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Effect of the Antitumor Drug *cis*-Diamminedichloroplatinum(II) and Related Platinum Complexes on Eukaryotic DNA Replication†

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ABSTRACT: An SV40-based in vitro replication system has been used to examine the effects of platinum compounds on eukaryotic DNA replication. Plasmid templates containing the SV40 origin of replication were modified with the anticancer drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP, cisplatin) or the inactive analogues $[Pt(dien)Cl]^+$ and *trans*-DDP. The platinated plasmids were used as templates for DNA synthesis by the DNA polymerases present in cytosolic extracts prepared from human cell lines HeLa and 293. Bifunctional adducts formed by *cis*- and *trans*-DDP inhibited DNA replication by 95% at a bound drug to nucleotide ratio $[(D/N)_b]$ of $<9 \times 10^{-4}$, in contrast to the monofunctional $[Pt(dien)Cl]^+$ analogues, which required a $(D/N)_b$ of 3.4×10^{-3} for 62% inhibition of DNA replication. An average of two platinum adducts per genome was sufficient for inhibition of DNA replication by cisplatin. When *trans*-DDP-modified, but not *cis*-DDP-modified, SV40 origin containing plasmids $[(D/N)_b = 1.7 \times 10^{-3}]$ were allowed to incubate in the 293 cytosolic extracts for 1 h prior to addition of T-antigen to initiate replication, DNA synthesis was restored to 30% of control. This result suggested the presence of an activity in the extracts that reactivates *trans*-DDP-modified DNA templates for replication. This hypothesis was confirmed by an in vitro nucleotide excision repair assay that revealed activity in 293 and HeLa cell extracts selective for *trans*-DDP-modified plasmid DNAs. Such selective repair of *trans*-DDP-damaged DNA in human cells would contribute to its lack of antitumor activity.

An accepted mode of action for many anticancer drugs is the inhibition of DNA synthesis by drug–DNA adducts that impair the ability of polymerases to traverse the lesion. In mammalian cells, it has been very difficult to uncover the precise mechanism by which adducts functionally inactivate template DNA owing, in part, to the complexity of the replication apparatus. In the case of cisplatin, the major DNA adducts are intrastrand cross-links between N7 atoms of adjacent guanines or, at a lower frequency, adenine and guanine bases [for a review, see Sherman and Lippard (1987)]. These cross-links inhibit DNA polymerases and have been postulated to lead to the cytotoxic effects of the drug (Harder & Rosenberg, 1970; Howle & Gale, 1970).

Replication mapping experiments using both purified prokaryotic (Pinto & Lippard, 1985) and eukaryotic (Villani et al., 1988) DNA polymerases on single-stranded DNA templates have demonstrated the ability of *cis*-DDP[1] adducts to block the polymerases, although not without some translesion synthesis (Burstyn, Comess, and Lippard, unpublished results). We have been able to assess the effect of sequence context on the relative *cis*-DDP blockage sites by using bacteriophage T7 polymerase on double-stranded DNA (Burstyn, Heiger-Bernays, Essigmann, and Lippard unpublished results). Mapping experiments such as these and others examining different lesions (Moore et al., 1980; Rouet & Essigmann, 1985; Hayes & LeClerc, 1986; Gralla et al., 1987; Taylor & O'Day, 1990) can provide information regarding the influence of adducts on the activity of DNA polymerases.

In order to address the role of DNA-damaging agents on mammalian DNA replication, specifically the effects of a variety of platinum drugs in a biologically relevant milieu, we have employed an SV40-based in vitro replication system (Li & Kelly, 1984; Stillmann & Gluzman, 1985). This system utilizes soluble extracts from the cytoplasm of human cell lines that are capable of supporting efficient replication and supercoiling of exogenous plasmid templates containing the minimal SV40 origin of replication. All of the replication machinery is provided by the cytosolic extract with the exception of the viral protein, large T-antigen. In this initial study we have investigated three platinum compounds having different chemical properties and clinical efficacies. Specifically, we describe results for cisplatin and its clinically inactive isomer *trans*-DDP, both of which form intrastrand cross-links as their principal DNA lesions. We also examine the monofunctionally binding, antitumor-inactive compound [Pt-

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[1] Abbreviations: DDP, diamminedichloroplatinum(II); dien, diethylenetriamine; SV40, simian virus 40; kbp, kilobase pairs; $(D/N)_b$, bound drug to nucleotide ratio; AAS, atomic absorption spectroscopy; form II, closed circular duplex DNA containing at least one single-stranded break; RI, replicative intermediates.

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(dien)Cl]$^+$. These studies demonstrate the power of the SV40 in vitro replication assay for determining the effects of DNA binding agents on eukaryotic DNA replication and further underscore that differential repair (Ciccarelli et al., 1985) may be a significant determinant in the biological activity of *cis*- versus *trans*-DDP.

Materials and Methods

Preparation of Cytosolic Cell Extracts, Whole Cell Extract, SV40 T-Antigen, and Plasmid DNAs. Cytosolic extracts were prepared (Stillman & Gluzman, 1985) from suspension cultures of human 293 and HeLa cells grown in Dulbecco's modified minimal essential medium supplemented with 10% calf serum (GIBCO Laboratories). Cells in late exponential phase [(4–6) × 10^5 cells/mL] were washed with cold hypotonic buffer (20 mM HEPES–KOH [*N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid], pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol). The cells were resuspended in 2.5 mL (per liter of cells) of the same buffer and allowed to swell for 20 min, after which they were broken by 10–20 strokes in a Dounce homogenizer (B pestle) and incubated for 30 min on ice. The lysate was centrifuged at 12000*g* for 10 min to pellet the nuclei; the supernatant was made 50 mM in NaCl and then centrifuged at 30000*g* for 1 h at 4 °C. The extract was stored at −120 °C.

HeLa cells grown under the conditions described were used to prepare whole cell extracts according to published procedures (Manley et al., 1983; Wood et al., 1989) with one minor modification: all dialysis buffers contained 1 mM phenylmethanesulfonic acid.

T-antigen was prepared from 293 cells infected with Ad5SVR115 (Stillman & Gluzman, 1985) virus at a multiplicity of infection of 5 PFU/cell and incubated at 37 °C for 22–24 h. The cells were harvested, and T-antigen was extracted and purified as described by Simanis and Lane (1985).

Plasmid DNAs were prepared as described by Stillman and Gluzman (1985). Plasmids pSVO$^+$ (SV40 origin containing) and pSVO$^-$ (SV40 origin lacking) contained the 0.2-kbp *Hin*dIII to *Sph*I fragments of pSV40 and p8-4, respectively, inserted into pAT153 (Twigg & Sherratt, 1980). pSVO11 contains the 0.2-kbp *Hin*dIII to *Sph*I fragment of SV40 in pUC18. pLac is a 3.2-kbp plasmid prepared by insertion of the *lacZ* gene of *Escherichia coli* into the *Cla*I site of pBR322 (Donahue and Essigmann, unpublished results). Restriction enzymes and T_4 DNA ligase were purchased from New England Biolabs and used according to the manufacturer's recommendations.

Platination of DNA. Plasmids containing the SV40 replication origin were incubated at 37 °C for 16 h in the presence of a platinum compound at several formal drug/nucleotide [$(D/N)_f$] ratios in 3 mM NaCl–1 mM Na_2HPO_4 (pH 7.5) or 10 mM Tris-HCl–1 mM Na_2EDTA (pH 7.6). Platinum complexes were freshly dissolved just prior to use. Unbound platinum was removed by ethanol precipitation of the DNA. The amount of platinum bound per nucleotide, $(D/N)_b$, was measured by atomic absorption spectroscopy (AAS), on a Varian 1475 spectrophotometer equipped with a GTA 95 graphite-tube atomization system and programmable sample dispenser.

Replication Assays. Reactions were carried out in a solution (50 μL) containing (final concentration) 40 mM HEPES–KOH (pH 7.5), 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 μM each of dTTP, dGTP, and dCTP, 25 μM [α-^{32}P]dATP, 200 μM each of CTP, GTP, and UTP, 4 mM ATP, 40 mM creatine phosphate, and 1 μg of creatine phosphokinase (rabbit muscle type I, Sigma Chemical Co.), to which was added 0.3 μg of plasmid DNA (either control or platinated), 0.5 μg of T-antigen, and 175–200 μg (protein) of 293 or HeLa cytosolic extract. Reaction mixtures were prepared on ice and incubated at 37 °C for 60 min, unless otherwise indicated. For some experiments, reactions lacking T-antigen were incubated at 37 °C for 60 min at which time T-antigen was added and the reactions were allowed to proceed at 37 °C for an additional 60 min.

Measurement of DNA Synthesis. Reactions were terminated by addition of Na_2EDTA (pH 8.0) to 10 mM and 50 μg of denatured, sheared calf thymus DNA as carrier and then mixed with 1 mL of 8% trichloroacetic acid–1% sodium pyrophosphate for 15 min on ice. The acid-insoluble material was collected by filtration onto Whatman GF-C glass fiber filters and then washed several times with 10 mL of ice-cold 8% trichloroacetic acid–1% sodium phosphate and twice with ice-cold ethanol. Radioactivity was determined by liquid scintillation counting.

Analysis of Replication Products. Reactions were terminated by the addition of Na_2EDTA (pH 8.0) to 10 mM, sodium dodecyl sulfate to 0.1%, and ribonuclease A to 20 mg/mL and incubation at 37 °C for 15 min at which time proteinase K was added to 0.1 mg/mL. The samples were kept at 37 °C for 1 h and then extracted with phenol, and the unincorporated nucleoside triphosphates were removed by spin dialysis (Maniatis et al., 1982) with Sephadex G-50 in a 1-mL syringe. The solution was extracted once with phenol and then with chloroform–isoamyl alcohol (24:1 v/v), and the DNA was ethanol precipitated. Samples were subjected to agarose gel electrophoresis in TBE buffer (89 mM Tris–borate, 89 mM boric acid). Gels were stained with 0.5 μg/mL ethidium bromide following electrophoresis for visualization of the products. The gels were dried onto Whatman 3MM paper and autoradiographed.

In Vitro Repair Reactions. Assays were performed as described by Wood et al. (1989), with a few modifications. Covalently closed circular plasmid DNA modified with *cis*- or *trans*-DDP was purified by electroelution prior to use in the repair assay. Reactions were performed in 50-μL solutions containing 300 ng of plasmid pSVO11 modified with either *cis*- or *trans*-DDP, 300 ng of unmodified pLac plasmid control DNA, 45 mM HEPES–KOH (pH 7.6), 60 mM KCl, 8.0 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM Na_2EDTA, 2 mM ATP, 20 μM each of dGTP, TTP, and dCTP, 8 μM dATP, 2 μCi of [α-^{32}P]dATP (3000 Ci/mmol), 40 mM creatine phosphate, 2 μg of creatine phosphokinase (type I, Sigma Chemical Co.), 3.4% glycerol, 18 μg of bovine serum albumin, and typically 120 μg of extract protein. These solutions were incubated at 30 °C for 3 h. Reactions were terminated by the addition of Na_2EDTA to 10 mM, sodium dodecyl sulfate to 0.1%, and ribonuclease A to 20 mg/mL and incubation at 37 °C for 15 min, at which time proteinase K was added to 0.1 mg/mL for an additional 30 min. The solution was extracted with phenol–chloroform, and the DNA was ethanol precipitated. Salt and unincorporated nucleotides were removed by filtration on a Centricon-10 (Amicon) microconcentrator. Plasmid DNAs were linearized with *Pst*I in 30 μL of buffer and electrophoresed in a 0.8% agarose gel containing 0.5 μg/mL ethidium bromide. The dried gel was subjected to autoradiography and densitometry with a Betascope 603 blot analyzer (Betagen).

Results

Effects of Platinum Compounds on SV40 Replication in Vitro. Double-stranded pSVO11 plasmid DNA was allowed

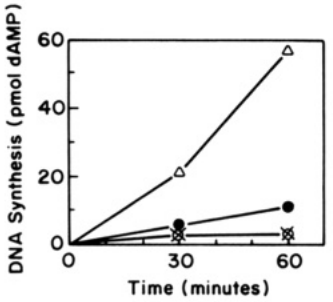

FIGURE 1: Effect of *cis*-DDP modification of an SV40-based plasmid on in vitro replication using 293 or HeLa cell cytosolic extracts. The symbols represent $(D/N)_b$ values of 1.7×10^{-3} (×), 0.9×10^{-3} (○), 0.4×10^{-3} (●), and 0.0 (△).

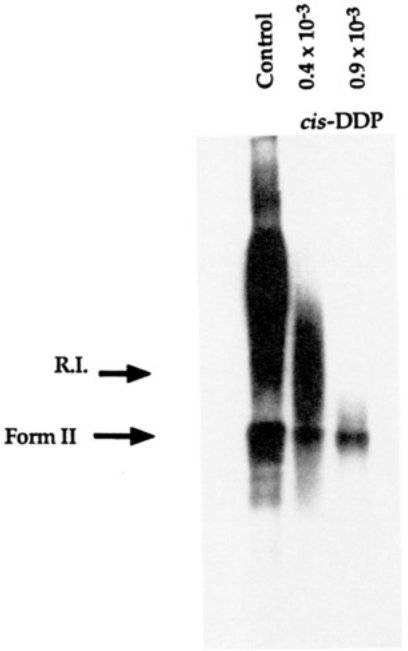

FIGURE 2: Replication products from *cis*-DDP-modified pSVO11 templates using HeLa cell cytosolic extract. Autoradiograph of a 0.8% agarose gel of purified DNA from 50-μL reactions. Left to right: unmodified DNA control; DNA modified to $(D/N)_b$ of 0.4×10^{-4}; DNA modified to $(D/N)_b$ of 0.9×10^{-4}. Abbreviations: RI, replicative intermediates; form II, circular duplex DNA containing at least one single-stranded nick.

to react with *cis*-DDP to achieve several $(D/N)_b$ levels. Figure 1 shows the time-dependent replication of plasmid templates at different levels of bound platinum. Replication was unaffected by the conditions used to platinate and subsequently to purify the plasmid DNA; control experiments revealed DNA synthesis to be the same as that for plasmid DNA that was not taken through the platination protocol (data not shown). At the $(D/N)_b$ level of 9.0×10^{-4} on the template, replication is inhibited by at least 95% of the control, which for these experiments is defined as the picomoles of dAMP incorporated after replication of undamaged plasmid. At a $(D/N)_b$ level of 4.0×10^{-4}, some replication occurs but is only 20% of the control. The products of replication at these $(D/N)_b$ levels are shown in Figure 2. We estimate the level of damage inhibiting replication to this extent to be 2.6 adducts per pSVO11 plasmid molecule.

In Table I, the influence of the chemotherapeutically inactive platinum complexes on in vitro replication of the templates is compared to results for *cis*-DDP-modified templates. At a $(D/N)_b$ level of 9.0×10^{-4}, *trans*-DDP is as effective as *cis*-DDP in inhibiting replication. By contrast, the monofunctional adducts formed by $[Pt(dien)Cl]^+$ at the same level of modification inhibit DNA replication by only 22% of the control. When the $(D/N)_b$ of $[Pt(dien)Cl]^+$ was increased to 3.4×10^{-3}, inhibition was increased to nearly 62%.

Table I: Effect of Platination on in Vitro DNA Replication from pSVO11 Templates Using 293 or HeLa Cell Cytosolic Extracts at $(D/N)_b$ Levels As Indicated

modification	$(D/N)_b$	pmol of dAMP h^{-1}	% control
unmodified	0	56.8	100
cis-DDP	1.7×10^{-3}	2.6	5
cis-DDP	0.9×10^{-3}	2.8	5
cis-DDP	0.4×10^{-3}	10.4	18
trans-DDP	1.7×10^{-3}	3.1	5
trans-DDP	0.4×10^{-3}	11.5	20
$[Pt(dien)Cl]^+$	0.9×10^{-3}	45.6	80
$[Pt(dien)Cl]^+$	3.4×10^{-3}	35.0	62

Table II: Effect of *cis*-DDP Modification of Selected Regions of pSVO11 Recombinant Molecules (Figure 3) on in Vitro DNA Synthesis

modification	pmol of dAMP h^{-1}
unmodified pSVO11	42.0 ± 2.9
cis-DDP-modified pSVO11 (plasmid A) (platinated origin)	5.2 ± 0.4
cis-DDP-modified pSVO11 (plasmid B) (unplatinated origin)	20.2 ± 1.4

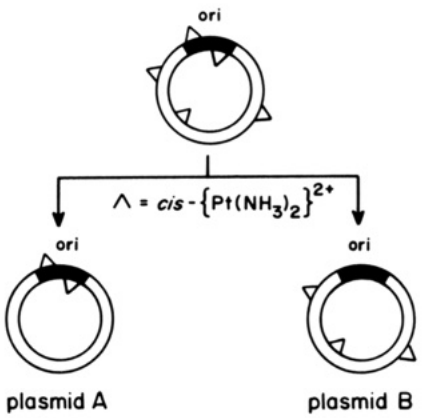

FIGURE 3: Recombinant plasmid molecules constructed as described in the text.

In Table I, the influence of the chemotherapeutically inactive platinum complexes on in vitro replication of the templates is compared to results for *cis*-DDP-modified templates. At a $(D/N)_b$ level of 9.0×10^{-4}, *trans*-DDP is as effective as *cis*-DDP in inhibiting replication. By contrast, the monofunctional adducts formed by $[Pt(dien)Cl]^+$ at the same level of modification inhibit DNA replication by only 22% of the control. When the $(D/N)_b$ of $[Pt(dien)Cl]^+$ was increased to 3.4×10^{-3}, inhibition was increased to nearly 62%.

Effect of Drug Binding to the SV40 Origin on DNA Replication. Experiments were performed to investigate whether the impaired DNA synthesis observed in the previous experiments was due specifically to inhibition of initiation of replication. The multiple oligo[d(G)] sequences at the origin of replication in SV40-containing plasmid should be especially favorable targets for the binding of platinum (Sherman & Lippard, 1987; Gralla et al., 1987). Accordingly, pSVO11 was modified with *cis*-DDP to a $(D/N)_b$ of 9.0×10^{-4} and digested with the restriction enzymes *Hin*dIII and *Sph*I, which cleave the plasmid into a 0.2-kbp origin-containing fragment and the pUC18 vector fragment. The 0.2-kbp *Hin*dIII/*Sph*I fragment from the unmodified plasmid was ligated by use of T4 DNA ligase into the platinated vector (Figure 3, plasmid A). This molecule was then introduced into the in vitro replication system; as shown in Table II, replication of this plasmid was almost completely inhibited. The *cis*-DDP-modified vector having an unmodified origin (Figure 3, plasmid B), however, inhibited DNA replication to only 48% of the value for unmodified, recombinant control template (Table II).

Partial Restoration of trans-DDP-Modified Templates following Preincubation with Cytosolic Extract. Previous results for *trans*-DDP binding to SV40-infected CV1 cells (Ciccarelli et al., 1985) suggested that a selective adduct repair might be present. On the basis of these observations we

Table III: DNA Replication following Preincubation of *cis*- and *trans*-DDP-Modified Templates with Human 293 Cell Cytosolic Extracts[a]

modification	preincubation (min)	T-antigen	ori(+) (pmol)	ori(−) (pmol)
unmodified	none	+	33.5 ± 0.8	3.2 ± 1.3
cis-DDP	none	+	3.7 ± 0.5	3.3 ± 1.5
trans-DDP	none	+	3.7 ± 1.1	2.9 ± 0.4
unmodified	60	+	33.9 ± 1.1	2.5 ± 0.5
cis-DDP	60	+	3.6 ± 0.1	3.0 ± 1.4
trans-DDP	60	+	10.0 ± 1.4	2.8 ± 1.3
unmodified	60	−	2.8 ± 0.2	2.5 ± 0.3
cis-DDP	60	−	2.4 ± 0.4	2.5 ± 0.3
trans-DDP	60	−	3.1 ± 0.4	2.8 ± 0.2

[a] Plasmids pSVO$^+$ [ori(+)] or pSVO$^-$ [ori(−)] were modified with either *cis*- or *trans*-DDP to a $(D/N)_b$ level of 1.7×10^{-3}. These plasmids were incubated in the absence or presence of 293 cell cytosolic extract for 60 min at 37 °C, at which time T-antigen was added, as described in the text.

suspected that such an activity might be present in the cytosolic extracts used in the present experiments that would be capable of modifying the *trans*-DDP-platinated templates to restore DNA replication. To address this possibility, *cis*- or *trans*-DDP-modified templates, $(D/N)_b$ of 1.7×10^{-3}, either containing (pSVO$^+$) or lacking (pSVO$^-$) the SV40 origin, were incubated as before. The results shown in Table III reveal that precincubation of the *trans*-DDP-platinated plasmid for 60 min with cytosolic extracts partially restored its ability to act as a functional template for DNA synthesis. Only templates modified with *trans*-DDP recovered their replicative ability following incubation in the cytosolic extract. Moreover, synthesis was recovered only in the plasmid containing the functional origin of replication, indicating the incorporation of radioactivity to be a consequence of DNA replication. A comparison of samples that were preincubated for 60 min and those that were not supports the conclusion that the reactivation was not due to nuclease activity as there was no difference in incorporation of labeled nucleotide between the these two samples (either *cis*- or *trans*-DDP-modified).

Presence of Repair Activity in 293 Cytosolic Extract on trans-DDP-Modified DNA. In order to determine whether the activity seen in the 293 cytosolic extract on *trans*-DDP-modified DNA was in fact due to DNA repair, an in vitro excision repair assay was performed (Wood et al., 1989). pSVO11 DNA that had been modified with *cis*- or *trans*-DDP to a $(D/N)_b$ of 1.7×10^{-3} was incubated with either HeLa whole cell, HeLa cytosolic, or 293 cytosolic extract. The data in Figure 4 illustrate the extent of DNA repair synthesis. Unmodified pLac DNA was included to reveal the level of background incorporation. Templates modified with *cis*-DDP demonstrated a low level, if any, of DNA repair in all of the extracts. This result was in contrast to data generated from the *trans*-DDP-modified templates. The greatest amount of activity was seen in the sample incubated with HeLa whole cell extract. This level was slightly higher than that obtained from the sample incubated with the 293 cytosolic extract. A lesser amount of incorporation occurs with the *trans*-DDP-modified plasmid incubated with HeLa cytosolic extract.

Discussion

The replication of platinated plasmid molecules was assessed with the SV40-based in vitro replication system. This system was chosen for several reasons, the most important being that it is thought to mimic closely events taking place in a eukaryotic cell during replication of nuclear DNA. All of the replication machinery is supplied by cytosolic extracts of human cell lines with the exception of large T-antigen and the SV40 template. In this system, DNA molecules form supercoiled, nucleosome-containing chromosomes not unlike those in eukaryotic nuclei. We were interested in exploring the extent to which this system might be used to evaluate the role of cytosolic components on the binding, processing, and, ultimately, the effect on DNA replication of anticancer drugs. The fidelity of polymerases in the system is far greater than observed for conventionally purified DNA polymerases α and β (Hauser et al., 1988), further underscoring the biological relevance of the approach.

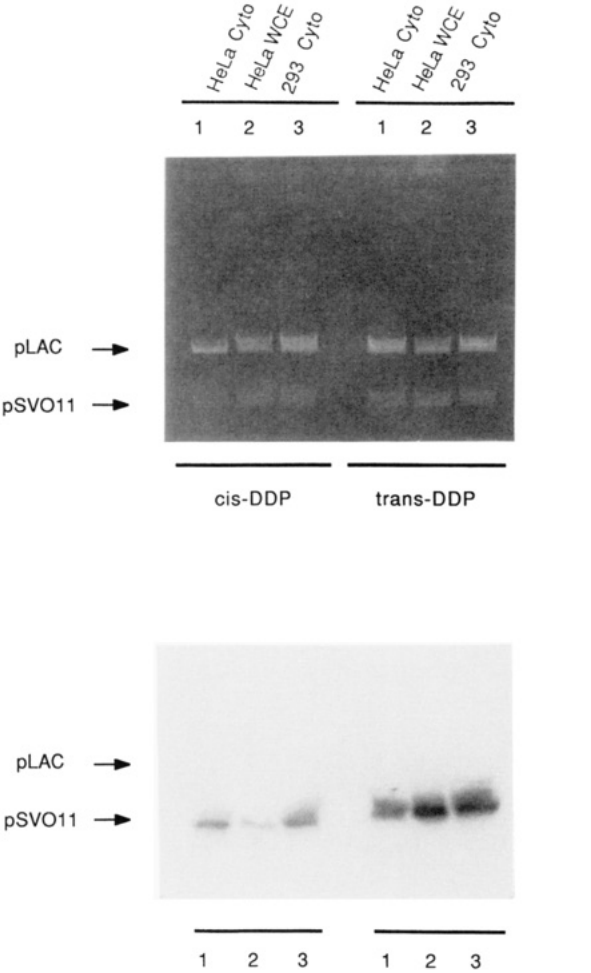

FIGURE 4: In vitro repair of pSVO11 plasmids modified with either *cis*-DDP or *trans*-DDP to a $(D/N)_b$ of 1.7×10^{-3}. Reactions contained 120 μg of extract protein (HeLa cytosolic extract, HeLa whole cell extract, or 293 cytosolic extract), 300 ng of pSVO11 DNA modified with *cis*-DDP or *trans*-DDP, and 300 ng of unmodified (pLac) plasmid DNA that had been purified by gel electrophoresis and electroelution to eliminate nicked material. After incubation at 30 °C for 3 h, the DNA was isolated, digested with *Pst*I, and subjected to agarose gel electrophoresis. A photograph of the ethidium bromide strained gel is shown in the upper panel, and an autoradiograph of the gel is shown in the lower panel. Abbreviations: Cyto, cytosol; WCE, whole cell extract.

The data reveal that, for *cis*- and *trans*-DDP, as few as 2.6 platinum adducts per template molecule inhibit DNA replication. At a lower $(D/N)_b$ of 4.0×10^{-4}, replication is partially restored to 20% of control. At this level of bound drug to plasmid pSVO11, only 10% of the DNA molecules contain no platinum adducts, assuming a Poisson distribution, and 23% of the population of molecules has at least one adduct. We therefore conclude that as few as one or two *cis*-DDP adducts per genome are sufficient to inhibit DNA synthesis. These findings led us to investigate the effect of platinum bound specifically to the SV40 origin. Previous work suggested that

the "GC box" regulatory elements, consiting of tandem repeats of the sequence d(GGGCGGA) near the origin, might be unusually sensitive targets for drugs such as cisplatin that bind selectively to guanine-rich sequences (Gralla et al., 1987). We therefore wondered whether this region might represent a uniquely sensitive target for cisplatin, accounting for its ability to inhibit replication at a level approaching one adduct per genome. Indeed, results obtained with a recombinant molecule containing platinum only in the origin fragment revealed that replication was nearly completely inhibited. On the other hand, the recombinant molecule having an unplatinated origin and a platinated vector still inhibited replication by 48% of control. It is clearly not essential, therefore, that *cis*-DDP bind to the origin in order to diminish DNA synthesis. The DNA molecules synthesized from plasmid B (Figure 3) were found to be less than full length (data not shown), consistent with replication being successfully initiated but inhibited at the elongation step.

A statistical analysis of the data for replication from plasmid A templates accounts well for the efficient inhibition of DNA synthesis on the portion of the vector that lacked the origin. In vitro replication mapping experiments, using pSVO11 plasmids modified to the same level as employed in the present recombinant molecules, have shown that *cis*-DDP binds preferentially to guanine-rich sequences, especially d(AGG) (Burstyn, Heiger-Bernays, Essigmann, and Lippard, unpublished results). Nine such sequences fall within the 0.2-kbp region containing the SV40 origin, and 18 lie outside that region. Assuming that *cis*-DDP binds only to d(AGG) sequences and using the Poisson analysis, we calculate that 17% of the recombinant plasmid A molecules would contain no platinum adducts at the origin. Considering the assumptions, this value is in reasonable agreement with the experimental level of replication, ~12%. If no sequence preference for platinum binding is assumed or if only d(AG) or d(GG) intrastrand cross-links are scored, 70% or 60%, respectively, of recombinant plasmid A molecules would have no platinum at the origin and thus support replication. These conclusions could in principle be tested by determining whether the $(D/N)_b$ for the origin region was different from that of the vector as a whole. Owing to the limit of detection by AAS, however, such a determination was not technically feasible. Nevertheless, it seems clear that the sensitivity of the SV40 origin to cisplatin can be adequately accounted for solely by the statistically greater number of d(AGG) target sequences present.

trans-DDP is ineffective as a chemotherapeutic agent for reasons that are still not fully understood. Much higher levels of intracellular *trans*-DDP are required to inhibit DNA synthesis in vivo compared to *cis*-DDP (Ciccarelli et al., 1985; Roberts et al., 1987). There are two possible explanations, not mutually exclusive, that account for its relatively lower toxicity to cells in culture, compared with that of *cis*-DDP. One is that, upon entering the cell, *trans*-DDP binds irreversibly to sulfur-containing molecules, such as glutathione (Eastman & Barry, 1988). The other is that, once bound to DNA in the nucleus, adducts of *trans*-DDP are preferentially removed (Ciccarelli et al., 1985). Irrespective of which mechanisms limit the toxicity of *trans*-DDP in vivo, it is noteworthy that its bifunctional adducts on DNA are fully as capable of blocking replication as those of *cis*-DDP. This conclusion is supported by the data in Table II and in our earlier in vivo SV40 replication studies in which, at equal $(D/N)_b$ ratios, the two isomers were found to inhibit DNA synthesis to identical extents (Ciccarelli et al., 1985). Our earlier work also suggested the possible existence of an activity within the cell that might be capable of removing *trans*-DDP lesions from DNA (Ciccarelli et al., 1985). This possibility was evaluated by measuring the effect of preincubating *trans*-DDP-modified templates for various times in the cytosolic extract prior to addition of T-antigen (Table III). This treatment, which partially restored the ability of the templates to support replication, led to a linear increase in incorporation of radioactivity over 30 min with saturation at 60 min (data not shown).

While this work was in progress, it was reported that a nucleotide excision repair activity exists in whole cell extracts that is capable of repairing *cis*- and *trans*-DDP-modified DNA (Hansson & Wood, 1989; Sibghat-Ullah et al., 1989). At similar levels of modification, *trans*-DDP induced at least a 2-fold higher level of repair synthesis than did *cis*-DDP (Hansson & Wood, 1989), but the potential biological implications of this finding were not discussed. In addition, the level of repair of the *trans*-DDP-modified plasmids was attributed to repair of the biologically irrelevant monoadduct formed by the drug with DNA. In this earlier work, *trans*-DDP was allowed to react with the plasmid DNA for greater than 12 h at 37 °C. Under these conditions, nearly 95% of the compound would have formed bifunctional DNA adducts (Bancroft et al., 1990), leaving an insignificant amount of monofunctional adducts. In order to demonstrate that the restoration of replication for *trans*-DDP-modified pSVO11 DNA following incubation in the cytosolic extracts was due to template repair, we carried out experiments analogous to those of Hansson and Wood (1989). At a $(D/N)_b$ of 1.7×10^{-3}, the same as used in our preincubation experiments, repair synthesis occurred in the *trans*-DDP-modified plasmid DNA when incubated with cytosolic extracts from 293 and HeLa cells and whole cell extracts from HeLa cells. These data support the hypothesis that there is an activity in mammalian cells capable of repairing *trans*-DDP-modified DNA at lower $(D/N)_b$ levels than for *cis*-DDP-modified DNA. Such repair synthesis activity was detectable by Wood et al. (1988) in cytosolic extracts but was not pursued owing to excessive background nuclease activity. Under the conditions reported here, this background was not of a magnitude that it interfered with out ability to make measurements of repair synthesis in cytosolic extracts.

It would be of interest to know the level of platination and the type of each platinum adduct present in the template prior to and following preincubation with cytosolic extract. The former information could not be obtained, however, owing to the insensitivity of platinum AAS on such small samples of DNA. The latter information would be best obtained by the use of site specifically platinated DNAs (Naser et al., 1988). Knowledge of this kind would enable us to determine whether restoration of template activity coincided with removal of a single type or subset of the full collection of *trans*-DDP DNA adducts. Further characterization of the activity is required before its biological function can be defined.

Taken together, the present results demonstrate the power of the in vitro replication system to assess the role of DNA binding agents in blocking replication. Bifunctional adducts of *trans*-DDP are as inhibitory as those of cisplatin. This result is significant, for it indicates that failure to form such adducts in vivo, or their selective repair, could be responsible in part for the differential biological activities of the two isomers. This information could lead to a rationale for designing drugs based on trans stereochemistry. The present studies also reinforce the notion that selective repair of *trans*-DDP-modified template

DNA can be an important determinant of the selective antitumor properties of *cis*- versus *trans*-DDP, while not excluding data that indicate inactivation of *trans*-DDP by sulfur-containing compounds in the cell (Eastman & Barry, 1987). Of additional significance is the ability of the present assay to reveal the binding and subsequent processing of platinum–DNA adducts by cellular components. Ultimately, the method should prove useful in the purification and characterization of these components and for studying other DNA-damaging agents.

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Registry No. *cis*-DDP, 15663-27-1; *trans*-DDP, 14913-33-8; $[Pt(dien)Cl)]^+$, 14215-58-8.

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