Pergamon

Biochemical Pharmacology, Vol. 48, No. 1, pp. 145–153, 1994.
Elsevier Science Ltd
Printed in Great Britain.

DIFFERENCES IN *IN VIVO* AND *IN VITRO* SEQUENCE-SPECIFIC SITES OF CISPLATIN–DNA ADDUCT FORMATION AND DETECTION OF A DOSE–RESPONSE RELATIONSHIP

GLENN J. BUBLEY,*† BEVERLY A. TEICHER,‡ GEORGE K. OGATA,*
LARUE S. SANDOVAL* and TETSUYA KUSUMOTO‡

*Oncology Division, Beth Israel Hospital, Boston, MA; and the ‡Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, U.S.A.

(*Received* 14 *October* 1993; *accepted* 14 *February* 1994)

Abstract—The cytotoxic and mutagenic properties of the anticancer drug *cis*-diammine-dichloroplatinum(II) (cisplatin) are mediated by bifunctional adducts between purines. Experiments performed in this study employed a new repetitive thermal-cycling technique to detect cisplatin adduct formation following exposure of cells in culture (*in vivo*) or following treatment of purified DNA (*in vitro* exposure). The initial goal of this study was to determine if cisplatin–DNA adduct formation could be measured accurately using phosphorimaging over a broad concentration range. If this proved possible, it would then be feasible to determine if adduct formation differed within chromatin compared with purified DNA. There were no significant differences in the cisplatin–DNA adduct pattern induced in closed circular or linear double-stranded plasmids *in vitro*, suggesting that this type of tertiary structural change does not affect the formation of adduct sites. Sequence-specific DNA adduct formation within a human repetitive DNA target sequence, alphoid DNA, following cisplatin treatment of prostate cancer cells in culture (*in vivo*) and treatment of purified DNA *in vitro* revealed consistent increases in adduct formation over a broad concentration range, validating the experimental technique. Comparing preferences for cisplatin adduct site formation under these different conditions of exposure demonstrated statistically significant differences. Similar differences were detected for cisplatin repair-deficient Xeroderma pigmentosum cells treated in cell culture, indicating that *in vivo/in vitro* preferences for adduct site formation are not the result of DNA repair *in vivo*.

Key words: cisplatin; adduct formation; prostate cells; *Taq* polymerase

Molecules that form adducts with DNA bases *in vivo* are widely recognized as carcinogens, mutagens, and toxins [1, 2]. A small subset of these molecules, among them cisplatin (*cis*-diamminedichloroplatinum II), has found utility as antitumor agents [3]. Elucidation of the cytotoxic and mutagenic properties of molecules, such as cisplatin, requires an understanding of the interaction of these agents with DNA. Many elegant studies have delineated the sequence-specificity of cisplatin–DNA adduct formation and have demonstrated that this agent forms bifunctional adducts predominantly at intrastrand GpG and ApG sites [4, 5]. Most of these studies were performed under *in vitro* conditions.

Understanding the action of chemotherapeutic agents, however, requires a knowledge of the interaction of the molecule with DNA within living cells. Cisplatin–DNA adducts within a specific sequence context can be determined through the replication-mapping or primer-extension method [5–8]. Until very recently, this method had not been sufficiently sensitive to detect adducts formed *in vivo*. However, as we [9] and others [10, 11] have shown recently, because the thermal stable polymerase, *Thermus aquaticus* (*Taq*), is effectively blocked by cisplatin adducts [12, 13], it is possible to detect cisplatin–DNA adducts within human alphoid satellite DNA from cells exposed to this agent by performing multiple rounds of replication-mapping (repetitive thermal-cycling). Alphoid DNA was chosen because it is abundant (1–5% of the genome), providing a large target for cisplatin–DNA adduct formation [14, 15].

The first question in this study was to determine if a dose–response relationship for sequence-specific cisplatin–DNA adduct formation could be detected accurately over a broad concentration range. If this proved possible, we could then use this technique to determine if there are factors, in addition to DNA sequence, that influence sites of cisplatin–DNA adduct formation. Sites of cisplatin–DNA adduct formation for different plasmid structures and for DNA from cells treated in cell culture (*in vivo*) and DNA treated following isolation and purification (*in vitro*) were compared over a broad concentration range. Adduct formation was detected and measured, using phosphorimaging techniques, for sites detected in the second monomer of a 342 bp alphoid DNA fragment.

† Corresponding author: Glenn J. Bubley, M.D., Department of Medicine, Division of Hematology/Oncology, Beth Israel Hospital, 330 Brookline Ave., Boston, MA 02215. Tel. (617) 735–2811; FAX (617) 735–3915.

MATERIALS AND METHODS

Cell lines and plasmids. The cell lines used in these

experiments were a human prostatic carcinoma cell line, PC-3 (from ATCC, Rockville, MD) [16], and an SV40-transformed Group A Xeroderma pigmentosum (XP) line (XP12BE) (Institute for Medical Research, Camden, NJ) [17]. Subconfluent cells (10^6) were treated in cell culture (*in vivo*) with various concentrations of cisplatin (Sigma Chemical Co., St. Louis, MO) under serum-free conditions for 2 hr, following which DNA was isolated by standard procedures [18]. DNA from untreated cells was extracted and either used as a control or treated with cisplatin following removal of DNA-binding proteins by proteinase K digestion and phenol extraction [18]. Conditions of cisplatin exposure were held constant in that the same amount of purified DNA (10 μg) or cells (10^6 cells) was exposed. Purified cellular or plasmid DNA was treated with cisplatin in TE buffer (Tris–HCl, 0.01 M; EDTA, 0.001 M; pH 7.6) for 2 hr at 37°. Following treatment of purified cellular or plasmid DNA, unbound cisplatin was removed by phenol extraction and ethanol precipitation [8, 19].

The plasmid used in these experiments, p342, was constructed by isolating 342 bp fragments from *Eco*RI digested human PC-3 cells and subcloning into the Bluescript II vector (Stratagene, La Jolla, CA), using standard techniques [18]. For experiments comparing sites of cisplatin–DNA adduct formation induced within different plasmid structures, p342 was treated with cisplatin either as a double-stranded circular DNA following cesium chloride purification [18] (Form I) or as a double-stranded linear template (Form III) following *Bam*HI digestion at a unique site in the plasmid.

Primers identical to those used for thermal-cycling experiments were employed to sequence, by the dideoxy method [18], the p342 plasmid. This permitted determination (within ±2 bp) of the location of cisplatin–DNA adduct sites within genomic DNA when both the DNA sequence and the thermal-cycling reaction products were loaded on the same gel. The alphoid DNA sequence for the depicted plasmid, as it is for any cloned alphoid fragment, is only a partial representation of the consensus sequence. Therefore, it is sometimes necessary to use this sequence as a ladder to deduce the exact sequence from the published consensus sequence [14].

Repetitive thermal-cycling assay. Substrates for repetitive thermal-cycling experiments were derived following *Eco*RI digestion of genomic or plasmid DNA. Digestion of cellular DNA was monitored by observation of the 342 bp and 684 bp fragments that are generated by *Eco*RI digestion of human genomic DNA [20]. In some experiments, the 342 bp alphoid fragment was isolated and purified from a low-melting point agarose (Bethesda Research Laboratories, Gaitherburg, MD). In other experiments, the entire *Eco*RI digest was used without purification of the 342 bp band as a substrate.

The oligonucleotide primer (No. 1) used in most of these experiments has the sequence 5′ AATTCTCAGTAACTTC 3′ and spans bp 1–16 within the alphoid fragment. The first 5 bases of this oligomer match the first 5 bases of the *Eco*RI recognition site, and its 16 bp sequence is an exact match of the first 16 bases of a 342 dimeric multimer of an alphoid RI subfamily. This subfamily is located primarily on chromosomes 1, 5 and 19 [20]. In other experiments, an oligonucleotide having the sequence 5′ GTGGAATTTGCAAGTGGA 3′ and spanning bp 89–106 within the alphoid first monomer was used as primer. This primer (No. 5) was used in similar experiments by Murray *et al.* [10] to detect cisplatin–DNA adducts on alphoid DNA.

Oligonucleotide primers (1 μg) were 5′ end-labeled with 50 μCi of [γ^{32}P]ATP (6000 Ci/mmol) (New England Nuclear, Boston, MA) using 2 units of T4-DNA kinase and the supplied buffer (New England Biolabs, Beverly, MA) according to standard conditions. Labeled primer (150 ng) was then added to plasmid or cellular DNA templates (1.5 μg for total *Eco*RI digested cellular DNA, 100 ng of the 342 bp fragment isolated from cellular DNA using low-melting-point agarose DNA, or 100 ng of plasmid DNA). Detection of specific cisplatin-induced polymerase arrest sites was enhanced by maintaining these primer:template ratios for each experiment. The primer and template were then mixed with 1 U *Taq* polymerase (Promega, Madison, WI), *Taq* polymerase buffer (Promega), a 200 μM concentration of each deoxynucleotide triphosphate (dNTP) (Promega), and 5% DMSO in a final volume of 20 μL. The reaction mixtures were placed within a DNA thermal-cycler heating block (Perkin-Elmer-Cetus, Norwalk, CT). Thermal cycles were performed at 94° for 20 sec, 35° for 1 min for primer No. 1 and 50° for primer No. 5 [10], and 72° for 1 min for 20 cycles (plasmid experiments) or 36 cycles (cellular experiments). Using the primer-to-template ratios described in this study, the autoradiographic signal-intensity represented at the end of the 342 bp molecule had not yet plateaued following this number of thermal cycles. Reaction products were mixed with 6 μL of stop buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, and 20 mM EDTA), and four μL of each reaction was resolved on a 6% acrylamide/8.3 M urea sequencing gel.

Determination of cisplatin bound per template and phosphorimaging. The amount of cisplatin bound per alphoid fragment was determined by measuring the amount of cisplatin in specific DNA samples with flameless AAS* performed in a Perkin-Elmer model 400 graphite furnace [21]. Determination of DNA concentrations was performed using a Beckman model DU-64 spectrophotometer.

A Molecular Dynamics Phosphorimager/Densitometer (model 400B) with ImageQuant software (Microsoft Corp.) was used to determine the intensity of specific bands on filters. The area of each band of interest was defined, and the area under the curve (AUC) was calculated following subtraction of an identical volume in a directly adjacent area on the same lane. The latter was necessary because the background was found to vary lane to lane even for the same experiment (see Figs. 1–4). This value was divided by the signal-intensity measured for the AUC produced at the end of the 342 bp alphoid fragment at the top of the autoradiograph. This value represents the ASIR and provides a mechanism for valid

* Abbreviations: AAS, atomic absorption spectroscopy; and ASIR, adduct-site intensity ratio.

comparison for measurements from other independent experiments. Since only a minority of alphoid fragments under all *in vivo* and most *in vitro* conditions of cisplatin exposure employed in this study had cisplatin adducts to inhibit the progression of the polymerase, this ratio also provided an additional control on accuracy of loading lanes with equal DNA concentrations.

The intensity of adduct formation for each of the 8 sites detected within the second monomer of the alphoid molecule was determined by deriving the ASIR for each site for both different concentrations and conditions of cisplatin exposure. The percentage of adduct formation within the second monomer was determined by dividing the ASIR for a single site by the total ASIR value for sites detected within the second monomer (and multiplying by 100). Comparisons were made for *in vivo* and *in vitro* substrates that had equivalent DNA adduct formation, as determined by AAS over a broad concentration range.

Statistical methods. Student's *t*-test was used to determine if there were statistical differences in cisplatin adduct formation following (i) treatment under different conditions of exposure, (ii) treatment of different plasmid structures, and (iii) treatment of PC-3 and XP cells *in vivo*.

RESULTS

Effect of plasmid configuration on sites of cisplatin–DNA adduct formation. As an initial step in understanding possible factors affecting cisplatin–DNA adduct formation, the effect of tertiary DNA structure was investigated through the use of separate, well-defined plasmid structures. The plasmid p342 containing a 342 bp alphoid DNA dimer sequence and existing either as a double-stranded covalent closed circle (Form I) or as a double-stranded linear form (Form III) was treated with cisplatin at two different concentrations. Following gel analysis, adducts were detected predominantly at GpG and ApG sites on the template (CpC and CpT on the sequence shown) (Fig. 1). Even under these *in vitro* conditions, there was a preference for adduct formation at specific dinucleotide purine sites, demonstrated by the observed variations in signal-intensity. Following phosphorimaging evaluations of three experiments, a dose–response increase was detected for cisplatin exposure (experiment in Fig. 1 is representative), but there were no significant differences found when comparing each site on Form I and III plasmids (data not shown).

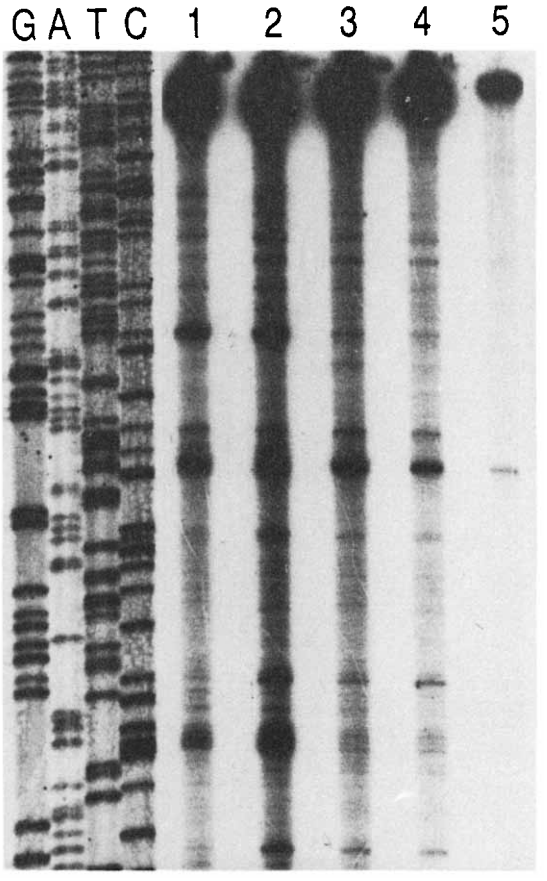

Fig. 1. Effect of plasmid conformation on cisplatin adduct sites *in vitro*. A plasmid containing a 342 bp alphoid fragment was treated with 100 nM cisplatin either as Form I DNA (lane 1) or Form III DNA (lane 2), or with 10 nM cisplatin as Form I (lane 3) or Form III (lane 4). These treatment conditions resulted in 1 cisplatin adduct per 2 alphoid fragments following treatment at 100 nM and 1 adduct per 20 fragments at 10 nM. Lane 5 was untreated plasmid DNA. Repetitive thermal-cycling was performed using primer No. 1 according to the conditions detailed in Materials and Methods. The DNA sequence represented on the left side of the figure results from annealing the plasmid with the identical primer used for repetitive thermal-cycling. As such, the DNA sequence shown represents the complementary strand. The intense signal at the top of the primer-extension lanes represents the end of the 342 bp fragment.

Detection of cisplatin–DNA adducts in vivo *and* in vitro *from PC-3 cells.* To determine the reproducibility and sensitivity of this method for detection of cisplatin–DNA adducts within living cells, the PC-3 prostatic carcinoma cell line was exposed in cell culture (*in vivo*) to different concentrations of cisplatin. No differences were observed in either the pattern or signal-intensity of adduct sites independent of whether 342 bp alphoid dimer fragments were isolated and purified, or the entire *Eco*RI digested DNA mixture was utilized (data not shown). Figure 2, using primer No. 1, shows a clear dose–response for DNA adduct formation at cisplatin concentrations ranging from 20 to 200 μM. Treatment of PC-3 cells in culture with these concentrations resulted in increasing cisplatin–DNA adduct formation, as measured using AAS (Table 1). As demonstrated in Table 2, although cisplatin dose-escalation resulted in a consistent increase in ASIR values, there were no significant changes detected in the percentage of adduct formation within the second monomer or the 342 bp alphoid fragment with increasing cisplatin concentration. The lowest concentration at which cisplatin adducts could be detected was 20 μM (Fig. 2, lane 2). Considering the template of the alphoid molecule alone, this treatment resulted in approximately 1 adduct per

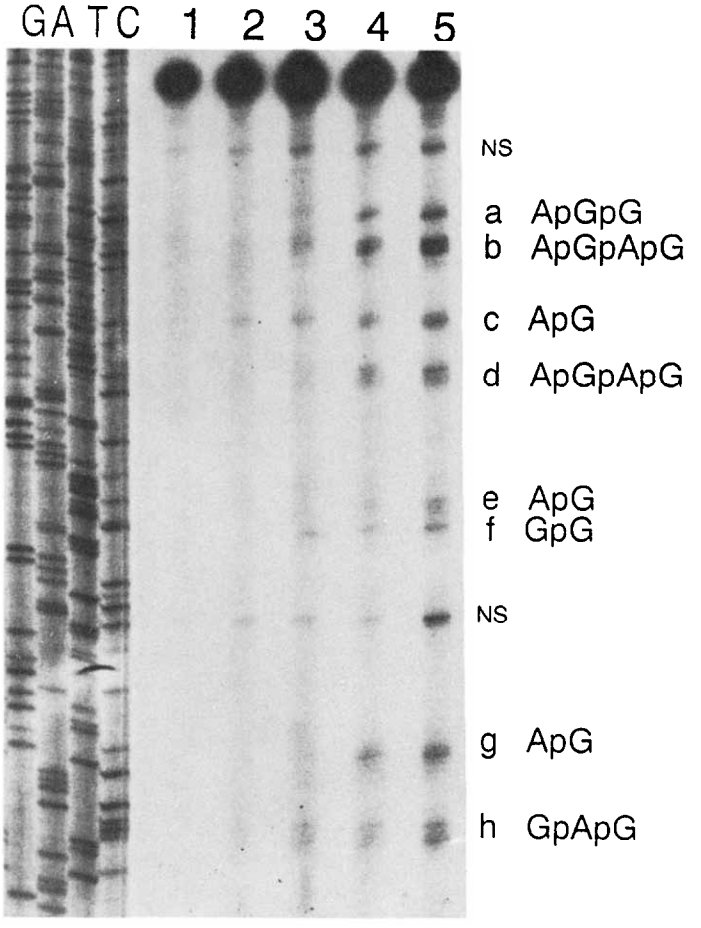

Fig. 2. Effect of cisplatin concentration on detection of cisplatin-induced adduct sites *in vivo*. Subconfluent PC-3 prostatic carcinoma cells were either untreated (lane 1) or exposed to 20 μM (lane 2), 50 μM (lane 3), 100 μM (lane 4) and 200 μM (lane 5) of cisplatin according to the conditions detailed in Materials and Methods, using primer No. 1. Reaction products were loaded adjacent to the sequence of a plasmid having 342 bp alphoid fragment (depicted on the left side of the figure) generated with the identical primer used in repetitive thermal-cycling. Sites of cisplatin adducts are depicted as (**a–h**), and the corresponding adduct sites on the DNA template (as determined from the consensus sequence [14]) are shown. The intense signal at the top of the primer-extension lanes represents the end of the 342 bp fragment. NS depicts non-specific sites of polymerase inhibition.

Table 1. Effect of cisplatin concentration on *in vivo* and *in vitro* adduct formation for PC-3 cell DNA

Cisplatin concentration (μM)	Cisplatin (pg/μg DNA)	Adducts/10 kb
In vivo		
10	61 ± 7*	1.3
20	79 ± 11	1.6
50	212 ± 14	4.7
100	425 ± 18	8.1
200	675 ± 24	14.4
In vitro		
2	84 ± 13	1.8
5	220 ± 17	4.8
10	440 ± 22	8.6
20	670 ± 31	14.3
50	952 ± 69	20.3

* Mean ± SEM for three separate AAS determinations.

40 single-stranded alphoid molecules. The lowest concentration at which all 8 adducts could be detected clearly was 50 μM, which resulted in approximately 1 adduct per 14 single-stranded molecules.

For *in vitro* treatment, genomic DNA from PC-3 cells was treated with various concentrations of cisplatin following DNA isolation and purification. With increasing concentrations of drug exposure there was an increase in overall adduct formation (Table 1). At the drug-to-DNA ratios of exposure used in this study, there was approximately a 1:10 ratio of adduct formation for *in vitro* compared with *in vivo* exposure (Table 1), as measured by AAS. As for *in vivo* treated substrates using primer No. 1, there was a consistent adduct pattern for *in vitro* treated DNA (Fig. 3) over a range of cisplatin exposures. Limits of *in vivo* and *in vitro* detection were equivalent considering the amount of cisplatin adduct formation per μg of DNA (Table 1, Figs. 2

Table 2. Cisplatin adduct formation in alphoid DNA detected with primer No. 1

	In vivo								
	PC-3*			XP†			*In vitro**		
Site	50 μM	100 μM	200 μM	50 μM	100 μM	200 μM	5 μM	10 μM	20 μM
a %	17.0	18.6	17.9	17.7	18.0	17.9	12.6	13.4	13.3
ASIR	0.0062	0.0099	0.0141	0.0069	0.0099	0.0143	0.0046	0.0076	0.0116
b %	28.6	28.1	29.3	29.2	30.1	31.1	20.4	21.7	22.7
ASIR	0.0106	0.0150	0.0269	0.0101	0.0161	0.0248	0.0076	0.0122	0.0197
c %	12.3	11.5	10.1	10.1	11.1	10.1	15.3	14.1	13.7
ASIR	0.0046	0.0062	0.0079	0.0035	0.0061	0.0080	0.0056	0.0080	0.0119
d %	11.6	11.6	11.5	11.7	10.9	10.5	14.2	14.4	15.3
ASIR	0.0043	0.0062	0.0090	0.0041	0.0060	0.0084	0.0052	0.0081	0.0133
e %	8.2	7.2	7.2	8.2	8.5	7.1	8.0	7.7	8.3
ASIR	0.0030	0.0038	0.0056	0.0028	0.0046	0.0056	0.0030	0.0044	0.0072
f %	8.5	8.1	7.7	8.7	8.5	8.1	8.7	8.7	7.9
ASIR	0.0032	0.0043	0.0060	0.0030	0.0046	0.0065	0.0032	0.0050	0.0068
g %	8.3	10.6	10.1	8.5	7.7	8.6	9.7	8.6	8.7
ASIR	0.0031	0.0056	0.0079	0.0029	0.0042	0.0068	0.0036	0.0049	0.0074
h %	7.8	7.1	7.1	5.9	5.2	5.6	11.1	11.4	10.1
ASIR	0.0029	0.0037	0.0054	0.0021	0.0028	0.0049	0.0041	0.0065	0.0087

*† The ASIR and the percentage of adduct formation values are means of *six or †three independent determinations for each concentration shown.

and 3). At the highest concentration of cisplatin exposure represented in Fig. 3 (50 μM, lane 6), there was a marked reduction in the signal representing the end of the 342 bp molecule at the top of the autoradiograph. This is because at this concentration a majority of alphoid molecules have adducts that retard the progression of the polymerase. Other than the DNA represented in this lane, with increasing cisplatin concentration, signal-intensity increased for all observed sites of adduct formation, as reflected by the increasing ASIR values. However, as was detected following *in vivo* treatment, there were no significant changes in the percentage of adduct formation at any site (**a–h**) in the second monomer with dose-escalation (Table 2).

Since there was no effect of drug concentration with respect to the percentage of adduct formation at any site in the second monomer, it was possible to consider data in the aggregate for PC-3-derived substrates annealed with primer No. 1. Eight sites (**a–h**) in the second monomer at three cisplatin concentrations were measured using phosphorimaging in six independent *in vivo* and *in vitro* experiments. These data revealed a significant difference in adduct formation for sites **b**, **c**, and **h** in that site **b** was favored *in vivo*, and sites **c** and **h** *in vitro* (Table 3).

Cisplatin adduct formation in XP cells. To determine if differences in adduct formation *in vivo* and *in vitro* were the result of selective repair *in vivo*, cisplatin–DNA adduct sites were detected for human fibroblasts derived from an individual with XP (group A). These cells have been shown to be deficient in cisplatin–DNA repair [17]. Treatment of subconfluent XP cells in culture resulted in levels of DNA modification that were comparable with those obtained for PC-3 cells treated in cell culture, as detected using AAS (data not shown). Also, overall ASIR values and percentage of adduct formation for the 8 sites detected using primer No. 1 were not statistically different from those observed for PC-3 cells treated in cell culture (Fig. 4, Tables 2 and 3).

Detection of cisplatin adducts within alphoid DNA using a different oligonucleotide primer. An 18 bp oligonucleotide was used by Murray *et al.* [10] in a similar study to determine the effect of cisplatin adduct formation for *in vivo* and *in vitro* treated HELA cells. Repetitive primer-extension was performed on *in vivo* and *in vitro* treated DNA samples obtained from PC-3 cells using specific activities of primer No. 5 equivalent to those used for primer No. 1. Under these conditions, an approximately 5-fold increase in the intensity of the 342 bp stop-site at the end of the alphoid dimer was detected in multiple experiments (data not shown). Furthermore, adduct sites could be detected after treatment with only 10 μM cisplatin, and all 8 sites could be detected after treatment with only 20 μM cisplatin (data not shown). As was true for primer No. 1, a clear dose–response for signal-intensity was detected but there were no significant differences for sites within *in vivo* and *in vitro* treated substrates detected with this primer.

DISCUSSION

Recently, this and other laboratories have demonstrated the feasibility of using alphoid DNA and PCR methodology to detect cisplatin–DNA adducts on DNA from cells treated in cell culture (*in vivo* conditions) [9–11]. As have others [10, 11], we concluded that the bands depicted in these autoradiographs are the result of cisplatin–DNA adducts because of the lack of bands within control untreated fragments and because, in reference to the consensus alphoid sequence [14], the sites observed in these experiments correspond to known sites of cisplatin–

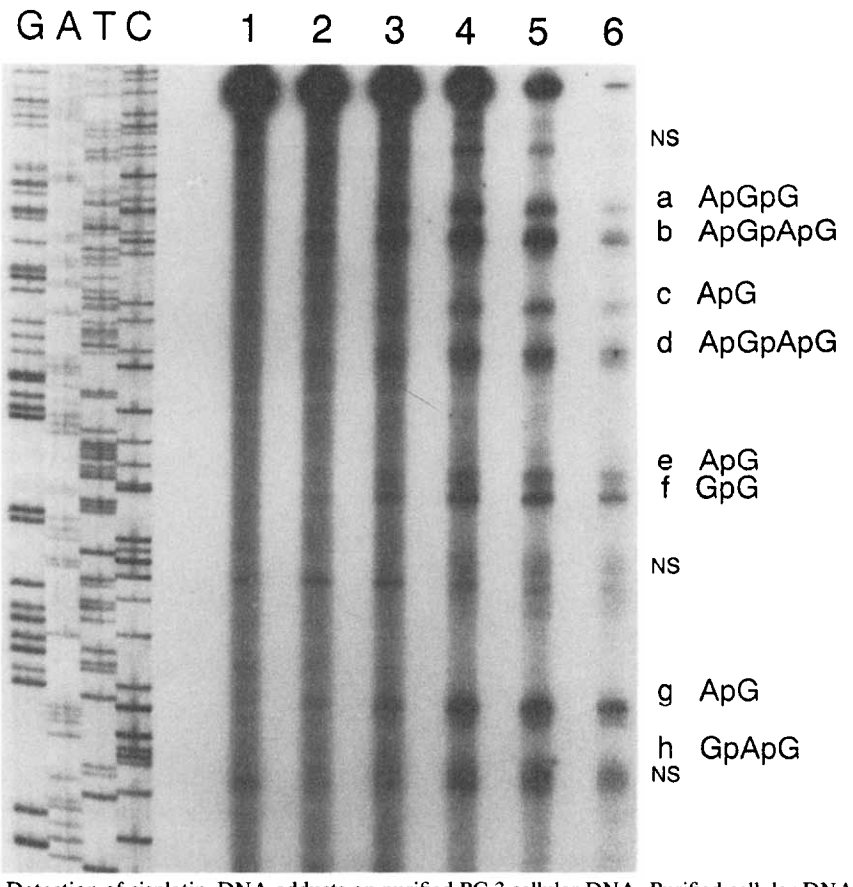

Fig. 3. Detection of cisplatin–DNA adducts on purified PC-3 cellular DNA. Purified cellular DNA was treated at identical drug-to-nucleotide ratios with 2 μM (lane 2), 5 μM (lane 3), 10 μM (lane 4), 20 μM (lane 5) or 50 μM (lane 6) and used for repetitive primer extension according to conditions detailed in Materials and Methods, using primer No. 1. Untreated purified DNA is represented in lane 1. Reaction products were loaded adjacent to the sequence of a plasmid having 342 bp alphoid fragment (depicted on the left side of the figure) generated with the identical primer used in repetitive thermal-cycling. Sites of cisplatin adducts are depicted as (**a–h**), and the corresponding adduct sites on the DNA template (as determined from the consensus sequence [14]) are shown. The intense signal at the top of the primer-extension lanes represents the end of the 342 bp fragment. NS depicts non-specific sites of polymerase inhibition.

Table 3. Cisplatin–DNA adduct formation within alphoid DNA*

Site†	DNA sequence†	*In vitro*‡	*In vivo*‡	XP§	*In vitro*‖	*In vivo*‖
a	ApGpG	15.88 ± 0.98	17.87 ± 0.44	17.85 ± 0.39	12.30 ± 0.87	12.55 ± 0.75
b	ApGpApG	18.51 ± 0.84	26.68 ± 0.59¶	29.96 ± 0.47	23.52 ± 0.72	23.32 ± 0.32
c	ApG	14.52 ± 0.56	11.32 ± 0.37¶	10.45 ± 0.75	11.90 ± 0.29	12.40 ± 0.29
d	ApGpApG	12.49 ± 0.84	11.63 ± 0.51	11.04 ± 0.57	10.45 ± 0.63	11.03 ± 0.83
e	ApG	8.39 ± 0.27	7.58 ± 0.33	7.93 ± 0.87	8.17 ± 0.80	7.51 ± 0.57
f	GpG	8.47 ± 0.24	8.16 ± 0.16	8.45 ± 0.65	11.06 ± 0.95	10.25 ± 1.07
g	ApG	9.86 ± 0.76	9.50 ± 0.76	8.35 ± 0.85	11.45 ± 1.20	11.07 ± 0.84
h	GpApG	10.77 ± 0.47	7.57 ± 0.38¶	5.95 ± 0.95	11.22 ± 0.58	12.00 ± 0.77

* Measured as percent adduct formation within the second monomer ± SEM.

† DNA site in the second monomer is from Choo *et al.* [14].

‡ Based on 6 separate experiments with PC-3 cells and PC-3 DNA at 3 concentrations for a total of 18 samples using primer No. 1.

‡ Based on 3 separate experiments at 3 concentrations for a total of 9 samples using primer No. 1. Results were not significant compared to *in vivo* PC-3 samples using primer No. 1.

‖ Based on 3 separate experiments in PC-3 cells and PC-3 DNA for a total of 9 samples using primer No. 5. Results were not statistically significant comparing *in vivo* and *in vitro* samples using primer No. 5

¶ Significant at the $P < 0.0005$ level, comparing *in vivo* and *in vitro* treated samples.

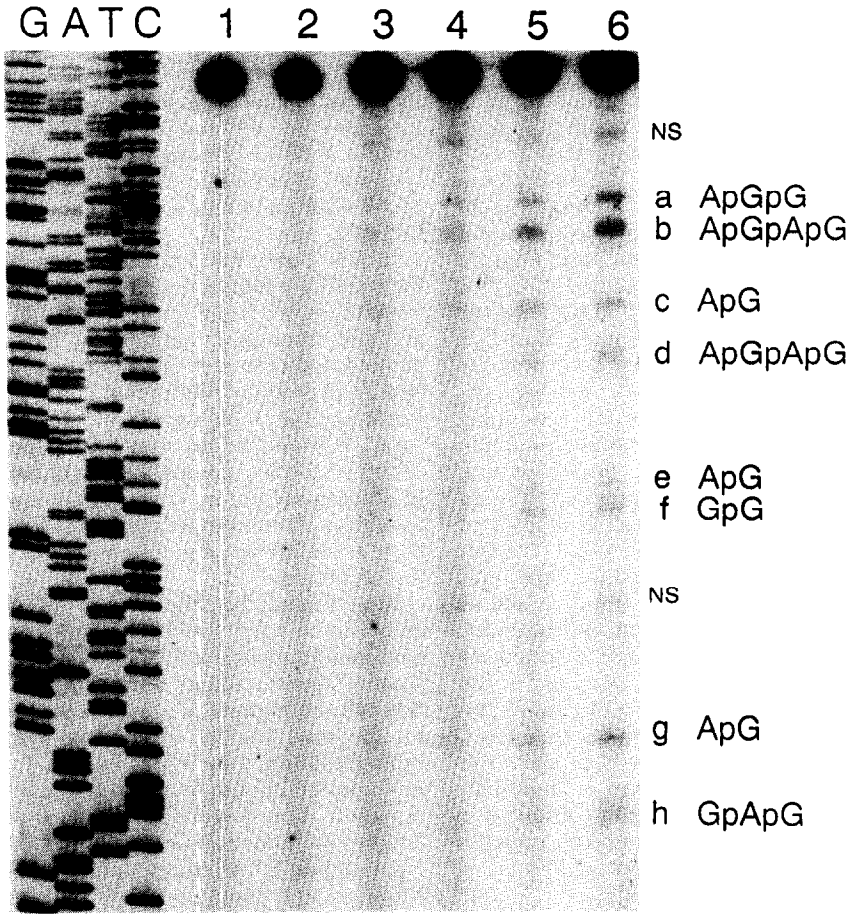

Fig. 4. Detection of cisplatin adducts in XP cells treated in cell culture. Subconfluent XP cells were treated in cell culture with either no treatment (lane 1) or cisplatin at 10 μM (lane 2), 20 μM (lane 3), 50 μM (lane 4), 100 μM (lane 5) or 200 μM (lane 6) and used for repetitive thermal-cycling using primer No. 1 according to the conditions detailed in Materials and Methods. Reaction products were loaded adjacent to the sequence of a plasmid having 342 bp alphoid fragment (depicted on the left side of the figure) generated with the identical primer used in repetitive thermal-cycling. Sites of cisplatin adducts are depicted as (**a–h**), and the corresponding adduct sites on the DNA template (as determined from the consensus sequence [14]) are shown. The intense signal at the top of the primer-extension lanes represents the end of the 342 bp fragment. NS depicts non-specific sites of polymerase inhibition.

DNA adduct formation [4–8] (Figs. 2–4). Also the demonstration in this investigation, for the first time, of an *in vivo* and *in vitro* dose–response relationship for signal-intensity at specific sites (Figs. 1–4) (as demonstrated by reproducibly increased ASIR values, Table 2) further confirms the validity of our method, since cisplatin adduct formation is known to increase with increasing levels of drug exposure (Table 1) [4, 12, 13]. Although it is not known with certainty whether all possible cisplatin–DNA adducts, especially the uncommonly formed interstrand adduct [4], are detected using this method, it has been observed that all cisplatin–DNA adducts may be capable of inhibiting *Taq* polymerase progression [12, 13].

In this study, relatively high concentrations of cisplatin were necessary to detect adduct sites *in vivo*. However, for both the purified DNA and chromatin substrates (Figs. 2–4), the pattern of cisplatin adduct formation was not altered over a broad concentration range, suggesting that cisplatin–DNA adduct sites are unlikely to be altered at lower levels of drug exposure. This consistent pattern of site detection also reaffirms that alphoid DNA is sufficiently homologous to serve as a target for detection of cisplatin–DNA adducts in these studies [9, 10].

Intrastrand GpG and ApG are the cisplatin–DNA adducts formed most frequently [4–10]. Considering these data, it is notable that 3 of 8 sites detected within the second monomer (**a**, **b**, and **d**) are sites in which more than one adduct might form. In fact, considering GAG intrastrand adducts in which an adduct forms between guanines [4], sites **b** and **d** are sites of three possible adducts. Although we are

unable to distinguish the proportion of different adducts formed at these sites, their radiographic intensity likely results from the sum of different adducts on separate alphoid molecules. However, this phenomenon alone does not fully account for the differences in adduct intensity under either *in vivo* or *in vitro* conditions. For example, there is over a 1.7-fold difference in adduct preference for ApG sites at positions **c** and **e** *in vitro*. As has been observed for plasmid substrates ([22], Fig. 1), there are preferences for adduct site formation on cellular DNA, possibly mediated by the influence of flanking DNA sequence [6].

Having demonstrated that a dose–response relationship existed for the detection and measurement of cisplatin–DNA adducts and that these are formed at a consistent frequency over a broad concentration range, it was feasible to ask how might their formation be affected by DNA conformation or *in vivo* and *in vitro* conditions of exposure. Experiments performed using circular as opposed to linear double-stranded plasmid substrates demonstrated that it is unlikely that conformational changes reflected by the difference between the structures of Forms I and III affect sites of cisplatin adduct formation (Fig. 1). In contrast, statistically significant differences in adduct formation were detected for sites **b**, **c**, and **h** (Table 3) for *in vivo* and *in vitro* treated substrates using primer No. 1. These differences are not likely to result from selective DNA repair, since XP cells demonstrated the same frequency of adduct formation following *in vivo* treatment (Table 3), further supporting the observation that the overall frequency of GpG and ApG cisplatin adducts is comparable in XP and normal fibroblasts immediately following exposure [17].

Murray *et al.* [10] recently published experiments that used similar methods to compare cisplatin–DNA adducts within DNA from HeLa cells treated in culture and naked DNA from these cells. In contrast to our findings, they did not detect a difference in adduct formation for *in vivo* and *in vitro* treated DNA. These seemingly dissimilar findings may result from the detection of partially distinct alphoid DNA subsets using different alphoid primers. The inclusion of the *Eco*RI site within primer No. 1 may result in the detection of a relatively specific subset of alphoid molecules in a more consistent DNA conformation or nucleosome phase [20]. A specific nucleosome phasing has been detected for a subset of alphoid molecules that have *Eco*RI sites from African Green Monkey DNA [23]. This subset, estimated to constitute only 8% of alphoid DNA [24], contains a linker segment spanning bp 111 to 142 on the second monomer [23], corresponding to the location of sites **a** and **b**, the only sites in which *in vivo* adduct formation is favored. Importantly, cisplatin [25, 26] and a number of other genotoxic agents [27, 28] form adducts preferentially within linker DNA.

Consistent with the possibility that the two primers detect different alphoid subsets, we did not detect a difference for *in vivo* and *in vitro* adduct site formation using the primer used by Murray *et al.* However, in contrast to their results, we could readily detect a dose–response for adduct site formation.

In addition to nucleosomal structure and DNA flanking sequence, there may be other factors that affect cisplatin–DNA adduct formation. For instance, DNA-binding proteins with a specific affinity to alphoid DNA have been isolated [23], and these may influence sites of drug–DNA adduct formation. Also, addition of single-stranded binding protein to M-13 plasmid DNA has been shown to alter sites of cisplatin adduct formation *in vitro* and allows cisplatin to bind to sites otherwise less accessible [29].

In this study, we detected a more minor effect for non-DNA sequence-related influence on adduct sites. However, it is possible that the *in vivo* effects are underestimated, perhaps by the detection of alphoid molecules that are in different nucleosomal phases, even using primer No. 1. To determine the importance of DNA binding proteins, including histones, it would be necessary to be able to detect sequence-specific cisplatin adducts on a single gene in human cells. However, it is not feasible to administer enough cisplatin to intact cells such that 1 in 40 single copy genes has an adduct, the limit of detection of this technique.

Nonetheless, the difference in the pattern of *in vivo* and *in vitro* cisplatin adduct formation may have important implications for this and other DNA-reactive agents. Adduct formation for cisplatin and other chemotherapeutic agents has been investigated for the most part on plasmid or synthetic DNA substrates [6–8]. Alterations in adduct formation *in vivo* may contribute to different cytotoxic profiles, especially if, as postulated, modification of nuclear DNA is the principal mediator of cytotoxicity [30]. For instance, if chromatin structure affects sites of DNA adduct formation, then the cytotoxicity of cisplatin and other chemotherapeutic agents may be determined, in part, by their ability to form adducts in the vicinity of genes crucial for a particular cell line. This possibility is supported by observations that cisplatin–DNA adducts, at least *in vitro*, effectively block transcription [31]. The ability to detect cisplatin DNA adducts produced *in vivo* may also be useful for detecting adducts from patient samples following treatment or for measuring *in vivo* cisplatin–DNA repair.

Acknowledgements—The authors would like to thank their colleagues, including E. Frei III, L. Schnipper, R. Drews, V. Chan, J. P. Eder, S. Balk, C. Yockey, M. Frantz and A. Ng, for their helpful reading of the manuscript, support, and encouragement. This work was supported by NCI Grants R29-CA51438 (G.J.B.) and RO1-CA 50174 (B.A.T.)

REFERENCES

1. Singer B and Grunberger DG, *Molecular Biology of Mutagens and Carcinogens*. Plenum Publishing Corp., New York, 1983.
2. Farber E, Chemical carcinogenesis. *N Engl J Med* **305**: 1379–1389, 1984.
3. Loehrer P and Einhorn L, Drugs five years later: Cisplatin. *Ann Intern Med* **100**: 704–713, 1984.
4. Fichtinger-Schepman AJ, van der Veer JL, den Hartog JHJ, Lohman PHM and Reedijk J, Adducts of the antitumor drug cisplatin with DNA; formation, identi-

fication and quantitation. *Biochemistry* **24**: 707–713, 1985.
5. Pinto AL and Lippard SJ, Sequence-dependent termination of *in vitro* DNA synthesis by *cis*- and *trans*-diamminedichloroplatinum(II). *Proc Natl Acad Sci USA* **82**: 4616–4619, 1985.
6. Lemaire M-A, Schwartz A, Rahmouni AR and Leng M, Interstrand cross-links are preferentially formed at the d(GC) sites in the reaction between *cis*-diamminedichloroplatinum(II) and DNA. *Proc Natl Acad Sci USA* **88**: 1982–1985, 1991.
7. Villani G, Hübscher U and Butour J-L, Sites of termination of *in vitro* DNA synthesis on *cis*-diamminedichloroplatinum(II) treated single-stranded DNA: A comparison between *E. coli* DNA polymerase I and eucaryotic DNA polymerases α. *Nucleic Acids Res* **16**: 4407–4417, 1988.
8. Hemminki K and Thilly WG, Binding of cisplatin to specific sequences of human DNA *in vitro*. *Mutat Res* **202**: 133–138, 1988.
9. Bubley GJ, Ashburner BA, Balk SP, Ara G, Varshaney A and Teicher BA, Identification of *cis*-diamminedichloroplatinum(II) adducts in DNA from drug-treated human cell nuclei. *Biotechniques* **12**: 694–696, 1992.
10. Murray V, Motyka H, England P, Wickham G, Lee HH, Denny WA and McFadyen WD, An investigation of the sequence-specific interaction of cisplatin and four analogs, including two acridine-tethered complexes, with DNA inside human cells. *Biochemistry* **31**: 11812–11817, 1992.
11. Murray V, Motyka H, England PR, Wickham G, Lee HH, Denny WA and McFadyen WD, The use of *Taq* DNA polymerase to determine the sequence specificity of DNA damage caused by *cis*-diamminedichloroplatinum(II), acridine-tethered platinum(II) diammine complexes or two analogues. *J Biol Chem* **267**: 18805–18809, 1992.
12. Jennerwein MM and Eastman A, Polymerase chain reaction-based method to detect cisplatin adducts in specific genes. *Nucleic Acids Res* **19**: 6209–6214, 1992.
13. Kalinowski D, Illenye S and van Houten B, Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative PCR assay. *Nucleic Acids Res* **20**: 3485–3494, 1992.
14. Choo KH, Vissel B, Nagy A, Earle E and Kalitsis P, A survey of the genomic distribution of alpha satellite DNA on all the human chromosomes, and derivation of a new consensus sequence. *Nucleic Acids Res* **19**: 1179–1183, 1989.
15. Waye JS and Willard HF, Nucleotide sequence heterogeneity of alpha satellite repetitive DNA: A survey of alphoid sequences from different human chromosomes. *Nucleic Acids Res* **15**: 7549–7569, 1987.
16. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF and Jones LW, Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* **17**: 16–23, 1979.
17. Dijt F, Fichtinger-Schepman A, Berends F and Reedijk J, Formation and repair of cisplatin-induced adducts to DNA in cultured normal and repair deficient human fibroblasts. *Cancer Res* **48**: 6058–6062, 1988.
18. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
19. Knox RJ, Friedlos F, Lydall DA and Roberts JJ, Mechanism of cytotoxicity of anticancer platinum drugs: Evidence that *cis*-diamminedichloroplatinum-(II) and *cis*-diammine-(1,1-cyclobutanedicarboxylato)-platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res* **46**: 1972–1975, 1986.
20. Baldini A, Smith D, Rocchi M, Miller OJ and Miller DA, A human alphoid DNA clone from the *Eco*RI dimeric family. *Genomic* **5**: 822–828, 1989.
21. Drummer OH, Proudfoot A, Howes L and Louis WJ, High-performance liquid chromatographic determination of platinum(II) in plasma ultrafiltrate and urine: Comparison with a flameless atomic absorption spectrometric method. *Clin Chim Acta* **136**: 65–74, 1984.
22. Bubley GJ, Ashburner BP and Teicher BA, Spectrum of *cis*-diamminedichloroplatinum(II) mutations induced in a shuttle vector propagated in human cells. *Mol Carcinog* **4**: 397–406, 1991.
23. Strauss F and Varshavsky A, A protein binds to a satellite DNA repeat at three specific sites that would be brought into mutual proximity by DNA folding in the nucleosome. *Cell* **37**: 889–901, 1984.
24. Wu KC, Strauss F and Varshavsky A, Nucleosome arrangement in green monkey α-satellite chromatin. Superimposition of non-random and apparently random patterns. *J Mol Biol* **170**: 93–117, 1983.
25. Foka M and Paoletti J, Interaction of *cis*-diamminedichloroplatinum(II) to chromatin: Specificity of the drug distribution. *Biochem Pharmacol* **35**: 3283–3291, 1986.
26. Hayes J and Scovell W, Cisplatin modified chromatin and nucleosomal core particle. *Biochim Biophys Acta* **1089**: 377–385, 1991.
27. Enright H, Miller W and Hebbel R, Nucleosomal histone protein protects DNA from iron-mediated damage. *Nucleic Acids Res* **20**: 3341–3346, 1992.
28. Moore CW, Internucleosomal cleavage and chromosomal degradation by bleomycin and phleomycin in yeast. *Cancer Res* **48**: 6837–6843, 1988.
29. Hoffmann J-S, Hübscher U, Michot B and Villani G, Interaction of *cis*-diamminedichloroplatinum(II) with single-stranded DNA in the presence or absence of *Escherichia coli* single-stranded binding protein. *Biochem Pharmacol* **42**: 1393–1398, 1991.
30. Zwelling LA and Kohn KW, Mechanism of action of *cis*-dichlorodiammineplatinum(II). *Cancer Treat Rep* **63**: 1439–1444, 1979.
31. Corda Y, Job C, Anin M-F, Leng M and Job D, Transcription by eucaryotic and procaryotic RNA polymerases of DNA modified at a d(GG) or a d(AG) site by the antitumor drug *cis*-diamminedichloroplatinum(II). *Biochemistry* **30**: 222–230, 1991.