Preferential Platination of an Activated Cellular Promoter by *cis*-Diamminedichloroplatinum†

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ABSTRACT: This study examines how accessibility to cisplatin on various genomic regions in T47D breast cancer cells, including the retinoic acid receptor β gene promoter and coding region and the dihydrofolate reductase gene promoter and coding region, is affected by treatment of the cells with 9-*cis* retinoic acid, a treatment that activates the retinoic acid receptor β gene promoter in these cells. A PCR-based assay was used to measure cisplatin adduct density based on the inhibition of PCR amplification of templates from cisplatin treated versus untreated cells. Treatment of cells with 9-*cis* retinoic acid enhanced accessibility to cisplatin on the retinoic acid receptor β gene promoter region, but not on the coding regions of that gene nor on the dihydrofolate reductase gene promoter or coding regions, where accessibilities to cisplatin remained 2−4 times lower than on the activated retinoic acid receptor β gene promoter. Examination of smaller regions within this promoter region showed a repression of platination in the 500 bp region surrounding the TATA box in cells prior to 9-*cis* retinoic acid treatment, which was abolished following promoter activation. Differences in sequence composition between the various regions could not fully account for differences in platination, suggesting that structural features such as bends in retinoic acid receptor β gene promoter DNA following gene activation, create energetically favorable sites for platination, and contribute to the cytotoxicity of the drug.

cis-Diamminedichloroplatinum (cisplatin) is a highly effective chemotherapeutic agent used in the treatment of a variety of human tumors (*1*). Its cytotoxicity derives from its ability to damage DNA, forming primarily bifunctional 1,2-intrastrand cross-links between adjacent guanines or adenine-guanine dinucleotides, as well as other minor adducts including interstrand cross-links (*2*). Adduct formation impedes DNA polymerase progression, inhibits DNA synthesis, and triggers apoptosis (*3*−*5*). While inhibition of DNA synthesis is believed to be a critical step in cisplatin's cytotoxicity, it does not by itself account for the marked antitumor efficacy of cisplatin. For example, adducts formed by the geometric isomer of cisplatin, *trans*-diamminedichloroplatinum (transplatin), also impede DNA polymerase progression and inhibit DNA synthesis (*6*). Yet transplatin does not induce apoptosis and has little antitumor activity. Furthermore, while cisplatin causes slowing of DNA synthesis, cells treated with cisplatin do not arrest in S phase, but progress and block in G2 (*7*). In addition, the extent to which DNA synthesis is slowed by cisplatin in sensitive versus resistant cells does not correlate with its relative toxicity to those cells (*8*). Several studies have pointed to transcription as a key target for cisplatin and have suggested that cisplatin's ability to target certain genetic regulatory elements and inhibit specific gene expression may contribute greatly to its toxicity (*9*−*12*).

A structural distortion in DNA occurs upon cisplatin binding that has been proposed to underlie the biological toxicity of cisplatin. Crystallographic studies reveal that the 1,2-cisplatin cross-link induces a bend of about 45° toward the major groove of the DNA helix accompanied by thermal destabilization (*13*). Such a bend appears to generate a structural motif with biological specificity, as certain chromosomal proteins bind with high affinity to these sites. These proteins include histone H1 (*14*), HMG1 (*15*), HMG2 (*16*), the human structure-specific recognition protein SSRP1(*17*, *18*), and the human transcription factor hUBF (*18*), all of which are proteins known to bend DNA and whose binding to DNA might be facilitated by the bends generated by cisplatin. In contrast, the structural effects of transplatin appear to be of greater variability (*19*), and transplatin adducts do not constitute high affinity binding sites for the above-mentioned chromosomal proteins (see ref *20* for review). Cisplatin's toxicity may therefore be related to the structural consequences of adduct formation, which could involve a disruption of normal binding of chromosomal proteins, or an impediment to conformational changes that are necessary for biological regulation.

In the studies reported here we have addressed the possibility that cisplatin may actually target chromosomal regions such as transcriptional promoters, where DNA bends or partial unwinding of DNA may occur following transcription factor recruitment to an activated promoter. If so, such a structural motif may provide a preferential target for cisplatin and contribute to the cytotoxicity of this drug. We have examined cisplatin adduct formation on promoter and

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downstream regions of the retinoic acid receptor β (RARβ)[1] gene in T47D breast cancer cells, where this gene undergoes retinoic-acid-dependent activation. Adduct formation as determined by a quantitative PCR-based assay was observed to be significantly higher on the activated RARβ promoter than on a downstream region of the same gene, as well as on the coding and promoter regions of the constitutively expressed housekeeping gene, dihydrofolate reductase (DHFR). Adduct formation on the DHFR promoter, which does not undergo retinoic-acid-dependent activation, did not increase following retinoic acid treatment of cells. Cisplatin's cytotoxicity may therefore derive in part from its ability to target and disrupt the function of certain genetic regulatory loci such as the RARβ promoter.

EXPERIMENTAL PROCEDURES

Cell Culture. T47D breast cancer cells were purchased from ATCC and maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 50 μg/mL gentamycin. Cell culture reagents were purchased from Irvine Scientific, Santa Ana, CA. All experiments were performed using charcoal-treated serum (600 mg of activated charcoal/50 mL of serum for 10 min at 4 °C, followed by filtration).

Analysis of RARβ and DHFR Gene Expression. About 5 $\times$ 10^5 T47D cells (at about 70% confluency) were treated as indicated and total cellular RNA was prepared using the Rneasy kit from Qiagen and following the manufacturer's procedure. One microgram of RNA was reverse transcribed into cDNA in a 20 μL reaction containing 0.5 mM dNTPs (Pharmacia), 100 μg/mL oligo dT (Promega), 2 units of RNAsin (Promega), 10 units of Moloney Murine leukemia virus reverse transcriptase (Promega), and reverse transcriptase buffer (Promega). This cDNA was then used as a template for quantitative PCR. Primers were chosen so as to amplify a 178 base region of the RARβ coding sequence encompassing parts of exons 4 and 5, as well as a 246 base region of the coding sequence of the dihydrofolate reductase from exons 1−4. The sequences are as follows: RARβ message (forward), 5′-GTGTACAAACCCTGCTTCGTCTGC-3′; (reverse) 5′-CTGGAGTCGACAGTATTGGCATCG-3′; DHFR message (forward), 5′-GGTTCGCTAAACTGCATCGTCGC-3′; (reverse) 5′-GTGGAGGTTCCTTGAGTTCTCTG − 3′.

Amplification conditions were as described below for the PCR-stop assay. Quantitative conditions were verified by preparing cDNA with 100 and 10 ng of RNA (in addition to 1 μg of RNA) and amplifying serial 2-fold dilutions of the cDNA from 2 to 0.25 μL of template to show that product formation was proportional to input template under our conditions. Following amplification, products were analyzed by agarose gel electrophoresis followed by band quantitation using a Kodak digital camera.

Cell Viability. Following treatments, cell viability was monitored by adding 10% Trypan blue and determining the fraction of cells that excluded the dye.

Cisplatin Treatments. Cells were plated at 50% confluency in six-well plates in medium supplemented with 10% charcoal-treated FBS in addition to the other standard additives described above. Following attachment, cultures were pretreated 24 h in the presence or absence of 1 μM 9-*cis* retinoic acid (Sigma, St. Louis, MO), followed by a 2 h incubation in the presence or absence of 1 mM cisplatin (Platinol, aqueous solution at 1 mg/mL, purchased from local pharmacies). The preincubation medium was then replaced, and genomic DNA was prepared 24 h later as described below. Although DNA repair has been observed to occur over 24 h in cells treated with lower doses of cisplatin (*21*−*22*), at the high levels used in our studies we observed an overall increase in adduct formation over 24 h, so that adduct densities approached 1−4 adducts/10 Kb and were therefore readily detectable in our assay. The increase in adduct formation over time suggests that residual cisplatin continued to cause DNA damage and that repair did not keep pace with adduct formation under our conditions.

Preparation of Genomic DNA. DNA was prepared from treated cultures using the QIAmp blood kit essentially following the manufacturer's protocol, except that cells were lysed directly on the plate in the presence of PBS, Qiagen protease, and lysis buffer supplied in the kit. Following purification, DNA was adjusted to 0.25 mg/mL in sterile water and stored at −20° C until use.

Analyses of DNA Damage by PCR Stop Assay. Quantitative PCR was used to compare cisplatin adduct formation on specific regions of DNA. The assay, known as the PCR stop assay, has been described (*23*). Because Taq polymerase is blocked at cisplatin adducts, the relative efficiency of PCR amplification of genomic DNA from cisplatin-treated versus control cells decreases in proportion to platination levels. The relative PCR efficiency is equivalent to the frequency (P) of undamaged strands within a population. P is related to the average number (n) of cisplatin adducts per fragment, by the Poisson formula: $P = e^{-n}$, or $-(\ln P) = n$. Adducts per Kb would then be equal to $-(\ln P)$/(size of fragment in Kbs). We found that fragment sizes of around 1 Kb provided a sufficiently large target size to enable measurement of adduct densities in the range 0.1−0.4 adducts/Kb observed here. A drop in the PCR signal of damaged DNA to 0.67 of the control signal would therefore reflect an average cisplatin adduct density of $-(\ln 0.67) = 0.4$ adducts/fragment. Standard deviations among triplicate PCRs were in the range of ±5%, meaning that adduct densities of less than about 0.05 adducts/fragment were undetectable. For each primer pair, we verified that product formation was directly proportional to input template DNA by performing a pilot experiment with serial 2-fold dilutions of template, followed by electrophoresis on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. Bands were quantitated using Kodak digital camera and analysis software. Depending on the primer set, the amount of template used in the PCR reaction ranged 0.03−0.25 μg/25 μL reaction. Reactions were performed in 25 μL containing template DNA, 25 pmol each of forward and reverse primer, 250 μM dNTPs (Pharmacia), 1.25 units of Taq polymerase (Qiagen), 1× buffer (Qiagen), and solution Q (Qiagen). The amplification program was as follows: 1 cycle (94 °C, 1 min 30 s); 25 cycles (94 °C, 1 min; 57 °C, 1 min; 70 °C, 2 min, 30 s); 1 cycle (94 °C, 1 min; 57 °C, 1 min; 70 °C, 7 min). Two independent templates

[1] Abbreviations: RARβ, retinoic acid receptor β; RARE, retinoic acid response element; PCR, polymerase chain reaction; DHFR, dihydrofolate reductase; ATCC, American type culture collection; PBS, phosphate-buffered saline; Kb, kilobase; bp, base pair; CDS, coding sequence.

Table 1: Primers Used for PCR-stop Assay of Genomic DNA

gene	region amplified	primer sequences (5′ → 3′)	product size (base pairs)
RAR β	RARβ Promoter (includes A,B,C below)	1: CGAGTGCAGTCAATTCAGCCAGG (for) 2: GCTTATCCTCTAGGTGTGGAGGC (rev)	1043
	(A) promoter, upstream and including TATA and RARE	1: see above (for) 3: CTTCCTACTACTTCTGTCACACAG (rev)	624
	(B) promoter region of TATA and RARE	4: GGGAGAGAAGTTGGTGCTCAACG (for) 5: CCTTCCGAATGCGTTCCGGATC (rev)	483
	(C) promoter, downstream and including TATA and RARE	6: GCTTTTGCAGGGCTGCTGGGAG (for) 2: see above (rev)	592
	RARβ CDS (1036 bp) (exon 10)	7: GGTGCAGAGCGTGTAATTACCTTG(for) 8: CTGCCTTGGAGGCTATCATTACTG(rev)	1036
	RARβ CDS (483 bp) (exon 10)	7: see above (for) 9: GGTCTTTGCCATGCATCTTGAGTG (rev)	483
DHFR	DHFR promoter	10: CAGAATGGGAGTCAGGAGACCTG (for) 11: GCAGAAATCAGCAACTGGGCCTC (rev)	1000
	DHFR CDS (1062 bp) (exons 1,2,adjacent introns)	12: CCGTAGACTGGAAGAATCGGCTC (for) 13: CAGTTGCCAATTCTGCCCCATGC (rev)	1062
	DHFR CDS (472 bp) (exon 1 and adjacent introns)	14: CAATTTCGCGCCAAACTTGACCG (for) 15: GAGCTCTAAGGCACCTGACAAAC (rev)	472
	DHFR CDS (272 bp) (exon 1 and adjacent introns) (internal control)	16: GGTTCGCTAAACTGCATCGTCGC (for) 17: CAGAAATCAGCAACTGGGCCTCC (rev)	272

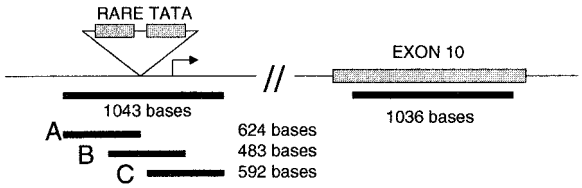

FIGURE 1: Location and sizes of PCR amplification products from the RARβ gene of T47D cells (see also Table 1). For the analysis in Figure 3, PCR primers were chosen so as to amplify a 1043 bp region of the RARβ promoter, including the retinoic acid response element (RARE) and TATA box, and a 1036 bp region of the RARβ coding sequence (CDS). For the analysis in Figure 4, PCR primers were chosen so as to amplify the subregions A, B, and C.

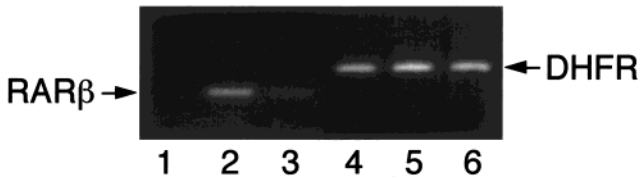

FIGURE 2: Quantitative RT-PCR analysis of RARβ gene expression (lanes 1−3) and DHFR gene expression (lanes 4−6) in T47D cells under various conditions. Lanes 1 and 4: untreated T47D cells. Lanes 2 and 5: T47D cells treated 24 h with 1 μM 9-*cis* retinoic acid. Lanes 3 and 6: cells treated 24 h with 9-*cis* retinoic acid, followed by 2 h with 200 μM cisplatin. Quantitative conditions for cDNA synthesis and PCR amplification were verified as described in the Experimental Procedures. A total of 10 and 2 μL, respectively, of the RARβ and DHFR PCR reactions were analyzed.

were prepared for each treatment condition, and each one was analyzed in triplicate. As an internal PCR control for each template, a 270 bp fragment of the dihydrofolate reductase gene was amplified. This fragment is too small to register significant levels of damage under the conditions we used, and its amplification product was seen to vary by less that 5% among the various templates. The primers 1−17 used for PCR amplification of various regions of genomic DNA are summarized in Table 1. Figure 1 shows a schematic representation of regions amplified from the RARβ gene.

RESULTS

Transcriptional Response of the RARβ Gene to 9-cis Retinoic Acid and Cisplatin. Figure 2 shows the results of an RT-PCR assay demonstrating retinoic-acid-dependent activation of the RARβ gene (lane 2 compared to lane 1) and cisplatin-mediated inhibition of this activation (lane 3). Under the same conditions, we observed little change in DHFR expression (lanes 4−6), indicating that expression of this gene is not retinoic acid dependent and that cisplatin has little immediate effect on its expression. This is consistent with another study in which treatment with cisplatin did not alter levels of DHFR message (*24*), although long term in vitro selection for cisplatin-resistant ovarian carcinoma cell lines by repeated exposure to cisplatin has been reported to generate variants that overexpress DHFR (*25*). Genomic DNA from cells before and after treatment with 9-*cis* retinoic acid was then used to analyze platination of the RARβ gene in its active and inactive state.

Preferential Cisplatin Adduct Formation on the Activated RARβ Gene Promoter. We performed a PCR stop assay to examine cisplatin adduct density on genomic DNA templates from 9-*cis*-retinoic-acid-treated (RA+) or -untreated (RA−) cells. Regions spanning about 1 Kb in length of the RARβ promoter (1043 bp), the RARβ coding sequence (1036 bp), the DHFR promoter (1000 bp), and the DHFR coding sequence (1062 bp) were examined as defined by primers listed in Table 1. PCR products were analyzed on agarose gels (Figure 3A) and bands were quantitated in order to determine the relative PCR efficiencies from platinated versus unplatinated templates. Using templates from (RA−) cells (Figure 3A, lanes 1 and 2), the relative PCR efficiencies from platinated versus unplatinated templates (lane 2 versus lane 1) were as follows: RARβ promoter (0.88), RARβ coding sequence (0.85), DHFR promoter (0.85), DHFR coding sequence (0.87). Using templates from (RA+) cells (lanes 3 and 4), the relative PCR efficiencies from platinated versus unplatinated templates (lane 4 versus lane 3) were as follows: RARβ promoter (0.65), RARβ coding sequence (0.88), DHFR promoter (0.9), DHFR coding sequence (0.88). On the basis of the PCR results, the cisplatin adducts densities were calculated by the Poisson equation described above. The results plotted in Figure 3B represent the averages

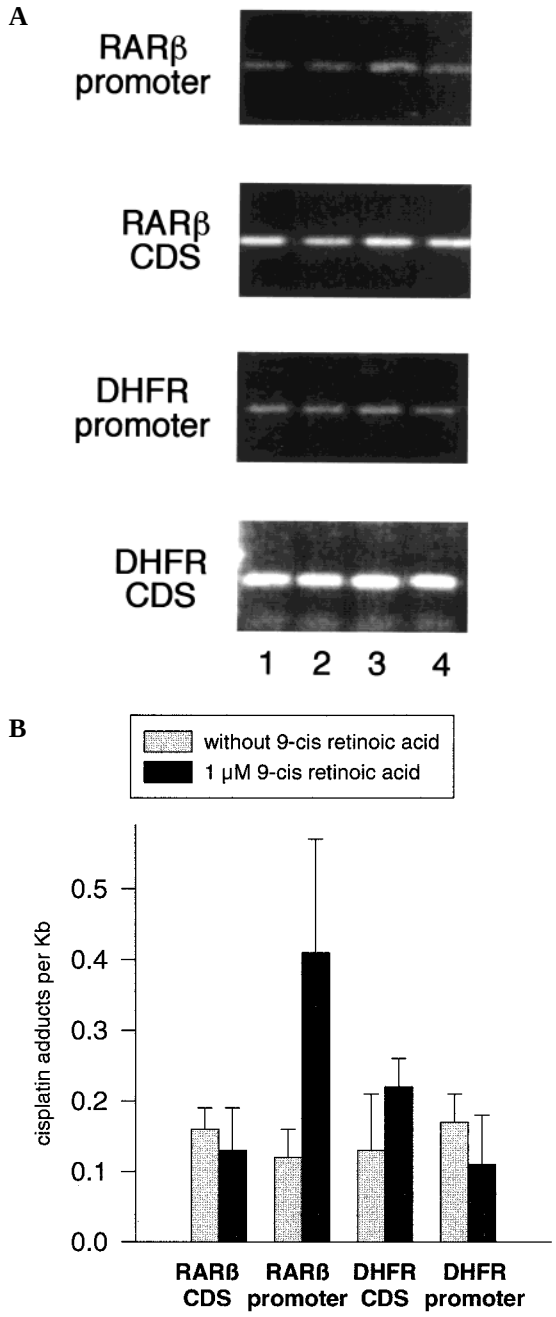

FIGURE 3: Cisplatin adduct formation on various regions of T47D cell genomic DNA as determined by the PCR stop assay. Regions analyzed were the RARβ CDS (1036 bp), the RARβ promoter (1043 bp), the DHFR CDS (1062 bp), and the DHFR promoter (1000 bp). Genomic DNA was prepared from cells pretreated in the presence (RA+) or absence (RA−) of 9-*cis* retinoic acid for 24 h followed by 2 h in the presence or absence of 1 mM cisplatin. (A) Agarose gel analysis of PCR products from one experiment. Lanes 1 and 2 (RA− cells). Lanes 3 and 4 (RA+ cells). Lanes 1 and 3 (no cisplatin). Lanes 2 and 4 (plus cisplatin). (B) Bar graph showing cisplatin adducts per Kb as calculated from the PCR results as described in the Experimental Procedures. The results represent the averages and standard deviations of two separate experiments with independently prepared templates, with each experiment being performed in triplicate. For each experiment, results were corrected for variations in the 270 bp PCR product of the DHFR gene.

and standard deviations of two separate experiments with independently prepared templates, with each experiment being performed in triplicate and corrected for variations in the 270 bp PCR product of the DHFR gene.

As shown in Figure 3B, in the case of the RARβ CDS (1036 bp), the DHFR CDS (1062 bp), and the DHFR promoter (1000 bp), treatment of cells with 9-*cis* retinoic acid has little effect on cisplatin adduct density. Thus, adduct densities (in adducts per Kb) on these regions in untreated cells (RA−) are observed to be 0.16 ± 0.03, 0.13 ± 0.08, and 0.17 ± 0.04, respectively. Adduct densities on these same regions in 9-*cis*-retinoic-acid-treated cells (RA+) are observed to be 0.13 ± 0.06, 0.2 ± 0.04, and 0.11 ± 0.07, respectively.

In contrast, cisplatin adduct density on the RARβ promoter (1043 bp) is enhanced in cells following treatment of cells with 9-*cis* retinoic acid (Figure 3B). For this region, adduct densities increase from 0.12 ± 0.04 adducts/Kb in untreated cells (RA−) to 0.4 ± 0.16 adducts/Kb in treated cells (RA+). Cisplatin adduct density measured on the active RARβ promoter region in RA+ cells is therefore about 2 times as high as on the DHFR CDS (1062 bp) or DHFR promoter regions in RA+ cells, and nearly 3.5 times as high as on the inactive RARβ promoter in RA− cells. Cisplatin adduct density on the constitutively expressed DHFR promoter does not increase following 9-*cis* retinoic acid treatment (Figure 3B).

To determine the extent to which differences in sequence composition might account for differences in adduct density, we examined the number of GG and AG dinucleotide pairs (the major sites of adduct formation in DNA) in the various DNA regions studied, as summarized in Table 2 (column 2). Clusters of three or four Gs were scored as one site. The number of potential sites for adduct formation was in turn used to estimate the relative frequencies of cisplatin targets per Kb compared to the DHFR CDS (1062 bp) region (Table 2, column 5), given that AG is targeted by cisplatin only about 40% as frequently as GG dinucleotides (*2*). We find that cisplatin adducts form on the RARβ promoter (1043 bp) about two times as frequently as we would anticipate on the basis of sequence composition alone. That is, the estimated frequency of targets is about the same for the RARβ promoter (1043 bp) region and the DHFR CDS (1062 bp) region, yet the observed adduct frequency on the promoter region is about two times what it is on the DHFR CDS (1062 bp) region (0.4 ± 0 0.16 versus 0.2 ± 0.04 adducts/Kb). Similarly, the estimated frequency of targets on the RARβ promoter (1043 bp) is about two times the estimated frequency on RARβ CDS (1036 bp), yet the observed adduct frequency is three times greater on the promoter compared to the CDS (0.4 ± 0 0.16 versus 0.13 ± 0.06 adducts/Kb). This suggests that other factors also contribute to the preferential targeting of cisplatin to the induced RARβ promoter.

Location of Cisplatin Adducts within the Promoter Region. To further localize adduct formation within the promoter, we subdivided the RARβ promoter (1043 bp) region into three overlapping regions A (624 bp), B (483 bp), and C (592 bp) shown schematically in Figure 1, and defined by primers listed in Table 1. As shown in Figure 4, in the absence of promoter activation with 9-*cis* retinoic acid, adduct formation is virtually undetectable in the central region (B) encompassing the TATA box and RARE (retinoic acid response element) and approximately equivalent in regions A and C in adducts per Kb to what we observe on the larger 1043 base region of the promoter (0.17 adducts/

Table 2: Comparison of Expected versus Observed Adduct Density in Various Gene Fragments from Cells Treated with 9-*cis* Retinoic Acid and 1 mM Cisplatin

1	2	3		4	5	6	7
gene fragment	no. of GG and AG sites	cisplatin targets per fragment[a] (a)	(b) sum	targets/Kb; column3(b) ÷ fragment length (Kb)	targets/Kb relative to DHFR; column 4 ÷ 83	expected adducts per Kb based on observed DHFR[b]; column 5 x (0.2)	observed adducts per Kb[c]
DHFR CDS	GG 77	GG 77	88	83	1.0	0.2	0.2 ± 0.04
1062 bases	AG 26	AG 11					
RARβ CDS	GG 25	GG 25	47	45	0.54	0.11	0.13 ± 0.06
(1036 bp)	AG 56	AG 22					
RARβ promoter	GG 72	GG 72	92	88	1.1	0.21	0.40 ± 0.16
(1043 bp)	AG 50	AG 20					
DHFR promoter	GG 84	GG 84	106	106	1.3	0.26	0.11 ± 0.07
(1000 bp)	AG 55	AG 22					

[a] AG adducts are about 40% as likely to form as GG adducts (*16*); therefore, AG targets (column 3a) = AG sites (column 2) × 0.4. [b] From column 7. [c] Calculated from PCR data in Table 2 using Poisson equation relating adducts per fragment to PCR signal, and correcting for fragment size.

Kb on each of regions A, C versus 0.12 adducts/Kb on the larger fragment overall). This suggests that some portion of the central region of the promoter included in fragment B is protected from platination in the uninduced state.

Following promoter activation, all three regions A, B, and C sustain elevated levels of damage, (0.34 ± 0.07, 0.43 ± 0.07, and 0.27 ± 0.11 adducts/Kb, respectively), which is on the average what we observe on the larger 1043 base region of the activated promoter (0.4 ± 0.16 adducts/Kb). This confirms the measurement of adduct density on the larger 1043 base region (Figure 3 and Table 2) and supports the conclusion drawn from the comparison of sequence composition that the activated RARβ promoter sustains levels of cisplatin damage greater than would be expected on the basis of base composition alone. Because regions A, B, and C do not differ significantly between themselves in GG and AG sequence compositions, the result here suggests that cisplatin adducts are distributed evenly over the activated promoter and that protection from platination in region B is abolished by promoter activation.

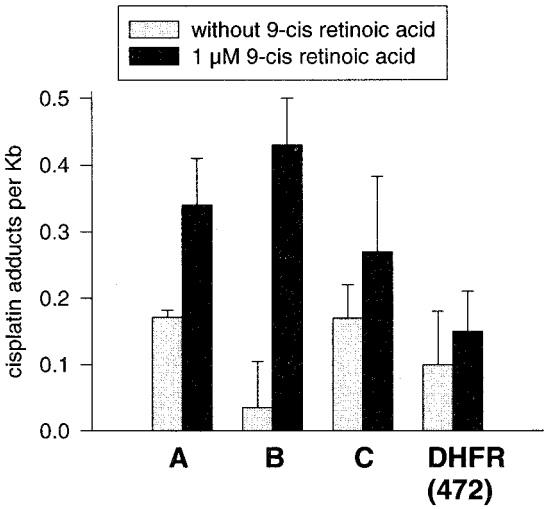

FIGURE 4: Cisplatin adduct formation on three overlapping regions A (624 bp), B (483 bp), and C (592 bp) of the RARβ promoter 1043 bp region shown schematically in Figure 1. Primers used are listed in Table 1. Cells were treated as in Figure 3. Results represent the average of triplicate assays.

DISCUSSION

In these studies, we have used a PCR-based assay to detect platination on genomic regions encompassing about 1 Kb of the RARβ promoter, the RARβ coding sequence (CDS), the DHFR promoter, and the DHFR CDS in T47D breast cancer cells. We see that treatment of these cells with 9-*cis* retinoic acid, a treatment that activates the RARβ promoter but not the DHFR promoter, results in a 3-fold increase in accessibility of the RARβ promoter region to cisplatin (0.4 ± 0.16 versus 0.12 ± 0.04 adducts/Kb). In contrast, the accessibilites to cisplatin of the DHFR promoter, DHFR CDS, and RARβ CDS are not significantly altered by treatment of cells with 9-*cis* retinoic acid, and observed adduct densities remain about 2−4-fold lower than on the activated RARβ promoter (0.11 ± 0.07, 0.2 ± 0.04, and 0.13 ± 0.06 adducts/Kb, respectively). The activated RARβ promoter appears therefore to be hypersensitive to cisplatin adduct formation. Preferential platination of the RARβ promoter relative to the other regions examined could be attributed only in part to differences in sequence composition, suggesting that structural factors also play a role in directing adduct formation to certain sites.

The possibility that promoter-specific structural changes influence reactivity with cisplatin is further supported by the analysis of subregions of the larger RARβ promoter fragment, where we see that accessibility to cisplatin increases over the entire promoter region following activation of the promoter. Furthermore, the accessibility to cisplatin of the central region of the promoter, including the TATA box and RARE appears to be suppressed in the inactive state, and this suppression is relieved upon promoter activation. In this case, inhibition of accessibility to cisplatin might be due to steric hindrance due to the possible positioning of a nucleosome at or near the start site of transcription. A recent analysis of a large set of unrelated RNA polymerase II promoters, both TATA-containing and TATA-less, has revealed a common bendability profile of DNA just downstream of the start site of transcription, suggesting that the DNA could wrap around protein in a nucleosome (*26*). In addition, the complementary triplet pair, CAG/CTG, shown to correlate with nucleosome positioning (*27*, *28*) is over-represented in the region just downstream of the transcription start site (*26*). Complexation with nucleosomes near the transcription start site of the inactive promoter might

therefore impede adduct formation by cisplatin, and displacement or destabilization of nucleosomes by the transcription initiation complex might remove this impediment.

Following transcriptional activation, the RARβ promoter not only becomes more accessible to cisplatin than it was prior to transcriptional activation but also appears to bind more cisplatin than would be expected based on sequence composition alone. This suggests that some structural feature of the DNA of the activated promoter might make it an energetically favorable site for cisplatin adduct formation. One such feature might be an induced bend in the promoter DNA generated as a result of protein−protein interactions between transcription factors bound to distant sites (see ref *29* for review). Such a bend might provide the stored energy required for strand separation and initiation of transcription, and this process could be blocked by the formation of a cisplatin adduct.

The retinoids *all-trans*-retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis* RA), as well as several synthetic analogues of these natural retinoids, have attracted considerable attention as potential chemotherapeutic agents for the treatment of promyelocytic leukemia (*30*) and other cancers (*31*−*33*). As ligands for nuclear retinoic acid receptors (RARα, β, or γ) and retinoid X receptors (RXRα, β, or γ), retinoids regulate the expression of sets of overlapping genes involved in the regulation of cell proliferation, differentiation, and apoptosis (*34*−*36*). *all-trans*-Retinoic acid, binding to RAR α, β, or γ, and 9-*cis* retinoic acid, binding to either RAR α, β, or γ or RXR α, β, or γ, promote the heterodimerization of these receptors and facilitate the binding of the heterodimeric complex to specific response elements (RAREs) in the promoter regions of retinoic-acid-responsive genes. This binding is believed to facilitate the recruitment of the components of the basal transcription complex to the promoter and promote chromatin remodeling required for the onset of transcription. While these events may by themselves lead to partial suppression of some solid tumors, in most examples studied, retinoids showed more potential when used in combination with DNA-damaging chemotherapies such as cisplatin, etoposide, and 5-fluorouracil (*37*−*39*). A synergistic activity between retinoids and cisplatin has been observed only when retinoid treatment preceded cisplatin treatment, and this correlated with a 1.5-fold increase in cisplatin adduct content of DNA without a change in cisplatin uptake (*39*). These data suggest that retinoid treatment induces an event or events that directly enhance cisplatin cytotoxicity and are consistent with the possibility suggested by our data that retinoids may generate cisplatin-hypersensitive structures that contribute to an enhanced response to cisplatin.

These data implicate chromatin organization as a component influencing the toxicity of cisplatin and add further support to a growing body of evidence pointing to the transcription process as a target for cisplatin mediated cytotoxicity. Cisplatin adduct formation may disrupt transcription through several mechanisms, including the following. (a) Cisplatin adducts may generate inappropriate binding sites for transcription factors and chromosomal proteins with HMG domains, titrating them away from their natural sites (*18*). A variety of HMG-box chromosomal proteins involved in the transcription complex bind to cisplatin adducts (*14*−*18*). (b) Cisplatin may inhibit transcription factor binding to certain promoters, as has been shown for a stably integrated mouse mammary tumor virus promoter-driven reporter gene (*40*). In this case, cisplatin treatment prior to hormonal induction prevented activation of the reporter gene and blocked recruitment of the transcription complex to the promoter. (c) By targeting GG dinucleotides, cisplatin may inhibit certain regulatory elements rich in strings of guanosines, leading to selective inhibition of such promoters (*9*, *10*). (d) As suggested by our results, cisplatin may target a DNA structure generated in the active promoters of certain genes, such as the RARβ gene. Assembled on activated promoters may be some of the factors needed to trigger apoptosis, including the transcription factor p53. Of interest is the recent identification of HMG-1, which is known to bind to cisplatin adducts, as a specific activator of p53 (*41*).

The differential toxicities of various platinum drugs may therefore be related to their respective abilities to mimic or disrupt chromatin structures critical to transcription. Further studies will be required to determine to what extent the observations made in this study extend to other promoters and to other DNA damaging agents.

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