Use of exonuclease III to determine the site of stable lesions in defined sequences of DNA: the cyclobutane pyrimidine dimer and cis and trans dichlorodiammine platinum II examples

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ABSTRACT A method to detect chemically stable lesions in DNA has been developed using Exonuclease III, a double strand specific nuclease, to digest 5'-end labeled DNA. The products, when analyzed on high resolution DNA sequencing gels, reveal the sites of DNA modification. Cyclobutane pyrimidine dimers induced by UV irradiation can be localized by comparison of the fragments produced by Exonuclease III digestion with fragments obtained after digestion of the DNA with UV specific endonuclease. The experiments demonstrate that Exonuclease III stops one base away from the cyclobutane pyrimidine dimers. Similar experiments with cis- and trans-dichlorodiammine-platinum (II) showed that modifications of DNA by these agents also impede Exonuclease III digestion. In general the same stop sites were found for cis- and trans-platinum adducts. They occur at sites of guanine bases. Additional stop sites were found for cis-platinum at sites of adjacent guanine bases. These results are in agreement with the model that cis-platinum forms intrastrand guanine-guanine dimers, whereas trans-platinum does not.

INTRODUCTION

The biological consequences of DNA damage include mutation, malignant transformation and cell death. An understanding of the effects on DNA by a particular agent requires knowledge of the sites of DNA damage as well as the enzymatic mechanisms for repair of the DNA lesions. Advances in DNA technology have made it possible to determine the sites of DNA damage for some classes of DNA damaging agents, particularly those that cause strand scission events. A strategy developed for the detection of damage by such agents is to expose a DNA fragment of known sequence to the agent under conditions that break the phosphodiester backbone (1,2). The sites of DNA breakage, and therefore the sites of DNA modification, can be determined by comparison of the electrophoretic mobility of the cleavage products created by treatment of the same DNA fragment with the standard DNA sequencing reactions of Maxam and Gilbert (3). The sites of DNA modifications by aflatoxin (2) and benzo (a) pyrene metabolites (4), nitrogen mustards (5), neocarzinostatin (1), bleomycin (1), and adriamycin (6) have been determined using this method.

This strategy is limited to agents that cause strand breaks in DNA directly or that create lesions that are susceptible to strand scission upon appropriate subsequent treatment. We wished to develop a means of localizing sites of DNA modification of agents that do not lead to strand scission. It seemed likely that some DNA modifications might impede degradation of DNA by exonuclease III of *E. coli* (7,8,9). Previous studies with this enzyme have demonstrated that it degrades double stranded DNA by successive hydrolysis starting at the 3' end of a double stranded DNA molecule (10). To determine the feasibility of this approach, exonuclease III was used to degrade 5' end labeled DNA fragments of defined sequence that contained cyclobutane pyrimidine dimers induced by ultraviolet light. The experiments reported here demonstrate that exonuclease III does stop at sites of pyrimidine dimers. This method was also used to determine whether or not differences could be detected in the sites of modification by the cis- and trans isomers of dichlorodiammine platinum II (11). The results of these experiments indicate that exonuclease III can be used as a general tool to determine the sites of stable lesions in DNA.

MATERIALS AND METHODS

DNA Substrates: The fragments of DNA used in the experiments were obtained from pLJ3 plasmid (12) that contains an insert of the lac p-o region of *E. coli*. The plasmid was digested with Eco RI, and the resulting DNA fragments were 5' end labeled in reactions that included polynucleotide kinase and (γ -³²P)-ATP as described by Berkner and Folk (13). The 5' end labeled fragments were further digested with restriction enzymes Hae III (which yields a 168 and 117 base pair fragment) or Hpa II (which yields a 45 and 58 base pair fragment) to obtain fragments which are terminally labeled at one end. The fragments were separated on a 4% polyacrylamide gel and eluted from the gel. The sequence of the fragments used is given below.

<u>lac p-o</u> 168		Hpa II site					
	1	10	20	30	40	50	60
5'	AATTCTGTTTCTGTGTGAAATTGTTATCCGCTCACAAATCCACACATTATACGAGCCGGAAGCATAAA						
3'	GACAAAGGACACACTTTAAACAATAGGCGAGTGTTAAGGTGTGTAATATGCTCGGCCTTCGTATT						
	70	80	90	99			
5'	GTGTAAGCCTGGGGTGCTAATGAGTGAG....						
3'	CACATTCGGACCCACGGATTACTCACTC....						

<u>lac p-o 117</u>		Hpa II site				
	1	10	20	30	40	
5'	AATTCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCGGCT					↓
3'	GAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGA					
	50	60	70	80	90	
5'	CGTATAATGTGTGGAATTGTGAGCGGATAACAATTCACAC...					
3'	GCATATTACACACCTTAACACTCGCCTATTGTTAAAGTGTG...					

UV Irradiation and Corendonuclease Digestion of DNA: DNA was irradiated with a germicidal lamp (primarily 254nm light) at a fluence of 8 J/m². DNA was treated with the G-75 fraction of the *M. luteus* UV-specific endonuclease (a gift of R. Grafstrom and L. Grossman, Johns Hopkins School of Public Health) in the presence of 50 mM NaCl, and 5 mM Tris-HCl pH 7.5 and 0.5 mM EDTA for 30 min at 37°C.

Platinum II Complexes: *cis*- and *trans*-DDP (dichlorodiammine-platinum II) were obtained from the Mathey Bishop, Inc., Malvern, PA 19355. Solutions were made in 0.1M NaClO₄ at a concentration of 200 µg/ml at a pH of 6.2. Platinum compounds were incubated with DNA in 0.1M NaClO₄ at 37°C in the dark. The reactions were stopped by the addition of NaCl to a concentration of 0.5M to inactivate unreacted *cis*- and *trans*-DDP.

Exonuclease Experiments: Exonuclease III (New England Biolabs) reactions were performed in a buffer that contained 20 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 10 mM DDT, 6 mM MgCl₂ and 10 mM KCl in a volume of 200 µl, at 37°C for 30 minutes. The optimal concentration of exonuclease III was determined for each labeled DNA fragment used. Usually a concentration of 264 U/ml was used. Reactions were stopped by the addition of an equal volume of 2x stop solution (0.1% SDS, 30 mM EDTA and 0.4 mg/ml carrier RNA).

3' Phosphatase Reaction: The presence of a 3' phosphate group on DNA was examined by incubation of DNA with 6 units of T4 polynucleotide kinase (New England Biolabs) in a buffer that contained 5 mM 2-mercaptoethanol, 100 mM Tris-HCl pH 6.5, 100 mM magnesium acetate for 12 hr at 37°C.

RESULTS

A DNA fragment of defined sequence was used as a probe to determine the sites of stable DNA adducts. The initial experiments were done with DNA that contained pyrimidine cyclobutane dimers as a target lesion. These lesions cause a local distortion in the DNA helix and for that reason are potentially

inhibitory to the processive action of exonuclease III. The pyrimidine cyclobutane dimers were formed by exposure of the DNA to ultraviolet light (14). The distribution of the pyrimidine dimers within a defined DNA sequence can be determined by treating the irradiated DNA with an ultraviolet light specific endonuclease enzyme purified from M. luteus (15). Our previous studies with this enzyme and with the T4 ultraviolet light specific endonuclease V of phage T4 demonstrated that these enzymes quantitatively cleave DNA at all dimer sites (16). Comparison of the products that result from exonuclease III digestion of ultraviolet light irradiated DNA with the fragments produced by digestion of the same substrate with the M. luteus enzyme allowed quantitative measurement of the frequency of exonuclease III termination at actual dimer sites. Moreover, comparison of the length of the DNA products with the length of the fragments produced by DNA sequencing reactions should allow identification of the precise site of exonuclease termination relative to the site of each pyrimidine dimer.

The experiments pictured in Figure 1 illustrate this approach. For these experiments DNA fragments of known sequence labeled at the 5' terminus of one strand were used. The 58 nucleotide long fragment was used as the substrate for the experiment pictured in Figure 1A, whereas the 117 base pair long fragment was used for that presented in Figure 1B. To determine the location of the pyrimidine dimers within the ultraviolet light irradiated substrate, the DNA was treated with the M. luteus enzyme. The amount of M. luteus enzyme used in these experiments was determined to be saturating, as addition of more enzyme did not lead to an increase in the number of breaks in the irradiated substrate. In the experiment pictured in Figure 1A, the DNA in lane 4 was treated with neocarcinostatin, a reagent that cleaves the DNA at positions of thymine and adenine (1). After treatment the DNA was denatured and layered onto high resolution denaturing polyacrylamide gels of the type used for DNA sequence analysis. The DNA products were visualized by autoradiography. The DNA sequence of the fragments is indicated to the side of the figures.

To determine if exonuclease III digestion of the labeled DNA terminated at the site of the pyrimidine dimers, parallel samples of ultraviolet light irradiated DNA and unirradiated DNA were digested with exonuclease III. The digestion conditions were chosen to optimize the degradation of the 5' end labeled DNA strand. Under the conditions used for these experiments, more than 80% of the unirradiated labeled DNA strand was digested to the point of acid solubility. In these experiments less than 5% of the input molecules

Figure 1

A: Analysis of the digestion products of a 5'-end labeled 58 base pair fragment with Exonuclease III on a 8% Urea-Polyacrylamide Gel. Lane 1, untreated control DNA; lane 2, UV irradiated DNA; lane 3, Exonuclease III digestion of unirradiated DNA; lane 4, neocarzinostatin treatment of DNA; lane 5, Exonuclease III digestion of irradiated DNA, the arrows point to two of several stop sites corresponding to dimer sites; lane 6, UV endonuclease digestion of UV irradiated DNA. B: Digestion products of a 5'-end labeled 117 base pair fragment. Lane 1, untreated DNA control; lane 2, UV irradiated DNA, treated with UV endonuclease; lane 3, UV irradiated DNA; lane 4, DNA treated with Exonuclease III; lane 5, UV irradiated DNA treated with Exonuclease III.

remained undigested as evidenced by the amount of radioactivity that migrated at the position of untreated DNA on the analytical gels.

Digestion of the unmodified DNA results in extensive degradation of the labeled strand; however some DNA products of intermediate length remain, as evidenced by discrete bands in these lanes. The pattern of exonuclease III terminations in unmodified DNA was characteristic for each substrate and was reproducible. These products probably represent kinetic barriers for the

exonuclease.

If cyclobutane pyrimidine dimers impede the activity of exonuclease III, digestion of ultraviolet irradiated DNA should produce a distinct series of partial digestion products that are not found upon digestion of unmodified DNA. Such products were present in the exonuclease III digestion of ultraviolet light irradiated DNA as indicated in Fig. 1. The exonuclease III sites in lane 5 are connected to their corresponding pyrimidine dimers, which are revealed by the site of cleavage of UV irradiated DNA by the *M.luteus* enzyme in lane 6. Inspection of the autoradiograms shows that both the number and the location of the additional partial digestion products corresponded to the sites of pyrimidine dimers. Since these products were not evident in unirradiated DNA, they are candidates for termination at the sites of the cyclobutane pyrimidine dimers.

The Site of Exonuclease III Termination

It should be possible to deduce the site of exonuclease III termination relative to the position of the dimer by comparison of the electrophoretic mobility of the digestion products with those produced by the DNA sequencing reactions. However, this comparison is not straightforward as the nature of the termini of the scission products affects the electrophoretic mobility of the molecule. It is likely that the Exo III digestion products will differ from the products of the DNA sequencing reactions in two respects:

1. Exonuclease III digestion should result in a 3' hydroxyl group, because it contains a 3'-phosphatase activity (10) whereas 3' phosphoryl termini are produced by the DNA sequencing reactions (3).
2. The exonuclease III partial digestion products of UV irradiated DNA will contain at least one pyrimidine dimer if termination events occur on the 3' side of the dimer.

The structure of the 3' termini of the exonuclease III digestion products of ultraviolet light irradiated DNA was examined. To determine if products produced by nuclease digestion of irradiated DNA contained terminal hydroxyl groups or whether they terminated with a phosphoryl group, we developed a method for removal of 3' terminal phosphates. This method relies upon the observation that at low pH the polynucleotide kinase purified from phage T4 infected *E. coli* possesses a 3' phosphatase activity (17).

DNA fragments produced by treatment of DNA with dimethylsulfate using the conditions for DNA sequencing as described by Maxam and Gilbert contain phosphoryl groups at both the 5' and 3' termini (3). Removal of the phosphate group from the 3' end of these fragments alters the mobility of these products, figure 2. However the electrophoretic mobility of the products of Exonuclease

III digestion of ultraviolet light irradiated DNA was unaffected, figure 2. These results demonstrate that the products obtained by Exonuclease III digestion of ultraviolet light irradiated DNA do not contain a 3' phosphate group. The experiments also demonstrate that a DNA fragment that contains a 3' hydroxyl terminus migrates more slowly than the same length fragment that contains a 3' phosphoryl group.

The use of T4 polynucleotide kinase as a specific 3' phosphatase should be a useful technique for analysis of DNA termini. In this regard it should be noted that the enzyme will also exchange the 5' terminal phosphate of polynucleotides with adenosine diphosphate or the 5' termini of other polynucleotides. Consequently, such analysis should be done in the absence of ATP or ADP and with a minimal amount of unlabeled polynucleotide carrier.

To analyse the second question of whether or not the presence of a cyclobutane pyrimidine dimer significantly alters the electrophoretic mobility of a DNA fragment, the mobility of a heavily irradiated ($5,000 \text{ J/m}^2$) sixteen nucleotide long DNA fragment was compared to that of unirradiated DNA on a 20% urea-containing polyacrylamide gel. Under these conditions at least 20% of

Figure 2

Analysis of the ends of fragments produced in sequencing and Exonuclease III reactions. A 58 base pair fragment was treated with the G+A specific sequencing reaction and thereafter incubated without, lane 1, or with, lane 2 polynucleotide kinase. Exonuclease III digestion of UV irradiated DNA, treated without, lane 3 and with, lane 4 polynucleotide kinase. UV endonuclease treatment of UV irradiated DNA is shown in lane 5.

the fragments contained pyrimidine dimers. (15,16) Nonetheless, all the radioactivity in both samples migrated as a single band (data not shown). Therefore, we conclude that the presence of the cyclobutane dimer does not significantly affect the electrophoretic mobility of the DNA.

What is the structure of the exonuclease III digestion products of UV-irradiated DNA? Two such products migrate very close to the positions indicated for T26 and C29 respectively. The mobility of these species is consistent with molecules that terminate in the sequences $^{32}\text{P} \dots \text{AATTG-OH}$ and $^{32}\text{P} \dots \text{AATTGTTA-OH}$ respectively. The experiments described above demonstrate that these products do not have 3' phosphoryl groups, and that the presence of the dimer does not significantly affect the mobility of DNA fragments of this length. It is likely that the structures assigned are correct. Inspection of Figure 1 demonstrates that similar products are found in the vicinity of every cyclobutane dimer site. We conclude that exonuclease III terminates one nucleotide before each dimer, leaving the product $^{32}\text{P} \dots \text{PyPy-N-OH}$.

The Frequency of Termination at Dimer Sites

The frequency of exonuclease III termination in the vicinity of potential dimer sites was determined by measurement of the fraction of the total radioactivity in each digestion product. A comparison of the frequency of exonuclease III termination with the actual distribution of dimers measured by digestion of the same DNA substrate with the M. luteus enzyme is shown in Table 1.

Thus, the frequency of termination of the exonuclease III digestion at dimer sites is in good agreement with the frequency of dimer formation as determined using the M. luteus enzyme. It is probable that each cyclobutane

Table 1

Quantitation of the amount of radioactivity in each band in comparison of the total radioactivity. Values determined by cutting out the bands from the gel shown in figure 1B and counting the cerenkov cpm/band as compared to cerenkov cpm for the total lane.

Sequence at dimer site in 117 lac p-o	UV irradiated treated with UV-specific enoo.	UV irradiated treated with exonuclease III
34 38 CTTTA	3.5	2.8
41 44 CTTC	1.1	2.2
65 68 ATTG	0.6	0.8

dimer creates a block to digestion of the DNA by exonuclease III.

Exonuclease III of DNA Modified by *cis*- and *trans* dichlorodiammine platinum II.

Cis dichlorodiammine platinum II is a drug that has successfully been introduced into several therapeutic protocols for the treatment of tumors (18). Both the cis and trans isomers of dichlorodiammine platinum (DDP) form stable covalent adducts with DNA. The reaction probably occurs by formation of a reactive "diaquo" form of the molecules followed by an attack at the nucleophilic sites of DNA (19).

To analyse the interaction of DDP with DNA we first incubated 5' end labeled DNA fragments with increasing concentrations of the compounds prior to analysis on polyacrylamide gels. Figure 3A shows that incubation of the DNA with increasing concentration of either the cis or trans compounds for 60 minutes resulted in formation of a DNA species that migrated more slowly than

Figure 3

A: 5'-end labeled DNA incubated with increasing amounts of cis-DDP (lanes 1-10) and trans-DDP (lanes 11-20) analyzed after denaturation on an 8% urea formamide sequencing gel. Concentration of the compounds in the lanes are as follows: 1, 1 µg/ml; 2, 2 µg/ml; 3, 8 µg/ml; 4, 16 µg/ml; 5, 32 µg/ml; 6, 64 µg/ml; 7, 100 µg/ml; 8, 200 µg/ml; 9, 400 µg/ml; 10, 800 µg/ml cis-DDP and 11, 1 µg/ml; 12, 2 µg/ml; 13, 8 µg/ml; 14, 16 µg/ml; 15, 32 µg/ml; 16, 64 µg/ml; 17, 100 µg/ml; 18, 200 µg/ml; 19, 400 µg/ml; 20, 800 µg/ml of trans-DDP and 21, untreated control.

B: reversion of the cis- and trans-DDP modification with thiourea. Lane 1, control DNA with thiourea treatment; 2, without thiourea treatment; lane 3 and 4, 8 µg/ml cis-DDP, 3 with and 4 without thiourea; lane 5 and 6, 64 µg/ml cis-DDP, 5 with and 6 without thiourea; lane 7 and 8, 400 µg/ml cis-DDP, 7 with and 8 without thiourea; 9, 800 µg/ml cis-DDP with thiourea; 10 and 11, 8 µg/ml trans-DDP, 10 with and 11 without thiourea; 12 and 13, 64 µg/ml, 12 with and 13 without thiourea; 14 and 15, 400 µg/ml trans-DDP, 14 with and 15 without thiourea.

does single stranded DNA on denaturing polyacrylamide gels. The formation of these species was dependent both upon the concentration of the dichlorodiammine platinum compounds and upon the time of incubation. Even the lowest concentration of the drugs (1 $\mu\text{g/ml}$) resulted in extensive formation of the slowly migrating DNA species if the reactions were incubated for 24 hours (not shown). Formation of the slow migrating DNA species can be completely reversed by treatment of the DNA with thiourea, a reagent that removes bound platinum from DNA (20)(Fig.3B).

To determine if modification of DNA by dichlorodiammine platinum II complexes created alkaline labile modifications of DNA, the modified DNA was subjected to alkaline hydrolysis. DNA that contained platinum adducts was broken neither by treatment at alkaline pH (1M piperidine 90°C, 30 min) nor by heat treatment at neutral pH followed by treatment at elevated pH (90°C, pH7.0 15 min followed by 0.1N NaOH 90°C 30 min). Thus neither the cis nor trans compounds create sites in the DNA that resulted in strand breaks. The modified DNA was also resistant to nicking by the M. luteus UV-specific endonuclease (experiments not shown).

We used exonuclease III to probe the sites of modification of DNA by both cis and trans isomers of platinum. Modification of the DNA with a concentration of either cis or trans dichlorodiammine platinum that results in intermolecular cross-linking, completely inhibited digestion of the DNA by exonuclease III. Figure 4A shows that modification of the DNA with low concentrations of the cis and trans platinum compounds resulted in partial digestion products that were not evident upon digestion of the unmodified DNA. No such partial digestion products were present when DNA modified by the platinum compounds was treated with thiourea before exonuclease III digestion (experiment not shown).

The sites of DNA modification can be deduced from the electrophoretic mobility of the partial exonuclease III digestion products. Analysis of the products at higher resolution is presented in Figure 4B. Termination events occur in the vicinity of adjacent guanine residues for DNA modified with either the cis or trans isomers. Additional termination events for the trans compound are evident at sites of some but not all isolated guanine bases. Some differences in the partial digestion products were observed for DNA modified by either the cis or trans compounds. For example, a series of at least five bands of apparent length 81-85 nucleotides long is evident in the digestion products in DNA treated with the cis derivative, whereas only two major products corresponding to DNA molecules 83 and 84 nucleotides long and

Figure 4

Analysis of Exonuclease III digestion products on an 8% sequencing gel. 5'-end labeled fragment 168 was treated with 20µg/ml of cis-or trans-DDP for 30 minutes and after that treated with Exonuclease III. Lanes 1-6 are the same in Figures A and B. Figure B is a longer run of the same experiment as in Figure A. Lane 1, C+T specific reaction; lane 2, G+A specific reaction; lane 3, 20µg/ml cis-DDP followed by digestion with Exonuclease III; lane 4, untreated DNA, digested with Exonuclease III; lane 5, DNA treated with 20µg/ml of trans-DDP followed by digestion with Exonuclease III and lane 6, untreated control DNA.

two minor products corresponding to molecules 81 and 82 nucleotides long are present in the exonuclease digestion products of DNA treated with the trans isomer.

The electrophoretic mobility of the digestion products is not altered by treatment of the digestion products with thiourea. We conclude that both the cis and trans isomers form adducts at guanine residues that impede the progress of exonuclease III, but that modification of DNA by the cis isomers creates an additional class of lesions not apparent in DNA modified by the

trans compound.

DISCUSSION

The experiments presented here demonstrate that exonuclease III can be used to identify sites of stable DNA modifications. Exonuclease III digestion of DNA was impeded by cyclobutane pyrimidine dimers. In this case each dimer created a block to further enzymatic digestion. Thus exonuclease III can also be used to provide quantitative information regarding the distribution of DNA damage within a defined sequence of DNA.

In the case of cyclobutane pyrimidine dimers, exonuclease III digestion terminates one base before the site of the dimer itself. It is likely that termination at the position occurs due to local denaturation of DNA in the vicinity of the cyclobutane pyrimidine dimers (21). Exonuclease III requires a double stranded substrate for activity (9). Thus local denaturation of the DNA in the vicinity of the dimer could impede the progress of the nuclease.

The observation that exonuclease III fails to excise cyclobutane pyrimidine dimers is consistent with genetic data regarding the role of the enzyme in repair of ultraviolet light induced damage. E. coli strains that are mutant in the structural gene for exonuclease III are not abnormally sensitive to the lethal effects of ultraviolet light (7).

The marked therapeutic effectiveness of the cis over the trans isomer of the dichlorodiammine platinum has not been fully explained. What are the similarities and differences between these compounds observed in our experiments? Treatment with high concentrations of either compound resulted in the formation of slow migrating material in denaturing gels. This material also migrated more slowly in non-denaturing polyacrylamide gels than did either the single or double stranded DNA molecules (not shown). From previous work (22,23) it is unlikely that the slow migrating components represent interhelix cross-links. However, both inter- and intrastrand cross-links have been described (24,25,26,27). Platinum binding has previously been shown to alter the electrophoretic mobility of superhelical DNA on agarose gels (28).

The data also shows that in some respects the platinum-compounds differ from other DNA alkylating agents commonly used in chemotherapy. Many of these agents such as cyclophosphamide and nitrogen mustard form adducts at the N⁷ position of guanine and N³ position of adenine. Such adducts introduce a positive charge into the imidazole ring of the purines hence weakening the N-glycosyl bond. We find no evidence for weakened N-glycosyl bonds in DNA modified by the platinum compounds, as no DNA breaks are apparent upon

treatment of the DNA at high temperature at neutral pH, followed by treatment at high temperatures at alkaline pH.

Extensive modification of DNA with either the cis or trans isomers completely inhibits the activity of exonuclease III. However, the lesions created by platinum appear to be fully reversible by treatment with thiourea. DNA modified by high concentrations of either the cis or trans compounds and then treated with thiourea is as sensitive to exonuclease III treatment as is unmodified DNA. Moreover, removal of the platinum adducts by thiourea does not produce strand breaks or result in alkaline labile lesions in the DNA.

Experiments with DNA modified with low concentrations of cis and trans isomers demonstrate that some of the platinum adducts impede exonuclease III digestion at specific sites. These sites differ somewhat for the cis and trans compounds. In general, the termination events observed for DNA modified by trans isomers are a subset of those observed for DNA treated with the cis compound. In both cases the modifications occur in the vicinity of guanine bases. The electrophoretic mobility of the digestion products suggests that termination occurs at the site of the modified guanine itself. The presence of the platinum adduct on these products does not significantly alter the electrophoretic mobility, as no change in mobility of these products was observed after removal of the bound platinum by treatment with thiourea. The termination events that occur when two or more adjacent guanine residues are found in a sequence are different for DNA modified by cis as compared to the trans isomer. In the case of the trans compound, one termination event occurs at every guanine in the sequence. In the case of the cis isomer additional termination events occur. The electrophoretic mobility of the additional termination products observed at sequences of adjacent guanines is consistent with termination of digestion one nucleotide before the first guanine in the sequence.

These results suggest that both cis and trans isomers form stable adducts at guanine residues. Guanine adducts of both compounds have been reported previously (29,30). At single guanine bases, termination occurs at the site of adduct formation. At sites of adjacent guanine residues an additional product is formed by the cis but not by the trans isomer. The cis-ODP modification could be an intrastrand guanine-guanine dimer. This guanine-guanine dimer would block the action of Exonuclease III one base before the modification as was observed in the case of pyrimidine dimers. Others have suggested that the geometry of the coordination complex of the cis isomer would permit dimer formation (N^7 to N^7 of adjacent guanines),

whereas the geometry of the trans isomer would not favor dimer formation. In this regard it is noteworthy that the reactive chloride groups are located about 3.4 Å apart in the cis isomer (close to the interbase distance in DNA), whereas the reactive groups in the trans compound are further apart (29). Intrastrand dimers between guanine bases have also been postulated by Cohen et al., who used an assay to determine inhibition of the restriction enzyme Pst I on pSM1-DNA. One of the four recognition sequences is flanked by GG sequences. This site is lost first after binding of cis- but not trans-platinum (31). In any event, the observation that the cis isomer creates a different class of DNA modifications than does the trans isomer may provide some clue as to the basis for the differential therapeutic effectiveness of the cis and trans compounds.

In summary, a new method for detection and characterization of stable DNA lesions using exonuclease III is presented. The method has been applied to study the distribution of cyclobutane pyrimidine dimers and sites of cis and trans platinum modification. The method should be useful for studies of modification of DNA by other carcinogenic and chemotherapeutic agents. The experiments presented here are in agreement with the hypothesis that intrastrand guanine-guanine cross-links are responsible for the anti-tumor activity of the cis-platinum compound (30,31). These lesions are not formed by trans-platinum and may be less easily repaired than monoadducts.

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