DNA Adducts of *cis*-Diamminedichloroplatinum(II) and Its Trans Isomer Inhibit RNA Polymerase II Differentially *in Vivo*[†]

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Received June 16, 1995; Revised Manuscript Received September 5, 1995[⊗]

ABSTRACT: The effects of *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and *trans*-DDP adducts on mammalian transcription *in vivo* have been investigated. A plasmid containing the β-galactosidase (β-gal) reporter gene was modified with either of the two platinum compounds and transfected into human or hamster cell lines. A 2−3-fold higher level of transcription was observed in both cell lines from plasmids containing *trans*-DDP adducts as compared to plasmids modified by *cis*-DDP. This difference in transcriptional activity was not decreased in human and rodent nucleotide excision repair deficient cell lines, indicating that more efficient excision repair of the *trans*-DDP adducts was not the cause of its lower ability to block transcription in this assay. For this conclusion to be valid, it is assumed that *trans*-DDP adducts are repaired primarily by the nucleotide excision repair pathway, as is the case with the adducts of *cis*-DDP. The possibility that *trans*-DDP adducts are preferentially bypassed by RNA polymerase was examined by monitoring the elongation of β-gal mRNA on damaged templates *in vivo*. Nascent β-gal mRNA transcripts were recovered from excision repair deficient xeroderma pigmentosum A cells transfected with platinated plasmids, and the extent of RNA synthesis was measured by using ribonuclease protection. Fourfold more *trans*-DDP than *cis*-DDP adducts were required to inhibit transcription elongation by 63%. RNA polymerase II bypassed *cis*- and *trans*-DDP DNA adducts with efficiencies of 0−16% and 60−76%, respectively. These data provide insight into the differential toxicity of the two platinum isomers.

cis-Diamminedichloroplatinum(II) (*cis*-DDP, cisplatin)[1] is the principal chemotherapeutic agent in regimens directed against testicular cancer (Loehrer & Einhorn, 1984), affording remission rates in excess of 90% (Feuer et al., 1993). *cis*-DDP forms DNA lesions that are believed to be essential for the cytotoxic activity of the drug (Bruhn et al., 1990). The adducts of *cis*-DDP include intrastrand 1,2-d(GpG), 1,2-d(ApG), 1,3-d(GpNpG), and interstrand cross-links (Eastman, 1983; Fichtinger-Schepman et al., 1985). The trans isomer of *cis*-DDP, *trans*-diamminedichloroplatinum(II) (*trans*-DDP), is 20-fold less cytotoxic than *cis*-DDP (Pascoe & Roberts, 1974) and is ineffective against tumors. *trans*-DDP forms similar adducts to those of *cis*-DDP with the exception that it cannot form 1,2-intrastrand cross-links (Pinto & Lippard, 1985), which comprise greater than 90% of all adducts formed by *cis*-DDP.

Although the precise biochemical mechanisms by which *cis*-DDP selectively kills cancer cells are not fully known, the DNA adducts of this platinum compound block both replication and transcription. *cis*-DDP adducts inhibit DNA synthesis efficiently *in vitro* (Pinto & Lippard, 1985) and in bacterial and mammalian cells (Alazard et al., 1982; Salles et al., 1983; Ciccarelli et al., 1985). When equal numbers of adducts are present on DNA, however, the cis and trans isomers inhibit DNA synthesis equally, as has been shown in mammalian cells (Salles et al., 1983; Ciccarelli et al., 1985; Uchida et al., 1986) and in *in vitro* assays using both prokaryotic and eukaryotic DNA polymerases (Harder et al., 1976; Bernges & Holler, 1988; Heiger-Bernays et al., 1990). The similar levels of replication inhibition observed for *cis*- and *trans*-DDP contrast with their different toxicities, suggesting that additional mechanisms are operative *in vivo*.

Several non-mutually-exclusive models have been proposed to account for the differential toxicity of *cis*- and *trans*-DDP to cultured cells. One explanation is that *trans*-DDP, upon entering the cell, forms fewer adducts than *cis*-DDP, owing to inactivation by sulfur-containing molecules, such as glutathione (Eastman & Barry, 1987). A second hypothesis suggests that DNA adducts of *trans*-DDP, once formed, are preferentially repaired over those of *cis*-DDP (Ciccarelli et al., 1985; Hansson & Wood, 1989; Heiger-Bernays et al., 1990; see, however, Roberts & Friedlos, 1987). Another model to explain the greater toxicity of *cis*-DDP *in vivo* is that its adducts may selectively inhibit transcription. Rel-

[†] This investigation was supported by the National Institutes of Health (Grants CA52127 to J.M.E. and CA34992 to S.J.L.).

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[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1995.

[1] Abbreviations: bp, base pair(s); β-gal, β-galactosidase; βG3, antisense RNA probe complementary to sequence located at the 3′ end of β-gal mRNA; βG5, antisense RNA probe complementary to sequence located 1604 bp upstream of the 3′ end of β-gal mRNA; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; CMV, cytomegalovirus; *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); D_0, platinum adducts per genome that reduce the percentage of β-gal enzyme activity or mRNA by 63% along the exponential portion of the dose−response curve in transfected cell lines; MEM, minimal essential medium; r_b, measured platinum-to-nucleotide ratio; SV40, simian virus 40; XPA, xeroderma pigmentosum complementation group A. The DNA adducts formed by *cis*-DDP and *trans*-DDP are indicated as 1,2-d(GpG) for *cis*-[Pt(NH$_3$)$_2$\{d(GpG)-*N*7(1),*N*7(2)\}]; 1,2-d(ApG) for *cis*-[Pt(NH$_3$)$_2$\{d(ApG)-*N*7(1),*N*7(2)\}]; and 1,3-d(GpNpG) for *cis*- or *trans*-[Pt(NH$_3$)$_2$\{d(GpNpG)-*N*7(1),*N*7(3)\}] (where N = any nucleotide).

0006-2960/95/0434-14783$09.00/0

evant to this model is evidence suggesting that *cis*-DDP causes L1210 cells to arrest in the G2 phase of the cell cycle and subsequently induces cell death by apoptosis (Sorenson & Eastman, 1988a; Sorenson et al., 1990). It is proposed that platinum−DNA adducts may trigger the apoptotic pathway by inhibiting either overall gene expression or that of a gene required for mitosis (Sorenson & Eastman, 1988b).

cis-DDP treatment of cells in culture influences gene expression both positively and negatively and to varying extents, depending on the promoter (Evans & Gralla, 1992a,b). These differential effects are not observed for *trans*-DDP. Direct evidence that *cis*-DDP can inhibit transcription initiation comes from a recent study in which *cis*-DDP treatment of cells inhibited binding of a transcription factor, NF1, to the mouse mammary tumor virus promoter present on a transiently introduced template (Mymryk et al., 1995). In these same studies, *cis*-DDP reduced the changes in nucleosomal organization required for transcription factor access, but *trans*-DDP did not. The relative number of platinum DNA adducts formed in cells after treatment with each platinum isomer was not determined. To date, the most definitive comparison of the effects of *cis*- and *trans*-DDP adducts on transcription has focused on the blocking of RNA polymerase processivity. Duplex DNAs containing platinum adducts were used as substrates for transcription by either *Escherichia coli* or wheat germ RNA polymerases. The intrastrand 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpTpG) adducts of *cis*-DDP as well as the infrequent interstrand cross-link formed by either compound irreversibly block elongation of nascent RNA molecules by both polymerases. In contrast, the intrastrand 1,3-d(GpTpG) adduct of the trans isomer could be bypassed by RNA polymerases, allowing elongation of the nascent RNA (Corda et al., 1991, 1993; Brabec & Leng, 1993). These observations *in vitro* support the possibility that inhibition of transcription may contribute to the selective toxicity of the cis isomer *in vivo*.

The focus of the present work was to compare directly the effects of *cis*- and *trans*-DDP DNA adducts on RNA synthesis in mammalian cells. A nonreplicating transient expression vector harboring a reporter gene, modified *in vitro* to defined levels with either platinum compound, was used to monitor inhibition of RNA synthesis *in vivo*. The use of both repair proficient and deficient cell lines allowed us to examine excision repair and transcriptional bypass independently for each platinum compound. By using this approach, we have determined the efficiency with which RNA polymerase II bypasses *cis*- and *trans*-DDP DNA adducts *in vivo*.

MATERIALS AND METHODS

Cells and Culture Conditions. HeLa cells (P. Sharp, MIT) were maintained in suspension culture in Joklik-modified minimal essential medium (MEM) (Gibco), supplemented with 5% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The Chinese hamster ovary (CHO) cells designated AA8 and UV20, obtained from the American Type Culture Collection, were maintained in suspension culture in α-MEM (Gibco) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. UV20 was derived by mutation of the AA8 cells (Thompson et al., 1981) and belongs to complementation group 1 (formerly group 2) (Collins, 1993). Human lymphoblastoid cell lines transformed with Epstein-Barr virus were obtained from the NIGMS Human Genetic Mutant Cell Repository. Cell line GM02250D is from a donor homozygous for xeroderma pigmentosum belonging to complementation group A (XPA), and GM05567 is from the proband's apparently unaffected, but possibly heterozygous, brother. Cells were maintained in suspension culture in RPMI 1640 medium (Gibco) supplemented with 15% fetal bovine serum, penicillin, and streptomycin.

Plasmids. All plasmids used were incompetent for replication but allowed for the transient expression of a prokaryotic reporter gene in mammalian cells. CMV-β-gal (7330 bp; provided by R. Tepper, Harvard Medical School) contains the prokaryotic β-galactosidase (β-gal) gene inserted between the eukaryotic cytomegalovirus (CMV) promoter and simian virus 40 (SV40) splice and polyadenylation sequence. PSV2-CAT (5003 bp) harbors the bacterial chloramphenicol acetyltransferase (CAT) gene flanked by the SV40 early promoter and splice and polyadenylation sequence (Gorman et al., 1982). pcDNA3-CAT (6230 bp; Invitrogen) contains the CAT gene between the CMV promoter and the bovine growth hormone polyadenylation sequence. All plasmids were isolated from *E. coli* DH5α cultures by using the maxi- or mega-plasmid purification kits of Qiagen, Inc.

Platination reactions were carried out in 3 mM NaCl and 1 mM Na_2HPO_4, pH 7.4, with 200 μg/mL DNA and appropriate platinum compound/DNA molar ratios by incubating at 37 °C for 16 h. Unreacted platinum compound was removed by dialysis (14 h) against 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. To serve as an unmodified control, plasmid DNA was subjected to the same treatment in the absence of platinum compound. Levels of platinum modification were determined by flameless atomic absorption spectroscopy on a Varian AA1475 instrument equipped with a GTA95 graphite furnace.

Transfections. Platinated DNA was transfected into cells by electroporation with a BTX Electro Cell Manipulator 600. Electroporation conditions for each cell line were optimized by independently varying the voltage and capacitance settings; conditions were chosen that yielded the greatest levels of β-gal expression per microgram of protein of cell extracts made from the transfected cells. Using optimized electroporation conditions, duplicate samples of DNA at each platinum level were transfected into cells. Specifically, HeLa cells in logarithmic growth [$(2-4) \times 10^5$ cells/mL] were concentrated by centrifugation and resuspended at 1.4×10^7 cells/mL in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Plasmid DNA (20 μg) and 5×10^6 cells (350 μL) were mixed and transferred to a sterile 0.2 cm electroporation cuvette, and a single electrical pulse was immediately delivered to the cells at 245 V, 1150 μF, and 13 Ω. Similarly, XPA and normal human lymphoblasts [$(5-8) \times 10^5$ cells/mL] were transfected by mixing 40 μg of plasmid DNA with 2×10^7 cells (350 μL) and electroporating at 125 V, 2700 μF, and 72 Ω in RPMI supplemented with 15% fetal bovine serum, penicillin, and streptomycin. CHO cells [$(1-2) \times 10^5$ cells/mL] were transfected by mixing 5×10^6 cells (350 μL) in α-MEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin with 40 μg of DNA and electroporating at 165 V, 1150 μF, and 13 Ω. Following 10 min at room temperature, transfected cells were transferred to 10 mL of prewarmed complete medium and

incubated in a 10 cm culture dish at 37 °C for 15, 24, or 48 h (see Results). For RNase protection assays, XPA lymphoblasts were cotransfected with 32 μg of modified CMV-β-gal and 8 μg of pcDNA3-CAT, and cells were harvested 4 h after transfection.

Transient Expression Assays. Cells were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in 100 μL of 0.25 M Tris-HCl, pH 7.8. Cells were lysed by three cycles of freeze−thaw, and the lysate was centrifuged (14000*g*) for 15 min at 4 °C. The supernatant from cells transfected with CMV-β-gal was assayed for β-gal activity by the method of Eustice et al. (1991), with slight modifications. Up to 40 μL of cell lysate was assayed in a 200 μL solution containing 9 mM $MgCl_2$, 102 mM β-mercaptoethanol, 80 mM Na_2HPO_4, pH 7.3, and 9 mM chlorophenol red β-D-galactopyranoside (Boehringer-Mannheim). Assay mixtures were incubated for a minimum of 30 min at 37 °C and stopped by the addition of 300 μL of 0.5 M EDTA, and the absorbance at 570 nm was measured with a Beckman DU-65 spectrophotometer. Cell extracts prepared from mock-transfected cells served as negative controls. The protein concentration of cell extracts was determined by the Bio-Rad microassay with bovine serum albumin as the protein standard (Bradford, 1976). β-Gal activity for each sample was normalized to the amount of protein present and is expressed as a percent of the activity determined for an unmodified control. Percent β-gal activity for duplicate samples at each platinum level was graphed as a function of platinum adducts/genome. Curves exponentially fitted to the data were used to determine the platinum level effecting a 63% reduction in β-gal activity (D_0). In experiments where HeLa cells were transfected with PSV2-CAT, the amount of CAT protein present in cell extracts was measured by using an enzyme immunoassay for CAT (CAT-ELISA, Boehringer Mannheim) according to the protocol provided by the supplier.

RNA Isolation. Total RNA was isolated by a modification of the method of Chomczynski and Sacchi (1987). Cells were lysed with 0.5 mL of RNAzol B (Biotecx Laboratories), 50 μL of chloroform was added, and the lysate was centrifuged (14000*g*) for 15 min. RNA was purified from the aqueous phase with 0.5 volume of isopropyl alcohol and 0.05 volume of RNA Tack Resin (Biotecx). After two washes with 75% ethanol (v/v), RNA was eluted from the resin with 100 μL of H_2O. To remove possible contaminating plasmid DNA, all RNA samples were treated with RNase-free DNase (Boehringer Mannheim) for 20 min at room temperature.

Probes. A 215 bp DNA sequence that begins 233 bp and terminates 18 bp 5′ to the stop codon of the β-gal gene was PCR amplified and then inserted downstream of, and in opposite orientation to, the T7 promoter of pGEM-3zf(−) (Promega). This pGEM-3zf-β-gal3 construct was linearized with *Pst*I and served as template for the synthesis of a ^{32}P-labeled antisense RNA probe complementary to the 3′ end of the β-gal mRNA (βG3; 215 nucleotide protected fragment). Linearized transcription vector pTri-β-gal (Ambion) served as the template for a second β-gal antisense RNA probe complementary to an internal sequence extending from base 934 to 1235 of the 3072 bp of the β-gal gene (βG5; 301 nucleotide protected fragment). Antisense RNA probes for CAT (152 nucleotide protected fragment) and for human β-actin (127 nucleotide protected fragment) were synthesized from the linearized vectors pTri-CAT and pTri-β-actin-125-human (Ambion), respectively. All antisense radiolabeled RNA probes were transcribed *in vitro* by using Ambion MAXIscript. T7 RNA polymerase (10 units) was incubated with pGEM-3zf-β-gal3 (1 μg), 500 μM ATP, GTP, and TTP, and 5 μM [α-^{32}P]UTP (800 Ci/mmol, 50 μCi) (Amersham) in a 20 μL reaction at 37 °C for 1 h. This reaction produced a 246 nucleotide transcription product containing the 215 nucleotide sequence complementary to the β-gal gene. Template DNA was removed by adding RNase-free DNase (2 units) and incubating at 37 °C for an additional 15 min. pTri-CAT and pTri-β-gal were similarly transcribed with T7 and SP6 RNA polymerases, yielding 241 and 344 nucleotide transcription products, respectively. pTri-β-actin-125-human was transcribed by using SP6 RNA polymerase, yielding a transcription product of 218 nucleotides, except that the reaction was carried out in the presence of an additional 150 μM unlabeled UTP in order to produce a lower specific activity probe. All probes were immediately purified by electrophoresis on an 8 M urea−5% polyacrylamide gel and were stored at −20 °C.

Ribonuclease Protection Assay. Assays were carried out by using the RPA II kit (Ambion) according to the protocol of the supplier. Briefly, total RNA (20 μg) was hybridized simultaneously with the CAT (2×10^5 cpm), β-actin (1×10^5 cpm), and either βG5 or βG3 RNA probes (3.5×10^5 cpm) at 45 °C for 14 h. Hybridization mixtures were then digested with 1000 units/mL RNase T1 by incubating at 37 °C for 30 min. Following the simultaneous inactivation of the RNase T1 and precipitation of the protected radiolabeled RNA probes, the protected fragments were separated on an 8 M urea−5% polyacrylamide gel. Quantitative analysis was performed by using a PhosphorImager (Molecular Dynamics).

RESULTS

Host Cell Reactivation of cis- and trans-DDP-Damaged Plasmids in HeLa Cells. The relative effects of *cis*- and *trans*-DDP DNA adducts on transcription were examined by using the host cell reactivation assay. HeLa cells were transfected with *cis*- or *trans*-DDP damaged CMV-β-gal plasmid DNA, and after 24 h, β-gal activities and total protein content were determined. Relative to the β-gal activity from unmodified plasmid, which was defined as 100%, β-gal expression decreased exponentially with increasing platinum damage (Figure 1). A reference point used for comparison of the dose−response curves is D_0, or the level of DNA damage sufficient to reduce the β-gal activity to 37% of its original value along the exponential portion of the curve (Protic-Sabljic & Kraemer, 1985). Twofold more *trans*-DDP damage ($D_0 = 40$ adducts/genome; $r_b = 0.0027$) than *cis*-DDP damage ($D_0 = 21$ adducts/genome; $r_b = 0.0014$) was required to produce an equivalent reduction in β-gal activity.

Three testable models could explain the differential reactivation of plasmids modified by *cis*- and *trans*-DDP: (1) translesion synthesis by RNA polymerase may be more efficient on DNA modified by *trans*-DDP than *cis*-DDP; (2) *trans*-DDP adducts may be more rapidly repaired than *cis*-DDP adducts; and (3) there may be preferential formation of *cis*-DDP adducts compared with *trans*-DDP adducts within the β-gal gene.

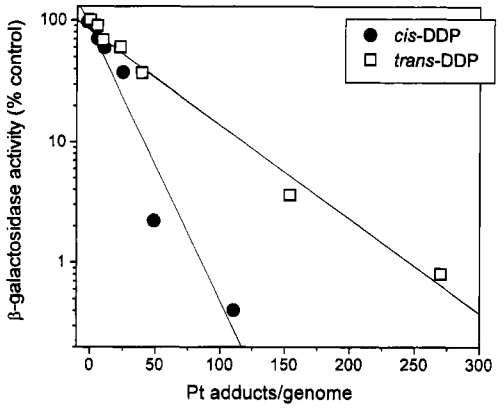

FIGURE 1: Dose-dependent decrease in β-gal gene expression in HeLa cells transfected with *cis*-DDP or *trans*-DDP damaged CMV-β-gal DNA. β-Gal activity in cell lysates was normalized for protein content and is expressed as a percent of the β-gal activity present in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one representative experiment. The relative standard deviation between experiments for the determination of percent β-gal activity was typically $\pm$10%.

In order to determine whether the differential reactivation effects observed in Figure 1 were a consequence of different distributions of *cis*- and *trans*-DDP DNA adducts, the reactivation assay was carried out in HeLa cells using a second plasmid, PSV2-CAT. PSV2-CAT differs from CMV-β-gal in that it contains different potential gene-inactivating target (i.e., promoter, coding, and splice/poly-adenylation sequences) as well as nontarget sequences. With PSV2-CAT, the D_0 value for *trans*-DDP was 38 adducts/genome (r_b = 0.0038), as compared to 19 adducts/genome for *cis*-DDP (r_b = 0.0019). This 2-fold difference in D_0 values is identical to that observed in the CMV-β-gal system. These observations argue against the possibility that the different levels of gene expression from *cis*- and *trans*-DDP modified DNA are a consequence of different platinum levels within the transcribed reporter gene.

Impact of Nucleotide Excision Repair Status on Reactivation of Platinated DNA. In order to assess the possible contribution of excision repair to the observed greater reactivation of *trans*-DDP modified DNA, the host cell reactivation assay was carried out in excision repair deficient XPA and apparently normal, consanguineous human lymphoblasts. Measurement of β-gal activity 24 h (~one cell doubling time) after transfection of both cell lines with *cis*- or *trans*-DDP damaged plasmid demonstrated the expected reduction in activity with increasing platinum damage (Figure 2). XPA cells exhibited a steeper dose response than did repair proficient cells. The 4-fold differential in D_0 values found for XPA and normal cells (Table 1) is comparable to differences previously observed for *cis*-DDP (Poll et al., 1984; Chu & Berg, 1987) and for UV damage (Lehmann & Oomen, 1985; Protic-Sabljic & Kraemer, 1985; Athas et al., 1991). A comparison of the two platinum compounds in normal lymphoblasts revealed a 1.6-fold greater D_0 value for *trans*- compared to *cis*-DDP, consistent with our results obtained in HeLa cells. Experiments in XPA cells, in which platinum adducts cannot be removed by excision repair, found an even larger differential of 2.7-fold between *cis*- and *trans*-DDP. If excision repair were contributing to the differential reactivation of *cis*- and *trans*-DDP damaged DNA observed in normal cells, one would have expected to find similar D_0 values for *cis*- and *trans*-DDP in excision repair deficient cells.

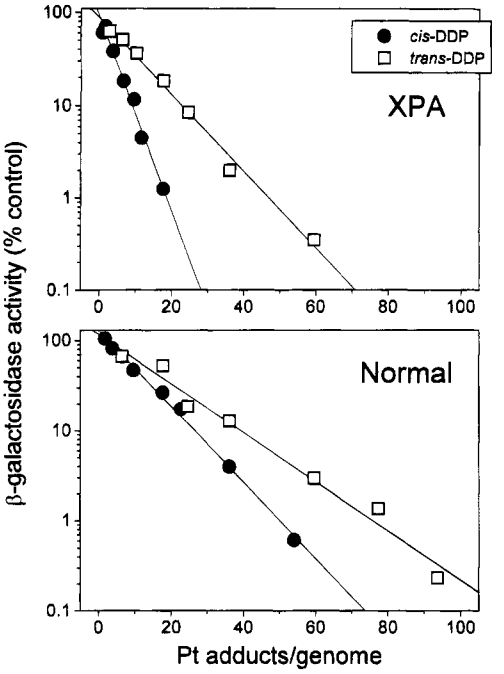

FIGURE 2: Dose-dependent decrease in β-gal gene expression in repair deficient XPA (top panel) and apparently normal (bottom panel) lymphoblastoid cell lines transfected with *cis*-DDP- or *trans*-DDP damaged CMV-β-gal DNA. β-Gal activity in cell lysates was normalized for protein content and is expressed as a percent of the β-gal activity present in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one representative experiment. The relative standard deviation between experiments for the determination of percent β-gal activity was typically $\pm$10%.

Table 1: Transcriptional Inactivation (D_0) by *cis*- and *trans*-Diamminedichloroplatinum(II) Adducts in CMV-β-Gal Genomes: Comparison of Repair Deficient and Repair Proficient Cell Lines[a]

	D_0 for the platinum derivative:			
	cis-DDP		*trans*-DDP	
DNA repair status:	XPA	normal	XPA	normal
24 h	3.6[b]	12.4	9.7	21.0
DNA repair status:	UV20	AA8	UV20	AA8
15 h	3.5[c]	3.6[c]	12.4[d]	13.5[d]
48 h	3.2[e]	11.0[c]	13.7[e]	35.0[c]

[a] D_0 values are the level of platinum compound in adducts per genome required to reduce expression of the β-galactosidase gene of CMV-β-gal to 37% of that of an unmodified control. D_0 values were determined as described in Materials and Methods. D_0 values can be converted to r_b by dividing by 14 660, the total number of nucleotides in the genome. Human lymphoblastoid lines were compared from an XPA patient and her apparently unaffected brother at 24 h after transfection. CHO repair deficient UV20 cells were compared with parental repair proficient AA8 cells at both 15 and 48 h after transfection. [b] Values represent the mean of D_0 values obtained from three separate experiments that together utilized two batches of independently platinated DNAs. The relative standard deviations for percent β-galactosidase activity between experiments were typically $\pm$10%. Typical data of one experiment are shown in Figure 2. [c] Values represent the D_0 from one experiment. [d] Values represent the mean of D_0 values obtained from two experiments. The relative standard deviations for percent β-galactosidase activity between experiments were typically $\pm$10%. Typical data of one experiment are shown in Figure 3. [e] Same as in footnote *d*, except that the values represent the mean of D_0 values obtained from three experiments.

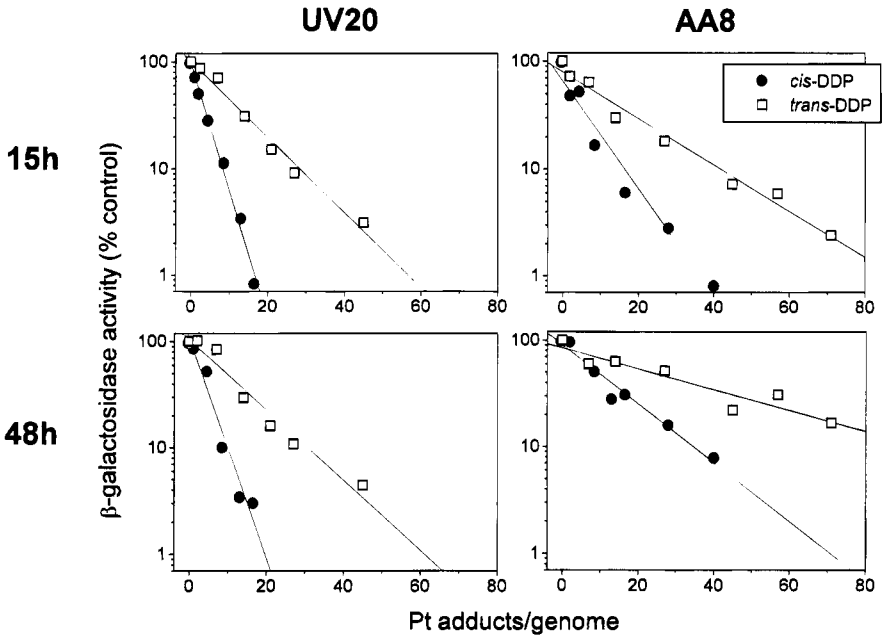

FIGURE 3: Dose-dependent decrease in β-gal gene expression in repair deficient UV20 (left panels) and repair proficient (right panels) CHO cell lines that had been transfected either 15 h (top panels) or 48 h (bottom panels) earlier with *cis*-DDP- or *trans*-DDP damaged CMV-β-gal DNA. β-Gal activity in cell lysates was normalized for protein content and is expressed as a percent of the β-gal activity present in lysates from cells transfected with unmodified plasmid. Each point represents the mean of values obtained from duplicate transfections of one representative experiment. The relative standard deviation between experiments for the determination of percent β-gal activity was typically $\pm 10\%$.

Additional experiments were performed to assess whether the observed differential effects of *cis*- and *trans*-DDP on transcription might be species or cell line specific. CHO repair proficient AA8 cells and excision repair deficient UV20 cells were transfected in parallel with *cis*- or *trans*-DDP damaged CMV-β-gal DNA, and β-gal activity was measured 15 h (~1 cell doubling time) after transfection. As shown in Figure 3, the transcriptional activity from platinated DNAs decreased exponentially with increasing dose. An ~3.5-fold enhanced reactivation of *trans*-DDP damaged plasmid was found in either cell line (Table 1). Interestingly, D_0 values obtained in AA8 cells were virtually identical to those found in UV20 cells (Table 1), suggesting that little or no repair of either platinum isomer, within the detection limits of this assay, had yet occurred in the repair proficient AA8 cells. β-Gal activity was next measured in either cell line 48 h after transfection. In UV20 cells, D_0 values found for *cis*- and *trans*-DDP at 48 h were identical to those found at 15 h. This result illustrates the repair deficient phenotype of UV20 cells and supports the view that excision repair is the primary mechanism of removal of platinum DNA adducts, although the action of other repair pathways on the platinum adducts before 15 h cannot be excluded. In contrast, in repair proficient AA8 cells, D_0 values for *cis*- and *trans*-DDP increased significantly and proportionately from 15 to 48 h, indicating excision repair of the adducts of both isomers. A comparison at 48 h of the two isomers in repair proficient cells revealed a 3.2-fold larger D_0 for *trans*-DDP than for *cis*-DDP; in the absence of repair, this difference was 4.3-fold. This trend in the changes of D_0 values obtained in CHO cells at 48 h paralleled closely that observed in human cells. Taken together, our results in CHO and human cells indicate that excision repair of *trans*-DDP lesions is not contributing to the greater reactivation of *trans*-DDP damaged DNA in these studies.

Effects of Platinum DNA Adducts on Transcription. Experiments were carried out to examine whether platinum DNA adducts were inhibiting β-gal gene expression by blocking RNA polymerase processivity *in vivo*. Blocking of mRNA synthesis within a defined region of the β-gal gene was measured directly by ribonuclease protection analysis. Repair deficient XPA cells were cotransfected with *cis*- or *trans*-DDP modified CMV-β-gal and a second unmodified plasmid, pcDNA3-CAT, to control for transfection efficiency. Total RNA was isolated 4 h after transfection in an effort to ensure that the effects of transcription, and not leaky excision repair, were being monitored. Northern analysis verified that total RNA samples were free of contaminating plasmid DNA (data not shown). Total RNA was hybridized simultaneously with antisense RNA probes for CAT, constitutively expressed β-actin, and one of two sequences within β-gal mRNA. One β-gal probe, βG3, complementary to a sequence located at the immediate 3′ end of β-gal mRNA (Figure 4A), was used to measure only full-length mRNA transcript. A second probe, βG5, was complementary to a sequence located 1604 bp upstream from the βG3 sequence (Figure 4A). βG5 detected full-length transcripts as well as those prematurely terminated due to platinum adducts within the 1604 bp sequence between βG3 and βG5. RNAs from the same sample but hybridized with either βG3 or βG5 were run in adjacent lanes. A representative autoradiograph is shown in Figure 4B.

Hybridizations with βG3 showed that amounts of full-length transcript decreased exponentially with increasing platinum modification (Figure 4B, B lanes). Curves generated from these data exhibit the same 3-fold differential in D_0 values for *cis*- and *trans*-DDP as was found in our previous experiments in XPA cells (curves not shown). Hybridizations with βG5 (Figure 4B, A lanes) were used to calculate the fraction of total β-gal mRNA (βG5) that was

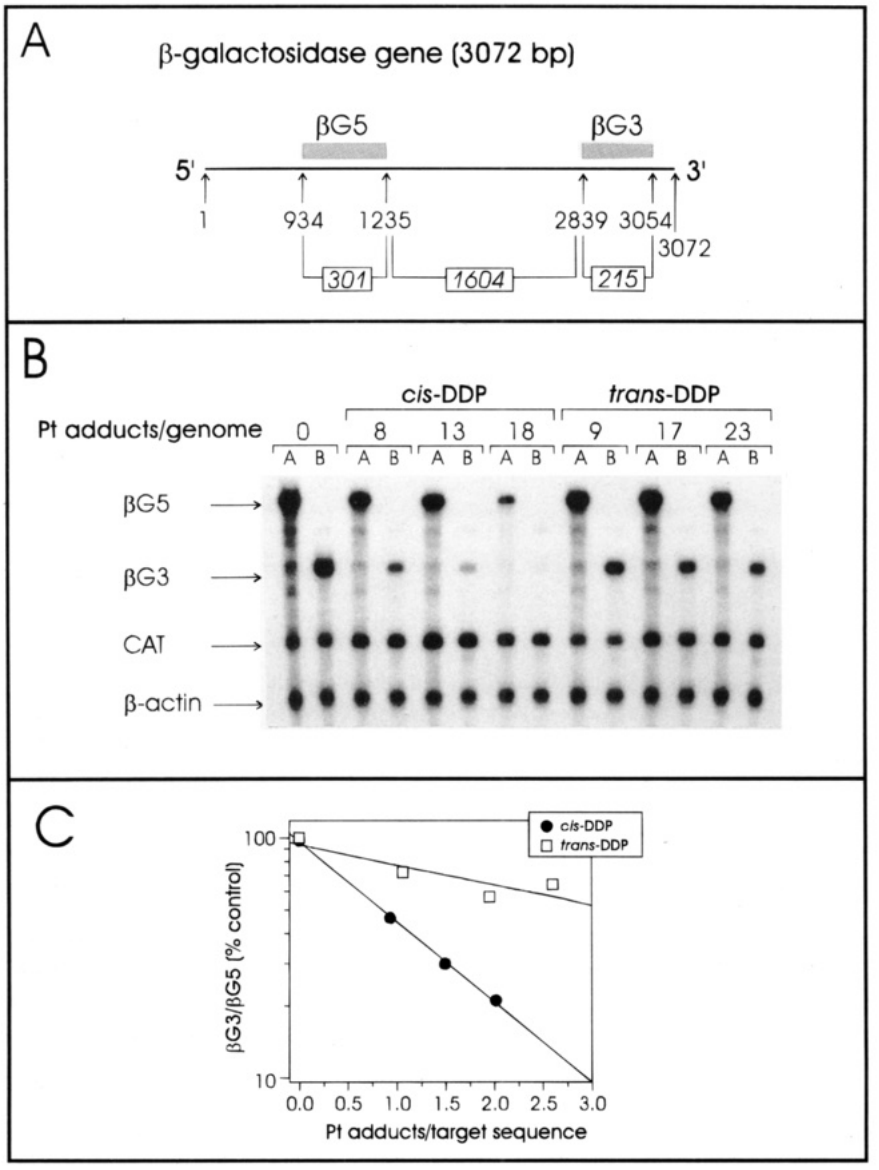

FIGURE 4: Blocking of RNA polymerase by platinum adducts was examined directly by ribonuclease protection. (A) Location of two antisense RNA probes that were used to analyze transcription blocking within a 1604 bp sequence of the β-gal coding region. βG5 and βG3 produced 301 and 215 nucleotide protected fragments, respectively. (B) Total RNA was isolated from XPA cells that had been cotransfected previously with *cis*- or *trans*-DDP damaged CMV-β-gal plasmid and a second unmodified vector pcDNA3-CAT. Ribonuclease protection assays on total RNA were carried out using antisense RNA probes for CAT, β-actin, and β-gal as described in Materials and Methods. The RNA probe used for β-gal was either βG5 (A lanes) or βG3 (B lanes). (C) Levels of CAT, β-actin, and β-gal mRNA were quantitated by PhosphorImaging. The PhosphorImaging intensity of the RNase-resistant portion of each probe was normalized by correcting for the number of radioactive nucleotides. The level of β-gal mRNA in each lane was additionally normalized to the level of CAT mRNA in order to control for variations in transfection efficiency. β-Actin protected bands demonstrate that equivalent amounts of total RNA were used for each assay. The fraction of β-gal mRNA that was successfully transcribed through the 1604 bp region to reach full length was calculated by the fraction of the normalized values βG3/βG5. This fraction is expressed as a percent of that fraction determined for the unmodified plasmid. Percentages are presented as a function of the number of adducts present, on average, within the 1604 nucleotide sequence.

successfully transcribed through the 1604 bp region to yield full-length transcript (βG3). Studies *in vitro* indicate that only platinum adducts present on the transcribed strand of DNA effectively block RNA polymerase processivity (Corda et al., 1991). The fraction βG3/βG5 therefore provides a measure of the blocking effected by platinum adducts on the transcribed strand of the 1604 bp region. Curves generated from these data (Figure 4C) revealed that 1.2 *cis*-DDP adducts were required in this 1604 nucleotide sequence to reduce full-length transcript to 37% of that synthesized from unmodified plasmid. At the levels of modification examined for *trans*-DDP, full-length transcript never fell below 56%. The slope of the dose response curve predicts, however, that ~5 *trans*-DDP adducts would be necessary to effect 37% inhibition. These results indicate that *cis*-DDP DNA adducts block RNA polymerase processivity more effectively than *trans*-DDP adducts *in vivo* by at least 4-fold.

DISCUSSION

The effects of the anticancer drug *cis*-DDP and its geometric isomer *trans*-DDP on RNA synthesis were investigated *in vivo* by using a nonreplicating transient expression vector in a host cell reactivation system. Transfection of HeLa cells with *cis*- or *trans*-DDP damaged plasmid revealed a 2-fold greater expression of the template modified by *trans*-DDP than by *cis*-DDP (Figure 1). These data suggested

greater inhibition of transcription by adducts of *cis*-DDP as compared to *trans*-DDP. The possibility existed, however, that preferential repair of *trans*-DDP adducts could have contributed to our observations.

Nucleotide excision repair is believed to be the main process by which platinum adducts are removed from DNA. More specifically, *cis*-DDP adducts are known substrates for transcription-coupled excision repair (Jones et al., 1991; May et al., 1993), of relevance in that the assay used in this study monitors adducts present in an actively transcribed gene. Cells from XPA individuals are defective for XPAC (XPA complementing) protein, which binds *cis*-DDP damaged DNA and functions as a key component in recognition of DNA damage during repair (Jones & Wood, 1993). XPA cells are deficient in the repair of *cis*-DDP (Chu & Berg, 1987; Dijt et al., 1988; Hansson et al., 1990) and *trans*-DDP DNA adducts (Hansson et al., 1990), as well as in the transcription-coupled repair of *cis*-DDP (Zhen et al., 1993) and UV (Evans et al., 1993) damage. Our experiments in XPA cells (Figure 2; Table 1) showed that the differential in D_0 values for *trans*-DDP compared to *cis*-DDP (2.7-fold) was enhanced relative to that found in a consanguineous normal cell line (1.6-fold). This result indicated that preferential excision repair of *trans*-DDP was not the cause of the enhanced expression found for *trans*-DDP damaged DNA.

Experiments carried out in CHO excision repair deficient UV20 cells (complementation group 1, formerly group 2) found remarkably similar results (Figure 3; Table 1). The CHO group 1 repair defect is partially complemented by the human ERCC1 gene (van Duin et al., 1988; Larminat & Bohr, 1994). The ERCC1 gene product, by comparison with its homologous protein Rad 10 in *Saccharomyces cerevisiae* (van Duin et al., 1986), is believed to function in the 5′ incision step of excision repair and in mitotic recombination (Bardwell et al., 1994). This point is relevant in that interstrand cross-links may require recombinational repair for their removal from DNA. CHO group 1 repair deficient cell lines have an increased sensitivity to both *cis*- and *trans*-DDP (Hoy et al., 1985), are deficient in the removal of interstrand (Meyn et al., 1982) as well as intrastrand *cis*-DDP cross-links (Larminat & Bohr, 1994), and have been shown to be deficient in the repair of *cis*-DDP adducts present in an actively transcribed gene (Larminat & Bohr, 1994). Our experiments showed that the differential in D_0 values for *trans*-DDP compared to *cis*-DDP was, again, slightly greater in the repair deficient UV20 (4.3-fold) than in the parental AA8 (3.7-fold) cell lines. Further, we observed significant repair of both isomers, as operationally defined by this assay, in the time that elapsed between 15 and 48 h after transfection in AA8 cells. By contrast, no repair of either isomer was evident during the same time period in the UV20 cells. The most straightforward explanation of these data is that excision repair is the primary mechanism of platinum adduct removal for both platinum isomers. The formal possibility exists that *trans*-DDP lesions could be removed by an additional process in excision repair deficient cells; to date, however, there is no evidence of such a process. The same differential between *cis*- and *trans*-DDP was found at 15 and 48 h, further supporting the view that excision repair did not contribute to the enhanced transcriptional activity from *trans*-DDP damaged templates. Taken together, the results from human and rodent repair deficient cell lines thus suggest that *cis*-DDP adducts inhibit transcription more efficiently than adducts of *trans*-DDP *in vivo*.

Table 2: RNA Polymerase Bypass Efficiency (%) of DNA Adducts Formed by *cis*- and *trans*-Diamminedichloroplatinum(II)

	Pt derivative:	
	cis-DDP	*trans*-DDP
human XPA[a]	0−17	60−70
CHO-UV20[a]	0−16	69−76
human XPA[b]	16	ND

[a] RNA polymerase bypass efficiencies were calculated as follows. The number of platinum adducts required within the gene inactivation target to reduce β-gal enzyme activity to 37% of an unmodified control (D_{0T}) in repair deficient cell lines were determined from D_0 values (Table 1) by using the size of the gene inactivation target. This putative target was considered to include either the CMV promoter and transcribed strand of the β-gal coding sequence and SV40 splice/polyadenylation sequence (5120 nucleotides of 14 976 total nucleotides in plasmid) or alternatively only the transcribed strand of the coding and polyadenylation sequence (3936 out of 14 976 nucleotides). All D_{0T} values were assumed to follow a Poisson distribution. A polynomial equation summarizing the individual contributions of each population of adducts described by the Poisson distribution to the total transcription blocking observed was solved iteratively to obtain a bypass efficiency of RNA polymerase for a single adduct of either platinum isomer. [b] Bypass efficiencies were calculated as in foonote *a* except that D_{0T} values were determined from experiments analyzing transcription blocking by using the ribonuclease protection assay (Figure 4A,B). The number of platinum adducts required within the 1604 nucleotide sequence to allow only 37% full-length transcript (D_{0T}) was obtained from the curves exponentially fitted to the data (Figure 4C).

A statistical analysis of the data obtained in excision repair deficient cells, assuming that repair by another putative process is negligible, allows the calculation of an approximate efficiency of translesion synthesis by RNA polymerase. Inhibition of transcription by platinum adducts could be caused by the impeded translocation of RNA polymerase II along the transcribed strand of the DNA template (Corda et al., 1991). Gene inactivation could also be mediated at the level of initiation through the inhibition of transcription factor binding (Mymryk et al., 1995). In our analysis, we therefore considered the gene inactivation target sequence within CMV-β-gal to include the double-stranded promoter region as well as the transcribed strand of the coding and splice/polyadenylation sequence. We find that, on average, 1.2 *cis*-DDP DNA adducts within this putative target are required to reduce β-gal activity by 63% (D_{0T}) in XPA cells. In contrast, at least 3.3 DNA adducts of *trans*-DDP are necessary to produce the same level of inhibition. If gene inactivation at the promoter is small and can be ignored, the D_{0T} values obtained in repair deficient cell lines can then be used to calculate an approximate bypass efficiency of RNA polymerase for both *cis*- and *trans*-DDP. This calculation was accomplished by using the Poisson distribution of D_{0T} for either isomer. Inclusion of the promoter in the target sequence that ultimately determines D_{0T} may or may not be a valid assumption. By carrying out calculations for both situations, however, we can establish an upper and lower limit for the bypass rates of each isomer. We determined the bypass frequency by RNA polymerase for a single DNA adduct of *trans*-DDP to be in the range of 60−76%, whereas the rate of bypass of a single *cis*-DDP adduct was calculated to be between 0−17% (summarized in Table 2). The D_{0T} for *cis*-DDP of ~1 indicates, assuming a Poisson distribution

of adducts, that the observed gene expression from *cis*-DDP damaged template originated almost entirely from a DNA population that contained no platinum adducts within the target sequence.

The analysis detailed above relies on the assumption that adducts in the promoter region contributed negligibly to gene inactivation in our experiments. If adducts in the promoter region are important, then our putative bypass efficiencies may have been underestimated. This possibility led us to compare directly the relative abilities of *cis*- and *trans*-DDP DNA adducts to block transcription elongation. RNA synthesis through a specific portion of the β-gal coding sequence *in vivo* was monitored by ribonuclease protection analysis (Figure 4A). Results of this experiment revealed that 1.2 *cis*-DDP adducts, on average, were required in the transcribed strand of the monitored region to inhibit transcription elongation by 63% (Figure 4B,C). The slope of the dose response curve for *trans-DDP* predicts that ~5 adducts would be necessary to produce equivalent inhibition. These data, obtained by the direct examination of transcript elongation (Table 2), are consistent with those found by measuring overall β-gal expression, suggesting that *cis*-DDP adducts mediated transcriptional inactivation in our system primarily at the level of the coding region. The data further suggest that *cis*- and *trans*-DDP adducts block RNA polymerase II *in vivo* differentially by a minimum of 4−5-fold. An alternative but less likely scenario would be that *cis*-DDP adducts could form in a region of the promoter such that protein−DNA interactions necessary for efficient transcription are blocked.

The present results are in agreement with previous *in vitro* studies examining the effects of specific DNA adducts formed by *cis*- and *trans*-DDP on transcription elongation by purified eukaryotic RNA polymerases. Results of such studies show that each of the three predominant intrastrand adducts formed by *cis*-DDP, including the 1,2-d(GpG), 1,2-d(ApG), and 1,3-d(GpTpG) cross-links, as well as the interstrand cross-link at a d(GC) site, provides absolute blocks to RNA polymerases when present on the transcribed strand (Corda et al., 1991, 1993). Such adducts comprise ~97% of the total adduct spectrum of *cis*-DDP (Bruhn et al., 1990). Our range of bypass rates (0−17%) for *cis*-DDP *in vivo* are thus consistent with results from these *in vitro* studies. The intrastrand 1,3-d(GpTpG) adduct of *trans*-DDP, in contrast to that formed by *cis*-DDP, does not provide an absolute block to RNA polymerase *in vitro* (Corda et al., 1993). Although the *trans*-DDP adduct spectrum has not been as well characterized as that of *cis*-DDP, the 1,3-d(GpNpG) adduct could account for ~40% of the total adducts formed by the trans isomer (Eastman et al., 1988). Our results suggest that this lesion, and the bulk of the remaining intrastrand adducts formed by *trans*-DDP, are bypassed *in vivo*. The *trans*-DDP interstrand cross-link, which blocks transcription elongation effectively *in vitro* (Brabec & Leng, 1993) and represents up to 20% of the total *in vitro* adduct spectrum (Eastman et al., 1988; Brabec & Leng, 1993), could be responsible for a significant portion of the ~30% blocking efficiency we have found *in vivo*.

Differential transcription inhibition as demonstrated in this report is one of several effects that may act in concert to bring about the different toxicities of *cis*- and *trans*-DDP. For example, incubation of DNA templates modified with *cis*- or *trans*-DDP with mammalian cell-free extracts results in enhanced repair of *trans*-DDP damaged templates over those modified with *cis*-DDP, suggesting that preferential repair may explain, at least in part, the lower toxicity of *trans*-DDP (Hansson & Wood, 1989; Heiger-Bernays et al., 1990). Studies *in vivo*, however, have yielded conflicting views that may be attributable to technical differences in experimental design (Ciccarelli et al., 1985; Roberts & Friedlos, 1987). The results of the present study indicate that, in the system used, excision repair of *cis*- and *trans*-DDP adducts occurs to an equal extent. This conclusion is supported by the relatively proportionate increase in D_0 values for *cis*- and *trans*-DDP when determined in excision repair deficient and then repair proficient cell lines (Table 1).

The apparent discrepancy between the similar excision repair of *cis*- and *trans*-DDP observed in the present study and the evidence for preferential repair of *trans*-DDP in the bulk of the literature can be explained in several ways. One aforementioned possibility is that the trans isomer is additionally repaired by another pathway. Another possibility is that a significant population of *trans*-DDP adducts may be uninhibitory to transcription, and therefore these lesions would be undetectable by the host cell reactivation assay. If this adduct population were repaired preferentially, such repair would go undetected as well.

The results presented in this paper suggest that inhibition of transcription may play a role in the cytotoxicity of *cis*-DDP. With regard specifically to the antitumor activity of *cis*-DDP, RNA synthesis is more critical for a rapidly dividing tumor cell than for a stationary cell (Mauck & Green, 1973). Previous studies have suggested that *cis*-DDP effects cell death via apoptosis (Sorenson & Eastman, 1988b). It has been further proposed that a general disruption of gene expression, or the specific inhibition of a gene necessary for mitosis, may be a signal for the induction of apoptosis (Sorenson & Eastman, 1988b). If the inhibition of RNA synthesis is involved in signaling apoptosis, the present studies suggest that *trans*-DDP would be less likely to trigger this pathway.

ACKNOWLEDGMENT

We thank Z. Chen and D. Treiber for helpful discussions and D. Treiber and K. Yarema for critical reading of the manuscript.

REFERENCES

Alazard, R., Germanier, M., & Johnson, N. P. (1982) *Mutat. Res. 93*, 327.

Athas, W. F., Hedayati, M. A., Matanoski, G. M., Farmer, E. R., & Grossman, L. (1991) *Cancer Res. 51*, 5786.

Bardwell, J. A., Bardwell, L., Tomkinson, A. E., & Friedberg, E. C. (1994) *Science 265*, 2082.

Bernges, F., & Holler, E. (1988) *Biochemistry 27*, 6398.

Brabec, V., & Leng, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5345.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Bruhn, S. L., Toney, J. H., & Lippard, S. J. (1990) *Prog. Inorg. Chem. 38*, 477.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156.

Chu, G., & Berg, P. (1987) *Mol. Biol. Med. 4*, 277.

Ciccarelli, R. B., Solomon, M. J., Varshavsky, A., & Lippard, S. J. (1985) *Biochemistry 24*, 7533.

Collins, A. R. (1993) *Mutat. Res., DNA Repair 293*, 99.

Corda, Y., Job, C., Anin, M.-F., Leng, M., & Job, D. (1991) *Biochemistry 30*, 222.

Corda, Y., Job, C., Anin, M.-F., Leng, M., & Job, D. (1993) *Biochemistry 32*, 8582.
Dijt, F. J., Fichtinger-Schepman, A. M., Berends, F., & Reedijk, J. (1988) *Cancer Res. 48*, 6058.
Eastman, A. (1983) *Biochemistry 22*, 3927.
Eastman, A., & Barry, M. A. (1987) *Biochemistry 26*, 3303.
Eastman, A., Jennerwein, M. M., & Nagel, D. L. (1988) *Chem.-Biol. Interact. 67*, 71.
Eustice, D. C., Feldman, P. A., Colberg-Poley, A. M., Buckery, R. M., & Neubauer, R. H. (1991) *BioTechniques 11*, 739.
Evans, G. L., & Gralla, J. D. (1992a) *Biochem. Pharmacol. 44*, 107.
Evans, G. L., & Gralla, J. D. (1992b) *Biochem. Biophys. Res. Commun. 184*, 1.
Evans, M. K., Robbins, J. H., Ganges, M. B., Tarone, R. E., Nairn, R. S., & Bohr, V. A. (1993) *J. Biol. Chem. 268*, 4839.
Feuer, E. J., Brown, L. M., & Kaplan, R. S. (1993) in *SEER Cancer Statistics Review: 1973−1990* (Miller, B. A., Ries, L. A. G., Hankey, B. F., Kosary, C. L., Harras, A., Devesa, S. S. & Edwards, B. K., Eds.) pp XXIV.1−XXIV.13, NIH Publication No. 93-2789, National Cancer Institute, Bethesda, MD.
Fichtinger-Schepman, A. M., van der Veer, J. L., den Hartog, J. H., Lohman, P. H., & Reedijk, J. (1985) *Biochemistry 24*, 707.
Gorman, C. M., Moffat, L. F., & Howard, B. H. (1982) *Mol. Cell Biol. 2*, 1044.
Hansson, J., & Wood, R. D. (1989) *Nucleic Acids Res. 17*, 8073.
Hansson, J., Grossman, L., Lindahl, T., & Wood, R. D. (1990) *Nucleic Acids Res. 18*, 35.
Harder, H. C., Smith, R. G., & Leroy, A. F. (1976) *Cancer Res. 36*, 3821.
Heiger-Bernays, W. J., Essigmann, J. M., & Lippard, S. J. (1990) *Biochemistry 29*, 8461.
Hoy, C. A., Thompson, L. H., Mooney, C. L., & Salazar, E. P. (1985) *Cancer Res. 45*, 1737.
Jones, C. J., & Wood, R. D. (1993) *Biochemistry 32*, 12096.
Jones, J. C., Zhen, W., Reed, E., Parker, R. J., Sancar, A., & Bohr, V. A. (1991) *J. Biol. Chem. 266*, 7101.
Larminat, F., & Bohr, V. A. (1994) *Nucleic Acids Res. 22*, 3005.
Lehmann, A. R., & Oomen, A. (1985) *Nucleic Acids Res. 13*, 2087.
Loehrer, P. J., & Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704.
Mauck, J. C., & Green, H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2819.
May, A., Nairn, R. S., Okumoto, D. S., Wassermann, K., Stevnsner, T., Jones, J. C., & Bohr, V. A. (1993) *J. Biol. Chem. 268*, 1650.
Meyn, R. E., Jenkins, S. F., & Thompson, L. H. (1982) *Cancer Res. 42*, 3106.
Mymryk, J. S., Zaniewski, E., & Archer, T. K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 2076.
Pascoe, J. M., & Roberts, J. (1974) *Biochem. Pharmacol. 23*, 1345.
Pinto, A. L., & Lippard, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4616.
Poll, E. H. A., Abrahams, P. J., Arwert, F., & Eriksson, A. W. (1984) *Mutat. Res. 132*, 181.
Protic-Sabljic, M., & Kraemer, K. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6622.
Roberts, J. J., & Friedlos, F. (1987) *Biochemistry 47*, 31.
Salles, B., Butour, J. L., Lesca, C., & Macquet, J. P. (1983) *Biochem. Biophys. Res. Commun. 112*, 555.
Sorenson, C. M., & Eastman, A. (1988a) *Cancer Res. 48*, 4484.
Sorenson, C. M., & Eastman, A. (1988b) *Cancer Res. 48*, 6703.
Sorenson, C. M., Barry, M. A., & Eastman, A. (1990) *J. Natl. Cancer Inst. 82*, 749.
Thompson, L. H., Busch, D. B., Brookman, K., Mooney, C. L., & Glaser, D. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3734.
Uchida, K., Tanaka, Y., Nishimura, T., Hashimoto, Y., Watanabe, T., & Harada, I. (1986) *Biochem. Biophys. Res. Commun. 138*, 631.
van Duin, M., de Wit, J., Odijk, H., Westerveld, A., Yasui, A., Koken, M. H. M., Hoeijmakers, J. H. J., & Bootsma, D. (1986) *Cell 44*, 913.
van Duin, M., Janssen, J. H., de Wit, J., Hoeijmakers, J. H. J., Thompson, L. H., Bootsma, D., & Westerveld, A. (1988) *Mutat. Res. 193*, 123.
Zhen, W., Evans, M. K., Haggerty, C. M., & Bohr, V. A. (1993) *Carcinogenesis 14*, 919.

BI9513665