DNAs and such studies are underway.

The method described here should be applicable to DNA and RNA samples of about 10 to 120 nucleotides long. The investigation of gene length samples, 1000 nucleotides or so, could be done but there are real tradeoffs between having enough cutting at each site without any multiple cuts and sensitivity which suggest that gene length sequences might be better done in sections. The protocol presented here could be readily adapted to much lower and higher nucleic acid concentrations in the reaction mixtures. For example, lower reaction concentrations could just be carried out with a larger volume.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

The protocol is depicted in part A. The duplex DNA has a dT_n tail with a 5' fluorescent label. After cleavage the fragments are co-precipitated with poly(A) and the fragments are separated by gel electrophoresis. The bands are quantified by fluorescence detection. The gel results obtained for a sample using the standard reaction conditions are shown in B. The effect of poly(A) on the yield of the fragments generated in a hydroxyl cleavage reaction is shown in C as a function of poly(A) concentration. The results obtained as a function of hydrogen peroxide concentration are shown in part D. The results in D were obtained with 3 mM poly(A) as co-precipitant.

Fig 2.

The histograms depict the percent change in the cleavage in the presence of the CC mismatch relative to those obtained with the complementary duplex. The results in A were obtained with labeling of the top strand and in B for the bottom strand. The results for the AC mismatch are shown for the top strand in C and for the bottom strand in D. The results shown are the average of three independent experiments.

Fig 3. The histograms depict the percent change in the cleavage of the duplex DNA in the presence of DAPI with the results for the top strand in A and the bottom strand in B. The results on the cleavage of the top strand in the presence of Hoechst 33258 are shown in C and for the bottom strand in D.

Fig 4.

The histograms depict the percent change in the cleavage of the CC mismatch DNA in the presence of DAPI with the results for the top strand in A and the bottom strand in B. The results of the top strand in the presence of Hoechst 33258 are shown in C and for the bottom strand in D. The results are the percentage change in cleavage relative to the CC duplex.

Fig 5. The histograms depict the percent change in the solvent accessible surface area of the hydrogens of the sugars of each residue in the presence of DAPI with the top strand shown in A and the bottom strand in B. The surface areas were calculated using the crystal structure of the complex. The analogous results for the top strand in the presence of Hoechst 33258 are shown in C and for the bottom strand in D.