Biochemical Pharmacology, Vol. 44, No. 1, pp. 107–119, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

DIFFERENTIAL EFFECTS OF CISPLATIN ON THE EXPRESSION OF CHIMERIC MARKER GENES IN CV-1 CELLS

GREGORY L. EVANS and JAY D. GRALLA*
Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90024-1569, U.S.A.

(*Received* 13 *January* 1992; *accepted* 25 *March* 1992)

Abstract—The effect of the antitumor drug cisplatin on marker gene expression in CV-1 monkey cells was measured. When non-replicating test genes were introduced by transient transfection, there was strong differential inhibition caused by the drug. Expression of certain genes was relatively insensitive, but expression of others was inhibited as strongly as was DNA replication. Stronger promoters led to stronger inhibition. This selective inhibition was not observed with the pharmacologically inactive isomer transplatin. The results raise the possibility that inhibition of strongly expressed genes by cisplatin may contribute to the antitumor activity of the drug.

Introduced by Rosenberg and colleagues, *cis*-diamminedichloroplatinum(II) (*cis*-DDP†; cisplatin) is one of the most effective antitumor drugs now in clinical use (see Ref. 1). It is widely used both alone and in combination chemotherapy for the treatment of several types of human cancers. The principal target of attack of *cis*-DDP within the cell is generally believed to be DNA [2, 3]. When it attaches to DNA *in vivo*, it forms both monofunctional and bifunctional adducts (for a review, see Ref. 4). The latter lesions include cross-links where two guanosines on the same strand are connected via N(7) positions by a platinum bridge. These intrastrand cross-links at G-strings distort the structure of the DNA [5] and interfere with its function [6–8]. *Trans*-DDP (transplatin), an isomer of *cis*-DDP, is pharmacologically inactive and less cytotoxic and mutagenic than *cis*-DDP [9], probably because it is stereochemically incapable of forming intrastrand cross-links at adjacent guanosines [10].

The cellular function that is the primary target of *cis*-DDP attack has been thought to be DNA replication. In cultured human amnion AV3 cells and in fresh human lymphocytes, *cis*-DDP inhibits bulk DNA synthesis before inhibition of bulk RNA or protein synthesis can be detected [11, 12]. In cultured AV3 cells and in murine Ehrlich ascites tumor cells *in vivo*, DNA synthesis was also selectively inhibited at low doses of the drug [11, 13]. In addition, in the same two studies, DNA synthesis was never restored to high levels, unlike bulk RNA and protein synthesis. Largely for these reasons, it has been assumed that the drug functions by inhibiting DNA replication. The observed inhibition of gene expression at high drug doses was thought to be a secondary consequence of the inhibition of template replication. This was consistent with the results of other studies which showed only minimal effects of *cis*-DDP on the activity of individual enzymes (e.g. alkaline phosphatase, succinic dehydrogenase, and Na^+,K^+-ATPase) *in vivo* [14, 15]. Little is known about the effects of *cis*-DDP on the function of RNA *in vivo*, except that it is a strong physical target for modification by the drug (or *cis*-DDP) [2].

Although these observations are not in doubt, recent studies have questioned the central role of DNA synthesis arrest and raised the possibility that direct inhibition of gene expression could contribute importantly to the efficacy of the drug [7, 16–18]. If the inhibition of gene expression contributes significantly to the pharmacological activity of *cis*-DDP, it would have to be highly selective. That is, a subset of critical genes would have to be especially sensitive to drug attack since it is clear that the bulk of the inhibition of gene expression is rather insensitive to *cis*-DDP [11, 15]. We suggested previously that two properties associated with this selective sensitivity might be high promoter strength and strings of guanosines [7, 16]. Another variable might be gene length with longer genes providing more potential targets for drug modification.

We have used CV-1 African green monkey kidney cells as a model system to test the possibility that the expression of individual genes which differ in their regulatory regions or gene bodies can be differentially sensitive to inhibition by *cis*-DDP. These effects on individual genes were compared to effects on bulk cellular DNA synthesis, the proposed primary functional target of the drug, as well as cellular RNA and protein synthesis and long-term cell survival. Although CV-1 cells are not transformed or tumorigenic, they have been shown to respond to *cis*-DDP by incurring a typical pattern of lesions

* Corresponding author: Dr. Jay D. Gralla, Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, 405 Hilgard Ave., Los Angeles, CA 90024-1569. Tel. (310) 825-1620; FAX (310) 206-7286.

† Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II), cisplatin; *trans*-DDP, transplatin; TCA, trichloroacetic acid; PEG, polyethylene glycol; and ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

[19]. They have been used previously in several studies addressing the effects of *cis*-DDP on the genome of the DNA tumor virus SV40 for which they are a permissive host [16, 20].

We have tested the capacity of gene regulatory regions and gene bodies to confer *cis*-DDP sensitivity to expression of marker genes using a transient expression assay. Transient expression assays have been used previously to expose altered capacities for plasmid DNA repair in *cis*-DDP-sensitive Walker rat tumor cells [21], in *cis*-DDP-resistant murine L1210 leukemia cells [8], and in cells derived from xeroderma pigmentosum patients [22]. The regulatory regions of *ras* and certain other oncogenic viral genes, as well as the very long *Escherichia coli* β-galactosidase gene body, are shown to be associated with unusually great sensitivity of marker gene expression to *cis*-DDP in this system. Moreover, the IC_{50} values for expression of certain individual genes are comparable to that for bulk cellular DNA synthesis and long-term cell survival. These results support the possibility that inhibition of the expression of certain genes involved either in the control of cell proliferation or cell death (apoptosis) may contribute to *cis*-DDP-mediated cell death.

MATERIALS AND METHODS

Platinum drugs and treatment. *Cis*-DDP was obtained from Johnson-Matthey, West Chester, PA, and from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and purchased from the Sigma Chemical Co., St. Louis, MO. Similar effects on transient gene expression were obtained with *cis*-DDP from the three different sources. *Trans*-DDP was purchased from Sigma. Drugs were stored desiccated at room temperature in the dark and dissolved 2 hr before use in sterile 150 mM NaCl [20].

African green monkey CV-1 cells (a gift from Dr. J. Jordan, UCLA) were maintained on tissue culture dishes (Corning, Corning, NY) in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) with high glucose, without glutamine, and supplemented with penicillin (300 U/mL), streptomycin (300 U/mL), fungizone (1 μg/mL), and 5% calf serum (GIBCO). To achieve maximum transfection efficiencies for the transient expression experiments and maximum reproducible sensitivity to platinum drugs, cells were never allowed to become confluent.

Cell viability and clonogenicity. Cell viability was determined by the trypan blue dye exclusion method, according to the supplier's (GIBCO) recommendations. Clonogenicity (long-term survival) was measured in a standard colony formation assay as described [23]. Briefly, cells were replated in triplicate at several different densities (usually 100–10,000) in 6-cm dishes in growth medium. Approximately 17 days after plating, dishes were fixed and stained with 1% methylene blue in 70% methanol, and colonies with 50 or more cells were scored.

Measurement of cellular DNA, RNA, and protein synthesis. Approximately 2×10^5 cells were seeded on 6-cm dishes and incubated in 5 mL of growth medium plus 5 or 10% calf serum (as indicated in the figure legends) for 24 hr prior to drug addition. Duplicate or triplicate plates received at least eight different concentrations of drug, ranging from 0 to 25 μM for *cis*-DDP and 0 to 200 μM for *trans*-DDP. Cultures were incubated in the presence of external drug for 24 hr. For 24-hr time points, macromolecular synthesis was measured during the last 2 hr of this incubation. For 48-hr time points, drug-containing medium was removed after 24 hr and replaced with drug-free medium; synthesis was measured during the last 2 hr of the incubation. In some experiments, DNA synthesis was measured throughout the entire 48-hr incubation period. Cellular DNA, RNA, and protein synthesis were measured by incorporation of [*methyl*-^{3}H]thymidine (New England Nuclear, 80 Ci/mmol), [5-^{3}H]uridine (Amersham, 30 Ci/mmol), and [L-4,5-^{3}H]leucine (Amersham, 140 Ci/mmol), respectively, into trichloroacetic acid (TCA)-precipitable material, using the procedure of Harder and Rosenberg [11]. For protein synthesis experiments, aminoacyl tRNAs were then hydrolyzed in 2% hydrogen peroxide, 1 N NaOH for 10 min at 37°.

Nuclear RNA synthesis. Approximately 5×10^5 cells were seeded on 10-cm dishes and incubated in 10 mL of growth medium plus 5% calf serum for 24 hr. Fresh *cis*-DDP stock solution prepared as above as added to duplicate or triplicate plates to give at least seven final concentrations ranging from 0 to 25 μM. Nuclei were isolated 24 hr later using Method I of Landes and Martinson [24]. Nuclei isolated from one dish were resuspended in 400 μL of cold TGMED buffer [50 mM Tris–HCl, pH 7.9, 20% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol] [24] and used immediately for nuclear transcription *in vitro*. Nuclear transcription was assayed using a procedure combined from Landes and Martinson [24] and Ben-Zeev and Becker [25]. Nuclei in 200 μL TGMED buffer were preincubated in the presence or absence of 0.2 μg/mL α-amanitin (Boehringer–Mannheim, Indianapolis, IN) for 5 min on ice [25]. Nuclei were then added to 250 μL IVTM buffer [100 mM Hepes, pH 7.6, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 8 mM phosphoenol pyruvate, 15 ng/mL pyruvate kinase (Sigma), 2 mM 2-mercaptoethanol, 300 mM KCl, 1 mM each of ATP, GTP, and CTP] [24] and 30 μL of water. Reaction mixtures were further preincubated at 31° for 5 min and then initiated by the addition of 20 μCi (20 μL) of [5-^{3}H]uridine 5′-triphosphate (UTP; New England Nuclear, 25 Ci/mmol). RNA synthesis was allowed to proceed for 40 min at 31° [25]. Reactions were terminated by removal of samples to tubes on ice containing 5 mL of 10% TCA and 20 mM tetrasodium pyrophosphate [25]. After a 10-min incubation on ice, incorporation was measured by collection of TCA precipitates and scintillation counting as for bulk cellular biosynthesis experiments. Background was measured by adding radioactive precursor to nuclear transcription mixtures on ice and immediately processing samples for TCA precipitation as before.

Plasmid DNAs. Each CAT plasmid contained a chimeric gene consisting of a 5′ transcriptional fusion

of eukaryotic promoter region DNA to a core gene body formed of the bacterial *cat* coding sequences (700 bp) followed by SV40 small t intron (66 bp) and SV40 early polyadenylation sequences [26]. Each CAT plasmid encodes a 1.6 kb CAT mRNA. While this core transcription unit is identical in each plasmid, the background of adjacent plasmid maintenance DNA is not. For eight of the ten CAT transcription units, the background DNA is identical and the only DNA sequence differences lie in promoter region DNA. The other two transcription units, found in pE3cat and pHβAPr-1-cat, are different from the majority and from each other in this respect. In these two, as compared to the rest, there are DNA sequence differences in promoter DNA as well as in background DNA. For all of the promoters and plasmids used [26–34], mRNA initiation sites have been mapped in plasmid-based transient expression experiments in either CV-1 or HeLa cells.

The five BGAL plasmids used all contain 5′-transcriptional fusions of cellular or viral regulatory DNA to the bacterial *β-gal* gene in identical plasmid backgrounds. They were derived from pCH110 (a gift of C. P. Ordahl), which has been described previously [34]. This plasmid contains SVER fused to β-galactosidase coding sequences, followed by SV40 polyadenylation sequences, and does not contain any introns. It contains essentially the same plasmid background sequences as pSV2cat.

pHIVbgal was constructed as follows. A derivative of plasmid pUC19 which contained the HIV LTR (−177 to +83) region in its polylinker was cut with Eco RI, blunt-ended with DNA polymerase, ligated with Hind III linkers, and cut with Hind III to produce a 300 bp HIV fragment with Hind III ends. This fragment was inserted into pCH126 by standard cloning procedures. pE3bgal was constructed in the same way starting with the pUC19 precursor of pE3cat [32]. pβactbgal was constructed in a similar way starting with plasmid pHβAPr-1-cat, which contains the human β-actin promoter region bounded by Hind III and Eco RI sites. These BGAL plasmids encode mRNAs approximately 4.0 kb in length, of which 3.3 kb are *β-gal* coding sequences.

Transfections and treatment with platinum drugs. Transient transfections of CV-1 cells were performed using a calcium phosphate technique modified from Shen *et al.* [35]. Immediately after the addition of transfection mix, various concentrations of platinum drugs were added in duplicate to dishes of transfected cells. For each DNA used, transfected cells received a total of four to eight concentrations of drug, ranging from 0 to 20 μM for *cis*-DDP and 0 to 200 μM for *trans*-DDP. After incubation at 37° for 4 hr, drug containing medium was aspirated and transfected cells were subjected to a polyethylene glycol (PEG)-sucrose shock (2 mL of a solution of 40% PEG, 7% sucrose per dish) at room temperature for 2 min, as described [35]. After replacement of drug-containing medium (10 mL), dishes of transfected cells were incubated for approximately 18 hr at 37°. Drug-containing medium was again replaced with drug-free medium 24 hr after transfection and transfected cells were incubated for a further 24 hr at 37° until they were harvested. Early transfections were done with one test plasmid DNA while later ones included two or three.

Transfected cells were harvested 48 hr after transfection. CAT extracts were prepared and assayed for CAT activity as described [26], with each assay containing one-half of a 10-cm dish-equivalent of cell extract. BGAL extracts were prepared 48 hr after transfection as described [34], and activity was measured colorimetrically via $O.D._{420}$ using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate [34] and one-half of a dish-equivalent of cell extract.

RESULTS

As a prelude to measurement of the effects of *cis*-DDP on the transient expression of individual genes in CV-1 cells, we began with measurement of the cytotoxic effects of the drug under conditions compatible with the measurement of expression from transfected plasmids. The cytotoxicity was measured in cells transfected with plasmids to account for any effect of the transfection protocol on toxicity. Additionally, since expression from plasmid transfectants typically takes a variable number of hours to develop, we used a treatment protocol that would be long enough to allow reproducible modification in the transfected system. Cultured, exponentially growing CV-1 cells were transfected with plasmids bearing chimeric marker genes, and then were treated with various concentrations of *cis*-DDP for 24 hr. Cells were then incubated for a further 24 hr in the absence of drug, at which time the number of cells that excluded the dye trypan blue was counted. The results (Fig. 1A) show that under these conditions 50% of the cells remained viable at approximately 3 μM *cis*-DDP. In an identical experiment done in the presence of *trans*-DDP, the IC_{50} value of *trans*-DDP for cell viability was approximately 90 μM (data not shown). These relative viability values for the two drugs are in good agreement with those seen earlier in human HeLa cells using a different drug treatment protocol and a colony formation assay [2]. We conclude that the IC_{50} value of 3 μM for *cis*-DDP is a reliable indication of the sensitivity of cells to general toxicity induced by the drug.

To test the long-term survival under these conditions, cells were transfected and treated with *cis*-DDP as above, but were then assayed for colony formation, or clonogenic survival, using standard procedures. The results are shown in Fig. 1B. The IC_{50} value of *cis*-DDP for clonogenicity using this protocol was approximately 0.3 μM. Thus, while most cells remain viable at *cis*-DDP concentrations below 3 μM, these cells are destined to die. For measurement of gene expression the cells will be harvested at this same time and the expression will reflect the amount accumulated over the entire 48-hr period. Thus, at 48 hr expression, especially below approximately 3 μM drug, will have occurred in cells which retain membrane integrity but are destined to die.

To be able to compare expression of individual genes with average cell properties, we determined by quantitative analysis the effects of *cis*-DDP on

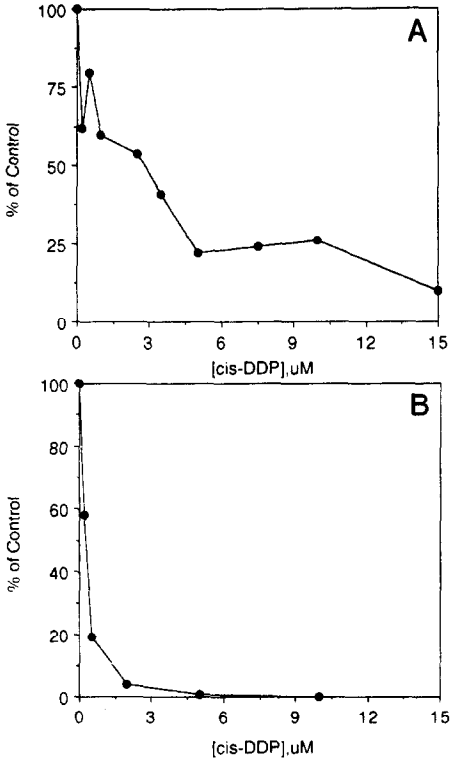


Fig. 1. Effect of *cis*-DDP on viability and long-term survival of transfected CV-1 cells. Cell viability (trypan blue dye exclusion) and clonogenicity (colony formation) were measured as a function of *cis*-DDP concentration as described in Materials and Methods. Transfected cells were incubated in medium containing *cis*-DDP for 24 hr, and then in drug-free medium for 24 hr before assay, as for transient expression experiments. (A) Viability. The control value was 110 ± 15 dye-excluding cells per large hemacytometer square. (B) Clonogenicity. The control value was 87 ± 20 colonies per 6-cm dish. Cell viability, clonogenicity, and transient expression assays were either done in the same experiment or in parallel experiments. The control value for one-half of a 6-cm dish equivalent of cell extract were 10,200 ± 1,300 cpm for DNA synthesis, 21,100 ± 3,000 cpm for RNA synthesis, and 8,200 ± 1,200 cpm for protein synthesis. The data shown represent the average of duplicate experiments for each assay.

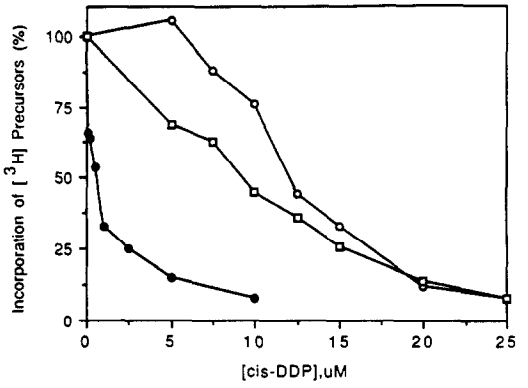


Fig. 2. Effect of *cis*-DDP on bulk cellular macromolecular synthesis in CV-1 cells. DNA (●), RNA (○), and protein (□) synthesis were measured by incorporation of [^{3}H]-thymidine, [^{3}H]uridine, and [^{3}H]leucine, respectively, in separate experiments. Cells in 5% serum were treated with drug for 24 hr and precursors were added for the last 2 hr of the incubation. The data shown represent averages from duplicate experiments. Background counts were typically less than 1% of sample counts in the presence or absence of drug.

the production of proteins and nucleic acids. A series of plates of exponentially growing CV-1 cells were treated with increasing amounts of *cis*-DDP for 22 hr. Radioactive precursors for macromolecular synthesis were then added for 2 hr to allow synthesis of radioactive macromolecules. The cells were then broken and the radioactive macromolecules were collected by acid precipitation, using standard procedures. In separate experiments, [^{3}H]leucine was used to assay for protein synthesis, [^{3}H]uridine for RNA synthesis, and [^{3}H]thymidine for DNA synthesis.

The results of these studies on bulk synthesis are displayed in Fig. 2. The data show that the half-inhibitory value (C) of *cis*-DDP for cellular DNA synthesis was approximately 0.6 μM added drug, very similar to the *cis*-DDP IC_{50} value for clonogenic survival (0.3 μM; Fig. 1B), and well below that for cell viability at 48 hr (3 μM; Fig. 1A). Similar results were seen when the effect of *cis*-DDP on DNA synthesis was measured with the radioactive precursor present over a 48-hr period (data not shown). By contrast, approximately 12 and 9 μM drug were required to half-inhibit bulk RNA and protein synthesis, respectively. Results from identical experiments carried out with *trans*-DDP also showed selective inhibition of cellular DNA synthesis at approximately 10-fold higher doses of drug (data not shown). The results demonstrate that in CV-1 cells bulk cellular RNA and protein synthesis are relatively insensitive to *cis*-DDP, in good agreement with the original more qualitative studies in human amnion AV3 cells [11]. We also remeasured these properties using a higher 10% serum concentration and found that the increase in serum led to a slightly increased sensitivity of bulk RNA and protein synthesis, but not DNA synthesis, to *cis*-DDP (data not shown); the transient expression studies described below are carried out under these conditions.

The interpretation of the RNA inhibition studies can be confusing since the measurement is dominated by the production of stable RNA, but includes mRNA as well. The effect of *cis*-DDP on the synthesis of mRNA specifically was measured since it had not been done previously and the possibility existed that it would be selectively inhibited by *cis*-DDP. This measurement was done by adapting the standard nuclear run-on transcription procedure for measuring mRNA to drug-treated CV-1 cells. Cells were treated with various concentrations of *cis*-DDP as described above, and after 24 hr nuclei were prepared. RNA synthesis that had been initiated *in vivo* was completed *in vitro* using radioactive

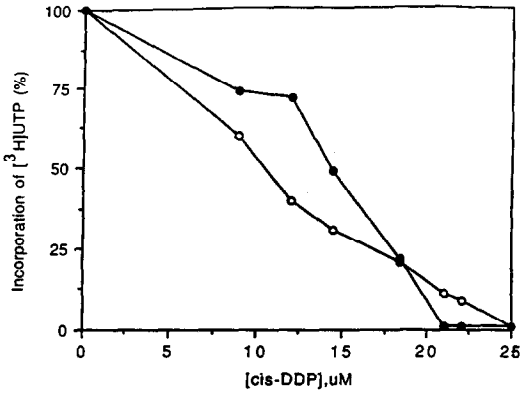


Fig. 3. Effect of *cis*-DDP on α-amanitin-sensitive and α-aminitin-resistant nuclear RNA synthesis. Cells in 5% serum were treated with drug for 24 hr, nuclei were prepared, preincubated with or without 0.2 μg/mL α-amanitin, and nuclear RNA synthesis was measured *in vitro* by incorporation of [^{3}H]uridine triphosphate (UTP). The control values were 13,300 ± 2,700 cpm for α-amanitin-sensitive RNA synthesis and 18,400 ± 3,700 cpm for α-amanitin-resistant RNA synthesis. The data shown for α-amanitin-sensitive (●) and α-amanitin-resistant (○) nuclear RNA synthesis represent averages of quadruplicate experiments. Background counts were typically less than 15% of sample counts in the presence or absence of drug.

nucleotides. Half of each sample of isolated nuclei had been preincubated with the RNA polymerase II-specific inhibitor α-amanitin. Thus, the synthesis of stable RNA and mRNA was distinguished by comparing α-amanitin-resistant and α-amanitin-sensitive runoff RNA synthesis [36]. Figure 3 shows the result of this experiment and indicates that neither mRNA synthesis (C 14 μM) nor stable RNA synthesis (C 11 μM) was significantly more sensitive to *cis*-DDP than bulk RNA synthesis. Similar results were reported recently for a related experiment carried out in murine L1210 leukemia cells [37], which are known to be very sensitive to *cis*-DDP *in vitro* and *in vivo*.

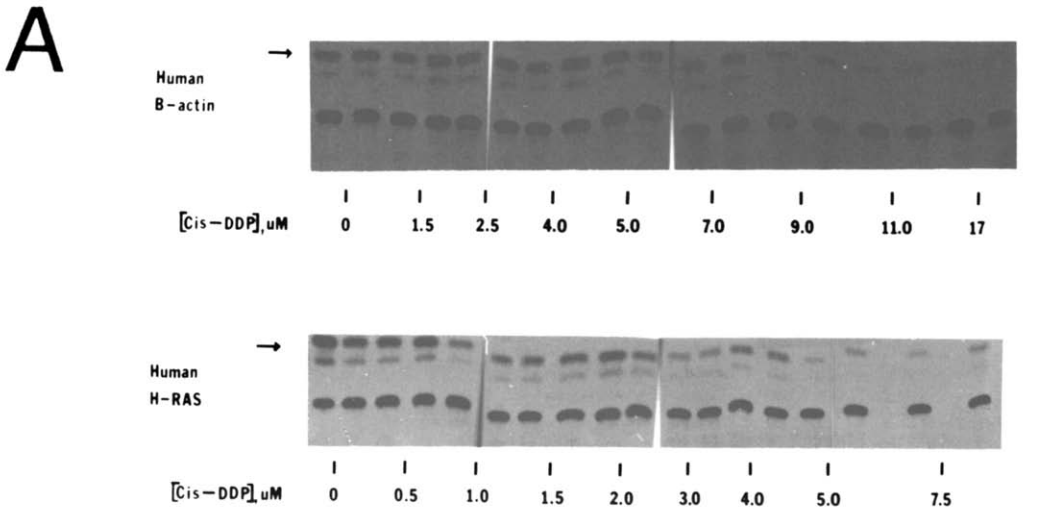
Differential inhibition of human ras *and actin constructs*. The possibility remained that expression from a minority of gene regulatory regions would be inhibited selectively by *cis*-DDP. To eliminate the possibility that such inhibition would be an indirect consequence of arrest of DNA synthesis of that gene, we introduced non-replicating plasmids into CV-1 cells. Initially, two plasmids were chosen for comparison of human *ras* and human actin [33, 38–40]. The plasmids were essentially identical (see Materials and Methods) except that each contained a different human cellular promoter region linked directly to the identical *E. coli* chloramphenicol acetyl transferase (*cat*) marker gene. Under these conditions, expression of CAT enzyme activity in these cells was dependent on gene expression initiated from the test promoter regions, as has been demonstrated previously in CV-1 and other mammalian cell lines [26, 27].

Plasmid DNAs were introduced into exponentially growing CV-1 cells by the calcium phosphate-mediated transfer procedure. Immediately afterward, transfected cells were treated with various amounts of *cis*-DDP for 24 hr. The external drug was then removed by replacement of medium and 24 hr later the cells were broken and plasmid-directed CAT activity was determined. The concentrations of drug used ranged from less than that required to inhibit bulk DNA synthesis to more than that required to inhibit bulk RNA synthesis.

Representative CAT assays for *ras*- and β-actin-directed CAT expression in the presence of drug are shown in Fig. 4A. These results show, and their quantitative comparison (Fig. 4B) confirms, that the two promoters conferred quite different sensitivities to *cis*-DDP. The β-actin driven gene was half-inhibited at approximately 5 μM drug, approximately 2-fold more sensitive than bulk RNA expression (C 12 μM; Fig. 2 and above). This difference was smaller when the bulk measurement was done in the same 10% serum used for the CAT measurement. By contrast, *ras*-driven expression was half-inhibited at a much lower *cis*-DDP concentration. Half-inhibition for *ras* was achieved at approximately 0.7 μM drug, a concentration similar to that required to half-inhibit clonogenic survival (0.3 μM; Fig. 1B), and cellular DNA synthesis in these cells (0.6 μM; Fig. 2 and above). The similarity with cellular DNA synthesis was also seen when DNA synthesis was measured in transfected cells with the drug protocol for transient expression and a 48-hr labeling period, conditions which may more closely mimic the transient expression experiments (data not shown). *ras*-CAT expression was similarly inhibited when drug was added 6–12 hr after transfection (data not shown). The comparison betwen plasmid-based and cellular inhibitions is somewhat uncertain since different assays with somewhat different requirements are used. However, the order of magnitude difference between *ras* and β-actin sensitivities must be due to different properties of the two regulatory regions.

Next, the possibility that this unusual sensitivity of the *ras* promoter was associated with the pharmacologically active form of the drug was tested. Identical experiments were done using *trans*-DDP, the pharmacologically inactive isomer of *cis*-DDP. As shown in Fig. 5, much higher concentrations of *trans*-DDP than *cis*-DDP were required to half-inhibit *ras*-driven CAT expression. The observed IC_{50} value of 20 μM *trans*-DDP was approximately 30-fold greater than that observed for the active form of the drug (0.7 μM), supporting the significance of the latter sensitivity. The data in Fig. 5 also show that the differential sensitivity of the *ras* and β-actin promoter regions virtually disappeared when the pharmacologically inactive isomer of the drug was used. Both promoters are associated with a sensitivity to *trans*-DDP of approximately 20 μM. Thus, *ras*-driven CAT expression was selectively sensitive only to the active form of the drug.

The greater activity of *cis*-DDP over *trans*-DDP (4-fold for β-actin, 30-fold for *ras*; Figs. 4 and 5) was of the same relative order as that seen in the bulk cellular protein synthesis experiments that did not involve plasmids (13-fold; Fig. 2 and data not shown) and was also about the same as that seen in



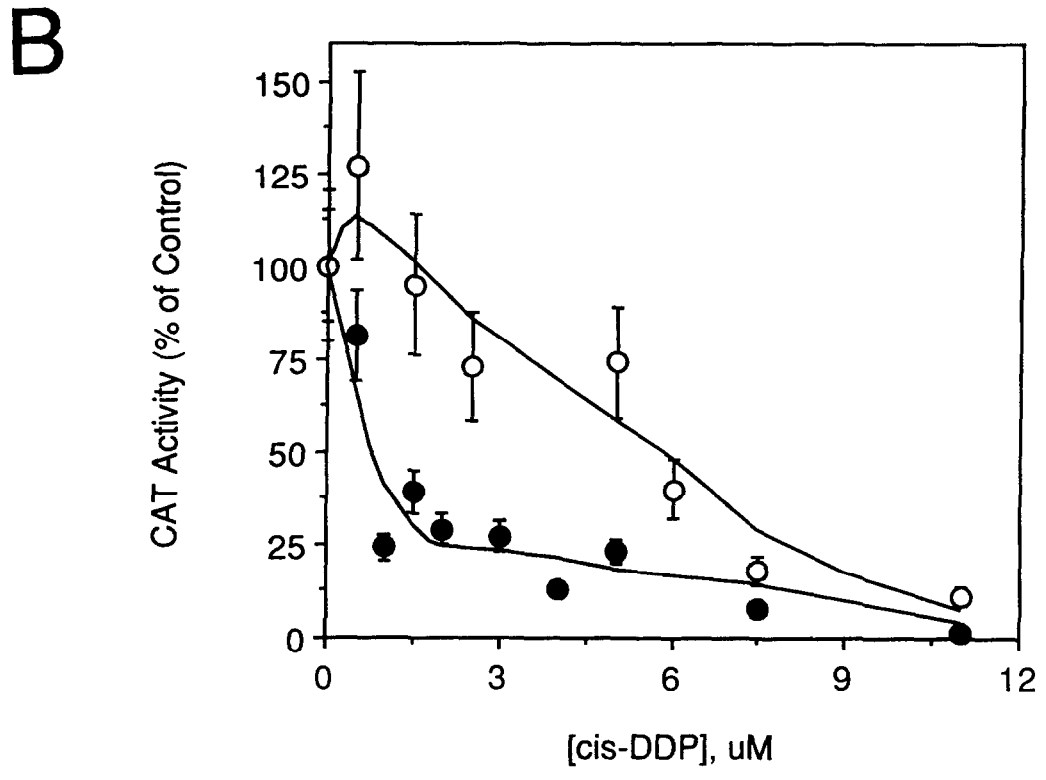


Fig. 4. Effect of *cis*-DDP on transient CAT expression driven by the human Harvey *ras* and β-actin promoters. Cells in 10% serum were transfected with *ras*-CAT (●) or β-actin-CAT (○) test genes, treated with drug for 24 hr, and then assayed for CAT activity 24 hr later. (A) CAT assays. (B) Quantitative analysis of assays represented in (A). The control values were 48.7 ± 7.3% conversion activity for *ras*-CAT and 15.3 ± 2.1% for β-actin-CAT. CAT activity typically varied by less than 20% between duplicate cell lysates within the same experiment. Data are shown as means ± SD of triplicate experiments for each construct.

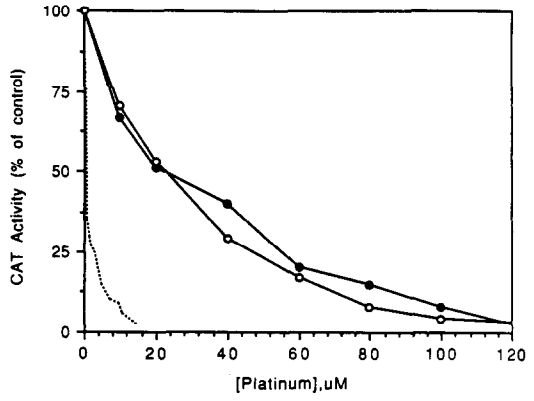


Fig. 5. Effect of *trans*-DDP on transient CAT expression driven by the human Harvey *ras* and β-actin promoters. *ras*- (●) and β-actin- (○) directed CAT activity were measured as a function of *trans*-DDP concentration. Experiments were performed as described in the legend to Fig. 4. Data for the effect of *cis*-DDP on *ras*-directed CAT activity (---) are reproduced from Fig. 4. The control value for the *trans*-DDP data was 52.1 ± 9.1% conversion activity. The data shown represent the average of two to four experiments.

the cell viability assay (30-fold; Fig. 1A and data not shown), and in the cellular assays for DNA synthesis (20-fold; Fig. 2 and data not shown).

Differential inhibition of a range of CAT constructs. As a complement to this comparison of human gene promoters, the sensitivity conferred by several other readily available regulatory regions was screened. These are active in CV-1 cells when used to drive the marker *cat* gene. They included the SV40 early region (SVER) containing the early promoter plus enhancer and the human immunodeficiency virus (HIV-1) LTR, both of which are functionally dependent on GC boxes [41, 42]. They also included the adenovirus 2 major late promoter (MLP), Rous sarcoma virus (RSV) LTR, adenovirus 5 E3 promoter, and the adenovirus 2 IVa2 promoter with the SV40 enhancer (IVa2PE) [32, 43–45]. Each of the regulatory regions was contained on essentially identical CAT plasmids (see Materials and Methods). The effect of *cis*-DDP on gene expression directed from each of these six additional plasmids was measured in the transient expression assay as before.

The results of these experiments (Table 1) indicate that a 20-fold range of sensitivity exists within this set of eight promoters. *Cis*-DDP IC_{50} values for CAT activity directed by these six, as well as the two plasmids discussed above, ranged from a low of 0.3 μM to a high of 6.5 μM. Five of the promoters conferred high sensitivity to *cis*-DDP, requiring between approximately 0.3 and 0.7 μM drug for half-inhibition. Three promoters were rather insensitive with half-inhibition values ranging from approximately 4.8 to 6.5 μM. By contrast, three of the *cis*-DDP-sensitive promoters and one of the insensitive promoters were equally insensitive to *trans*-DDP in this assay (Fig. 5 and data not shown). Also shown in Table 1 are the relative promoter strengths, estimated from CAT activity measured in control plates of CV-1 cells, and the relative density of potential (GG) drug targets in the transcriptional control elements of each of the eight promoters tested (discussed below). Collectively, this set of eight promoters is not likely representative of cellular promoters since many of them are viral, having been cloned and studied because of their involvement in transformation by these viruses. Nevertheless, the data demonstrate that the sensitivity to *cis*-DDP

Table 1. Inhibition of transient CAT expression in CV-1 cells by *cis*-DDP

Construct motifs	IC_{50} (μM)	Promoter strength	(GG) density in promoter
MLP-CAT	0.3 ± 0.1	460	6.6
SVER-CAT	0.3 ± 0.1	100	4.1
HIV-CAT	0.4 ± 0.1	30	5.7
RSV-CAT	0.6 ± 0.1	160	1.8
ras-CAT	0.7 ± 0.1	50	14.3
E3-CAT	4.8 ± 0.3	13	3.8
β-act-CAT	5.3 ± 1.5	11	3.8
IVa2PE-CAT	6.5 ± 0.5	10	4.5
E-CAT-O	ND*	2.5	ND
O-CAT	ND	1.0	ND

The IC_{50} values were determined from concentrations–response curves of the type shown for *ras*-CAT and actin-CAT in Fig. 4. Values are means ± SD, N = 3–7. The promoter strength is the estimated relative percent conversion of [^{14}C]chloramphenicol to its acetylated derivatives in 2 hr in equal plate fractions of lysates from cells transfected without platinum drugs. The absolute value for one-tenth of a plate-equivalent of MLP-CAT lysate was 48.1 ± 9.3%. The density of GG motifs was based on 25 bp of transcriptional control elements (60–210 bp) previously identified via DNA binding, *in vitro* transcription, and *in vivo* transcription studies.

* ND = not determined.

associated with the cellular human Harvey *ras* promoter region is mimicked by at least a subset of promoters active in mammalian cells.

Transient expression of the most sensitive promoters was half-inhibited at near 0.5 μM drug which is much lower than the concentrations required to half-inhibit bulk RNA and protein synthesis. This inhibition also occurred in cells that were 80–100% viable (see Fig. 1A) and was very similar to the inhibition of clonogenic survival (see Fig. 1B). The most insensitive promoters required an order of magnitude more drug, more like the amounts required to inhibit bulk RNA and protein synthesis. This latter dose was similar to that which half-inhibited cell viability, as measured by the dye exclusion assay. This rough coincidence suggests that the inhibition of this less sensitive class of promoters may be an indirect consequence of general drug toxicity, as proposed previously for all of gene expression [2, 11].

Table 1 shows that most of the sensitive promoters are quite strong. Thus, it was possible that the inhibition of sensitive CAT genes was an indirect consequence of accumulation of a large mass of test gene mRNA. If this were true, then the sensitivity should decrease if less DNA is introduced into the cell. To test these possibilities, transient expression assays were repeated under conditions of reduced input DNA for two of the sensitive CAT genes. For MLP-CAT expression (Fig. 6A) there was no change in sensitivity over a 100-fold range in the level of input DNA, corresponding to an approximately 40-fold range in CAT activity (data not shown). Thus, for MLP-CAT, the amount of CAT RNA and enzyme made does not determine *cis*-DDP sensitivity. At the HIV promoter the situation was slightly more complicated. The sensitivity (Fig. 6B) was altered slightly with reduced input DNA. This effect only occurred at concentrations that were higher than the C. Thus, for both constructs the accumulation of a high mass of CAT RNA and protein is not responsible for the low IC_{50} values.

Gene body effects on inhibition. These results have shown that the promoter can influence how sensitive gene expression is to inhibition. Next, we tested whether changing from the short CAT gene to the long β-galactosidase gene could also affect sensitivity. The coding region of the *E. coli* β-galactosidase gene is 3.3 kb in length compared to 0.7 kb for CAT [26, 34]. For the initial test, limited in part by plasmid availability, the effect of *cis*-DDP on BGAL expression directed by SVER and human β-actin was measured. The plasmids used, which do not replicate in these cells, contained these *cis*-DDP-sensitive and -insensitive promoter regions, respectively, as defined above, linked directly to the BGAL gene in identical plasmid backgrounds.

The results (Fig. 7) indicate that different gene bodies can confer different sensitivities to *cis*-DDP. BGAL genes driven by β-actin and SVER were half-inhibited at approximately 1.6 and 0.2 μM drug, respectively. In contrast, CAT genes driven by β-actin and SVER were half-inhibited at 5.3 and 0.3 μM drug, respectively (Table 1). Thus, there was an increased sensitivity to *cis*-DDP associated with BGAL test genes relative to CAT genes driven by

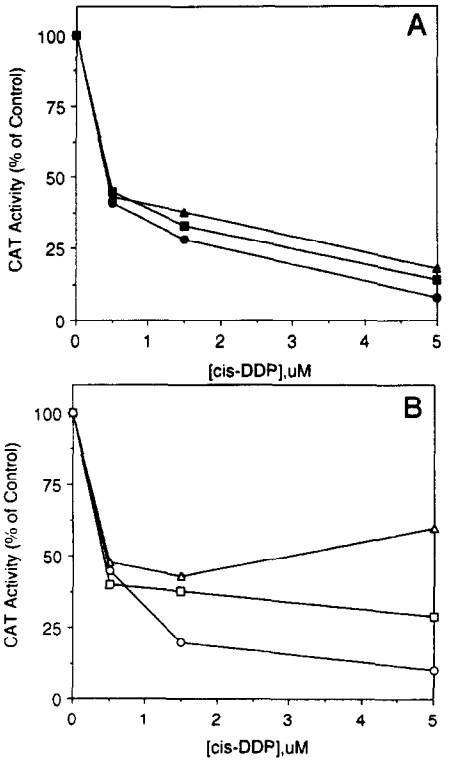


Fig. 6. Effect of input DNA level on *cis*-DDP sensitivity of MLP-CAT and HIV-CAT. MLP- (filled symbols) and HIV- (open symbols) directed CAT activities were measured as a function of *cis*-DDP concentration. Transient expression assays were carried out as described in the legend to Fig. 4 except with varying amounts of transfected test gene DNA. (A) Transfections with 0.1 (▲), 1.0 (■), and 10.0 (●) μg of MLP-CAT DNA. The control value was 49.2 ± 5.6% conversion activity. (B) Transfections with 1.0 (△), 3.0 (□), and 10.0 (○) μg of HIV-CAT DNA. The control value was 42.1 ± 7.7% conversion activity. Data shown are averages from duplicate experiments.

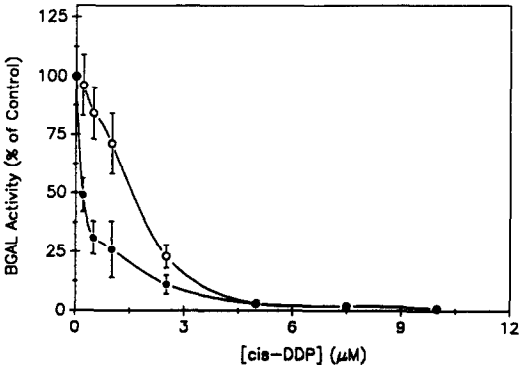


Fig. 7. Effect of *cis*-DDP on transient BGAL expression driven by the SVER and human β-actin promoters. SVER- (●) and β-actin- (○) directed transient BGAL activities were measured as a function of *cis*-DDP concentration. The control values were $O.D._{420}$ 0.580 ± 0.091 and 0.421 ± 0.075. Data are shown as averages ± range of duplicate experiments.

Table 2. Inhibition of transient BGAL expression in CV-1 cells by *cis*-DDP

Construct	IC_{50} (μM)	Promoter strength*
SVER-BGAL	0.2 ± 0.05	120
HIV-BGAL	0.3 ± 0.05	35
E3-BGAL	1.2 ± 0.2	15
β-act-BGAL	1.6 ± 0.3	10
O-BGAL†	ND‡	1

Values are averages ± range for two to three independent experiments for each test gene.

* Estimated relative control enzyme activity directed by each test gene. Absolute specific activity for SVER-BGAL was 153 ± 25 nmol ONPG cleaved/min/mg protein.

† BGAL activity directed by promoterless plasmid pCH126.

‡ ND = not determined.

these two promoters. In addition, as seen with the corresponding CAT genes, BGAL expression driven by SVER was about an order of magnitude more sensitive to drug than that driven by β-actin.

Concentration–response curves were also constructed for BGAL genes driven by one other sensitive (HIV) and one other insensitive (E3) promoter. In general (Table 2), the relative differences among promoter sensitivities were maintained. However, in all cases the longer BGAL constructs were more sensitive than the shorter CAT constructs. Since enzyme activities, i.e. active proteins, measured individually or in bulk are generally insensitive to inhibition by *cis*-DDP (see Introduction), it seems unlikely that the observed increased sensitivity of BGAL genes relative to CAT genes is mediated via selective drug attack on the BGAL protein, but this possibility cannot be ruled out completely.

DISCUSSION

These experiments have shown that the low concentrations of *cis*-DDP that are without significant effect on bulk gene expression can nevertheless inhibit expression in single-gene assays in mammalian cells. This effect occurred at pharmacologically relevant concentrations and was specific for the active form of the drug, the *trans* isomer being shown as non-inhibitory. The inhibition of gene expression in the transient expression assay occurred with an IC_{50} value as low as 0.2 μM added concentration, which compares to tissue concentrations as high as 33 μM in patients and 230 μM in animals (see Refs. 46 and 47) receiving therapeutic doses of *cis*-DDP. The low concentrations that inhibited gene expression selectively were of the same order as those which inhibited long-term cell survival of the same cells using the same protocol (Fig. 1B). The concentrations were also similar to those that led to inhibition of DNA replication (Fig. 2). These comparisons support the possibility [7, 17] that inhibition of the expression of a sub-set of especially sensitive genes could contribute to the cytotoxic effects of cisplatin on tumor cells.

These results were obtained using test plasmids containing two types of marker enzyme genes driven by eight different promoters. The plasmids were non-replicating and unintegrated and were assayed after transient transfection into cultured CV-1 monkey cells. Although several of these constructs were inhibited strongly at low concentrations of *cis*-DDP, several others were not. The inhibition of these least sensitive genes was similar to that of most cellular gene expression, as measured by parallel assay of bulk cellular RNA, mRNA, and protein synthesis under similar conditions in the same cells. Thus, the use of a single-gene assay revealed an unanticipated broad range of effects of *cis*-DDP, previously masked in studies where only bulk gene expression was measured.

As expected, the selective inhibition of expression was not an indirect consequence of drug-induced general cytotoxicity. That is, it occurred prior to the major effects of the drug on cell viability, as measured by parallel assay. By contrast, the inhibition of some plasmid genes, as typified by the human β-actin-CAT construct, as well as most cellular genes (bulk properties), occurred only when high concentrations of drug impaired cell viability. For these less sensitive genes, there was little difference in inhibition by *cis*- and *trans*-DDP, in contrast to the sensitive *ras* CAT gene where the active isomer was 30- to 40-fold more effective. Thus, inhibition of sensitive genes occurred only with the active form of the drug, at concentrations that did not inhibit bulk gene expression, and at times prior to the onset of changes leading to cell death. This contrasts with the inhibition of the bulk of cellular genes which may be an indirect consequences of general cell killing, as proposed previously [2, 11].

Similarly, the selective inhibition of certain genes cannot be an indirect consequences of inhibited replication of those genes since they are expressed from plasmids that do not replicate. Nevertheless, this inhibition occurred at concentrations that comparably inhibited cellular DNA replication in the same cells. Thus, the inhibition of expression of certain genes and of cellular DNA replication appears to occur independently at similar concentrations of *cis*-DDP. One possibility is that low doses of *cis*-DDP inhibit the production of proteins required for activation of both processes. These concentrations are much lower than those that inhibit bulk transcription. If cancer cells have a strong dependence for growth on the product of oncogenes with such sensitivities, then *cis*-DDP could interfere selectively with the growth of the tumor cell. High concentrations of *trans*-DDP, although equally toxic, did not exhibit this selectivity. Thus, *trans*-DDP toxicity is not caused by this phenomenon, consistent with its lack of selectivity towards tumor cells.

Contributions to gene sensitivity. Both the promoter region and the gene body contribute to the sensitivity of these genes to cisplatin. We consider first the effect of the promoter. We proposed previously that sensitive promoters might be those that are strongly expressed and contain strings of guanosines in their regulatory elements [7, 16]. Table 1 and 2 collect the measurements of promoter strength made in the

course of this study and the density of GG dinucleotides in the various promoters. In each table the promoters are arranged in order of decreasing sensitivity to *cis*-DDP. Qualitatively, the display indicates that the weaker promoters tend to be rather insensitive to inhibition and the stronger promoters tend to be more sensitive (see the quantitative analysis below). There appears to be no obvious correlation between the extent of sensitivity and the number of GG dinucleotides present in the promoter. Thus, superficially the prediction for the importance of promoter strength is supported but that for GG dinucleotides is not.

The importance of promoter strength can be seen independently in both the BGAL and CAT results. For the four BGAL constructs the hierarchy of decreasing promoter strength and decreasing drug sensitivity was the same (Table 2). For the CAT constructs (Table 1) the data for both sensitivity and promoter strength can be divided into two identical groups. The top group contains sensitive promoters and has promoter strengths ranging from relative 30 to 460. The bottom group contains insensitive promoters and has virtually identical relative promoter strengths ranging from 10 to 13. Since the hierarchy for promoter strength and drug sensitivity within the top group was not identical for the two variables, other influences on sensitivity are suggested. These may include the number of GG dinucleotides or other influences not proposed previously.

The data were analyzed further to search for semi-quantitative correlations. The IC_{50} values in these experiments were influenced by the loss of cells at moderate to high drug concentrations. To correct for this the activity was normalized to viable cell number, as determined in Fig. 1. For the sensitive CAT promoters the resulting curves were biphasic (not shown) with only about one-third of the cells being sensitive to very low concentrations and the remaining two-thirds much less sensitive. This second phase was similar to the relative *cis*-DDP insensitivity of bulk gene expression in viable cells. The phases may reflect different subpopulations of cells, perhaps in different phases of the cell cycle. In any case, we estimated the sensitivity of the most sensitive cells by determining an IC_{25} value from these curves. This collection of IC_{25} values was then used in regression analysis to search for correlations with promoter strength and GG dinucleotides.

The analysis showed no correlation with GG dinucleotides but did show a potential correlation between promoter strength and normalized *cis*-DDP sensitivity (Fig. 8). The correlation was excellent for the 4 BGAL constructs with a correlation coefficient (R^2) of 0.95. For the CAT constructs, six promoters lie perfectly on the line (but three separate promoters are collected as a point near the origin) and two promoters fall well off the line. These are HIV-CAT, which is about 30-fold hypersensitive to drug for its level of gene activity, and MLP-CAT, which is about 4-fold hyposensitive to *cis*-DDP for its level of gene activity. The MLP promoter directs the highest level of enzyme activity of all the CAT genes tested (Table 1). Thus, it may be that MLP displays the upper limit of sensitivity dictated by promoter

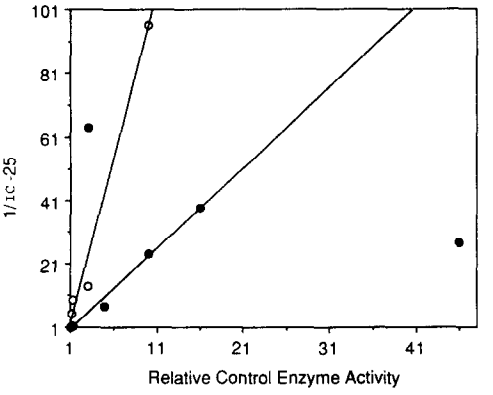


Fig. 8. Correlation between promoter strength and drug sensitivity. The promoter strength for CAT test genes (●) and BGAL test genes (○) was plotted against the inverse of their respective normalized *cis*-DDP IC_{25} values.

strength in this system and thus falls below the line. The cause of the unpredictably high sensitivity of HIV is not known but could reflect the slightly higher number of GG dinucleotides coupled with their appearance within regulatory motifs of established functional importance [42, 48]. These correlations are consistent with the suggestion that promoter strength can influence drug sensitivity through the well known phenomenon of the increased chromatin accessibility of actively transcribed genes (reviewed in Ref. 49). In this case, sensitivity would be mediated by direct drug attack on promoter DNA. However, our results do not exclude the possibility that *cis*-DDP is acting via a selective *trans*-regulatory effect, for example via alteration of the activity of certain transcription factors. Furthermore, they do not exclude a secondary influence related to the number of potential drug targets in the promoter.

Recall, however, that changing from the short CAT gene body to the longer BGAL gene body led to an increased drug sensitivity for all promoters tested. The analysis shows that this amounts to a 4-fold increase in sensitivity, as measured by the slopes of the curves of Fig. 8. This compares with a nearly 5-fold difference in the length of the BGAL (3.3 kb) and CAT (0.7 kb) coding regions inserted into these plasmids. Thus, the sensitivity parallels the size of the gene being transcribed. This may reflect the larger number of drug targets in the DNA of the gene or in its RNA transcript. Thus, the two properties of a gene that may induce sensitivity to cisplatin are being strongly transcribed and lying within a long transcription unit. For cellular genes a third property may be a short half-life for the protein product of the gene.* A further complication is that cisplatin can actually lead to promoter-selective induction of expression in human HeLa cells [50].

In addition, in order for a gene to contribute to *cis*-DDP-induced cytotoxicity, it should be important

* Evans GL and Gralla JD, manuscript submitted for publication.

for maintaining the proliferative state of the tumor cell. As an example, overexpression of c-*myc* mRNA is frequently seen in human hematologic and breast cancers [51, 52]; and overexpression of c-*erbB2*/HER-2/*neu* mRNA has been detected recently in human breast and ovarian cancers [53], the latter of which is one of the tumor types most responsive to *cis*-DDP therapy. Growth-related genes that are often deregulated in transformed cells include the glucose transporter, c-*jun* (encoding an AP-1-like transcription factor), and ornithine decarboxylase (ODC) genes [54–56]. We have observed recently selective inhibition of ODC enzyme activity in NIH 3T3 cells at low concentrations of *cis*-DDP coincident with those which effect selective inhibition of cellular DNA synthesis.* Therefore, some growth-related genes may be inherently sensitive to *cis*-DDP-like drugs, and inappropriate levels of expression in a tumor cell may be counteracted by inhibition by *cis*-DDP.

There have been few previous studies of the effects of *cis*-DDP on oncogenes and these do not fall into a consistent pattern. A correlation has been observed between inhibition of gene expression and antineoplastic activity for treatment of human acute lymphocytic and myeloid leukemia [57]. In this study, hematologic remission in acute leukemia patients was associated with significant reduction of *myc* oncogene and histone H3 mRNA levels measured 24 hr after treatment with antitumor drugs in various combinations. In contrast, there was no effect of *cis*-DDP on the level of c-H-*ras* proto-oncogene mRNA in murine L1210 leukemia cells [58] and c-*fos* mRNA is induced by *cis*-DDP and a large variety of agents that damage DNA [59, 60]. However, these studies involve mRNA measurements and leave open the possible selective inactivation of these mRNAs.*

Thus, it is too soon to identify the precise genes that could be functional targets of *cis*-DDP attack. These may include classical oncogenes, as just discussed, or genes involved in controlling (i.e. suppressing) *cis*-DDP-mediated apoptosis as suggested by the work of Barry *et al.* [61]. We would expect such genes to code for rapidly turned-over proteins, to be highly accessible when their promoters are activated, and to be associated with transcripts of significant size. If such genes can be identified it will be possible to consider redesign of existing drugs to attack them with greater effectiveness and selectivity.

Acknowledgement—This research was supported by a grant from the American Cancer Society.

REFERENCES

1. Loehrer PJ and Einhorn LH, Cisplatin. *Ann Intern Med* **100**: 704–713, 1984.
2. Pascoe JM and Roberts JJ, Interactions between mammalian cell DNA and inorganic platinum compounds–I. DNA interstrand cross-linking and cytotoxic properties of platinum(II) compounds. *Biochem Pharmacol* **23**: 1345–1357, 197.
3. Pinto AL and Lippard SJ, Binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (cisplatin) to DNA. *Biochim Biophys Acta* **780**: 167–180, 1985.
4. Sherman SE and Lippard SJ, Structural aspects of platinum anticancer drug interactions with DNA. *Chem Rev* **87**: 1153–1181, 1987.
5. Rice JA, Crothers DM, Pinto AL and Lippard SJ, The major adduct of the antitumor drug *cis*-diamminedichloroplatinum(II) with DNA bends the duplex by ≈40° toward the major groove. *Proc Natl Acad Sci USA* **85**: 4158–4161, 1988.
6. Pinto AL and Lippard SJ, Sequence-dependent termination of *in vitro* DNA synthesis by *cis*- and *trans*-diamminedichloroplatinum(II). *Proc Natl Acad Sci USA* **82**: 4616–4619, 1985.
7. Gralla JD, Sasse-Dwight S and Poljak LG, Formation of blocking lesions at identical DNA sequences by the nitrosourea and platinum classes of anticancer drugs. *Cancer Res* **47**: 5092–5096, 1987.
8. Sheibani N, Jennerwein MM and Eastman A, DNA repair in cells sensitive and resistant to *cis*-diamminedichloroplatinum(II). *Biochemistry* **28**: 3120–3124, 1989.
9. Plooy ACM, van Dijk M and Lohman PHM, Induction and repair of DNA cross-links in Chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity, and antitumor activity. *Cancer Res* **44**: 2043–2051, 1984.
10. Lepre CA, Strothkamp KG and Lippard SJ, Synthesis and ^{1}H NMR spectroscopic characterization of *trans*-[Pt(NH_3)$_2$(d(ApGpGpCpCpT)-*N*7-*A*(1),*N*7-*G*(3))]. *Biochemistry* **26**: 5651–5657, 1987.
11. Harder HC and Rosenberg B, Inhibitory effects of anti-tumor platinum compounds on DNA, RNA, and protein synthesis in mammalian cells *in vitro* *Int J Cancer* **6**: 207–216, 1970.
12. Howle JA, Thompson HS, Stone AE and Gale GR, *cis*-Dichlorodiammineplatinum[II]: Inhibition of nucleic acid synthesis in lymphocytes stimulated with phytohemagglutinin. *Proc Soc Exp Biol Med* **137**: 820–825, 1971.
13. Howle JA and Gale GR, *Cis*-dichlorodiammine-platinum(II). Persistent and selective inhibition of deoxyribonucleic acid synthesis *in vivo*. *Biochem Pharmacol* **19**: 2757–2762, 1970.
14. Borch RF, The platinum anti-tumour drugs. In: *Metabolism and Action of Anti-cancer Drugs* (Eds. Powis G and Prough RA), pp. 163–193. Taylor & Francis, London, 1987.
15. Tay LK, Bregman CL, Masters BA and Williams PD, Effects of *cis*-diamminedichloroplatinum(II) on rabbit kidney *in vivo* and on rabbit renal proximal tubule cells in culture. *Cancer Res* **48**: 2538–2543, 1988.
16. Buchanan RL and Gralla JD, Cisplatin resistance and mechanism in a viral test system: SV40 isolates that resist inhibition by the antitumor drug have lost regulatory DNA. *Biochemistry* **29**: 3436–3442, 1990.
17. Sorenson CM and Eastman A, Mechanism of *cis*-diamminedichloroplatinum(II)-induced cytotoxicity: Role of G_2 arrest and DNA double-strand breaks. *Cancer Res* **48**: 4484–4488, 1988.
18. Sorenson CM and Eastman A, Influence of *cis*-diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. *Cancer Res* **48**: 6703–6707, 1988.
19. Roberts JJ and Friedlos F, Differential toxicity of *cis*- and *trans*-diamminedichloroplatinum(II) toward mammalian cells: Lack of influence of any difference in the rates of loss of their DNA-bound adducts. *Cancer Res* **47**: 31–36, 1987.
20. Ciccarelli RB, Solomon MJ, Varshavsky A and

* Evans GL and Gralla JD, manuscript submitted for publication.

Lippard SJ, *In vivo* effects of *cis*- and *trans*-diamminedichloroplatinum(II) on SV40 chromosomes: Differential repair, DNA-protein cross-linking, and inhibition of replication. *Biochemistry* **24**: 7533–7540, 1985.
21. Knox RJ, Lydall DA, Friedlos F, Basham C and Roberts JJ, The effect of monofunctional or difunctional platinum adducts and of various other associated DNA damage on the expression of transfected DNA in mammalian cell lines sensitive or resistant to difunctional agents. *Biochim Biophys Acta* **908**: 214–223, 1987.
22. Chu G and Berg P, DNA cross-linked by cisplatin: A new probe for the DNA repair defect in xeroderma pigmentosum. *Mol Biol Med* **4**: 277–290, 1987.
23. Sklar MD, Increased resistance to *cis*-diamminedichloroplatinum(II) in NIH 3T3 cells transformed by *ras* oncogenes. *Cancer Res* **48**: 793–797, 1988.
24. Landes GM and Martinson HG, Transcriptional properties of chick embryonic erythroid nuclei *in vitro*. *J Biol Chem* **257**: 11002–11007, 1982.
25. Ben-Zeev A and Becker Y, Requirement of host cell RNA polymerase II in the replication of herpes simplex virus in α-amanitin-sensitive and -resistant cell lines. *Virology* **76**: 246–253, 1977.
26. Gorman CM, Moffat LF and Howard BH, Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2**: 1044–1051, 1982.
27. Gorman CM, Merlino GT, Willingham MC, Pastan I and Howard BH, The Rouse sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc Natl Acad Sci USA* **79**: 6777–6781, 1982.
28. Wu FK, Garcia JA, Harrich D and Gaynor RB, Purification of the human immunodeficiency virus type 1 enhancer and TAR binding proteins EBP-1 and UBP-1. *EMBO J* **7**: 2117–2129, 1988.
29. Natarajan V and Salzman N, Cis and trans activation of adenovirus IVa_2 gene transcription. *Nucleic Acids Res* **13**: 4067–4083, 1985.
30. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning—A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
31. Ishii S, Merlino GT and Pastan I, Promoter region of the human Harvey *ras* proto-oncogene: Similarity to the EGF receptor proto-oncogene promoter. *Science* **230**: 1378–1381, 1985.
32. Garcia J, Wu F and Gaynor R, Upstream regulatory regions required to stabilize binding to the TATA sequence in an adenovirus early promoter. *Nucleic Acids Res* **15**: 8367–8385, 1987.
33. Gunning P, Leavitt J, Muscat G, Ng S and Kedes L, A human β-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc Natl Acad Sci USA* **84**: 4831–4835, 1987.
34. Hall CV, Jacob PE, Ringold GM and Lee F, Expression and regulation of *Escherichia coli lac* gene fusions in mammalian cells. *J Mol Appl Gen* **2**: 101–109, 1983.
35. Shen Y, Hirschhorn RR, Mercer WE, Surmocz E, Tsutsui Y, Soprano KJ and Baserga R, Gene transfer: DNA microinjection compared with DNA transfection with a very high efficiency. *Mol Cell Biol* **2**: 1145–1154, 1982.
36. Weinmann R and Roeder RG, Role of DNA-dependent RNA polymerase II in the transcription of the tRNA and 5S RNA genes. *Proc Natl Acad Sci USA* **71**: 1790–1793, 1974.
37. Sorenson CM, Barry MA and Eastman A, Analysis of events associated with cell cycle arrest at G_2 phase and cell death induced by cisplatin. *J Natl Cancer Inst* **82**: 749–754, 1990.
38. Ishii S, Kadonaga JT, Tjian R, Brady JN, Merlino GT and Pastan I, Binding of the Spl transcription factor by the human Harvey *ras* 1 proto-oncogene promoter. *Science* **232**: 1410–1413, 1986.
39. Lowndes NF, Paul J, Wu J and Allan M, c-Ha-*ras* gene bidirectional promoter expressed *in vitro*: Location and regulation. *Mol Cell Biol* **9**: 3758–3770, 1989.
40. Frederickson RM, Micheau MR, Iwamoto A and Miyamoto NG, 5′ Flanking and first intro sequences of the human β-actin gene required for efficient promoter activity. *Nucleic Acids Res* **17**: 253–270, 1989.
41. Weiher HR, Konig M and Gruss P, Multiple point mutations affecting the Simian virus 40 enhancer. *Science* **219**: 626–631, 1983.
42. Harrich D, Garcia J, Wu F, Mitsuyasu R, Gonzalez J and Gaynor RB, Role of SP1-binding domains in *in vivo* transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. *J Virol* **63**: 2585–2591, 1989.
43. Hen R, Sassone-Corsi P, Corden J, Gaub MP and Chambon P, Sequences upstream from the T-A-T-A box are required *in vivo* and *in vitro* for efficient transcription from the adenovirus serotype 2 major late promoter. *Proc Natl Acad Sci USA* **79**: 7132–7136, 1982.
44. Cullen BR, Raymond K and Ju G, Functional analysis of the transcription control region located within the avian retroviral long terminal repeat. *Mol Cell Biol* **5**: 438–447, 1985.
45. Natarajan V, Madden MJ and Salzman NP, Proximal and distal domains that control *in vitro* transcription of the adenovirus *IVa2* gene. *Proc Natl Acad Sci USA* **81**: 6290–6294, 1984.
46. Choie DD, Del Campo AA and Guarino AM, Subcellular localization of *cis*-diamminedichloroplatinum(II) in rat kidney and liver. *Toxicol Appl Pharmacol* **55**: 245–252, 1980.
47. Belliveau JF, Posner MR, Cummings FJ, Wiemann MC, Crabtree GW, O'Leary GP, Savolainen A, Launder L and Calabresi P, Plasma emission spectroscopy: A simple and convenient method for the pharmacokinetic evaluation of cisplatin in tissues and body fluids. *Proc Am Assoc Cancer Res* **26**: 160, 1985.
48. Garcia JA, Wu FK, Mitsuyasu R and Gaynor RB, Interaction of cellular proteins involved in the transcriptional regulation of the human immunodeficiency virus. *EMBO J* **6**: 3761–3770, 1987.
49. Gross D and Garrard WT, Nuclease hypersensitive sites in chromatin. *Annu Rev Biochem* **57**: 159–197, 1988.
50. Evans GL and Gralla JD, Cisplatin-induced imbalances in the pattern of chimeric marker gene expression in HeLa cells. *Biochem Biophys Res Commun* **184**: 1–8, 1992.
51. Rotherberg PG, Erisman MD, Diehl RE, Rovigatti UG and Astrin SM, Structure and expression of the oncogene c-*myc* in fresh tumor material from patients with hematopoietic malignancies. *Mol Cell Biol* **4**: 1096–1103, 1984.
52. Escot C, Theillet C, Lidereau R, Spyratos F, Champeme M-H, Gest J and Callahan R, Genetic alteration of the c-*myc* protooncogene (*MYC*) in human primary breast carcinomas. *Proc Natl Acad Sci USA* **83**: 4834–4838, 1986.
53. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A and Press MF, Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science* **244**: 707–712, 1989.
54. Birnbaum MJ, Haspel HC and Rosen OM, Transformation of rat fibroblasts by FSV rapidly increases glucose transporter gene transcription. *Science* **235**: 1495–1498, 1987.

55. Sistonen L, Hölttä E, Mäkelä TP, Keski-Oja and Alitalo K, The cellular response to induction of the $p21^{c\text{-}Ha\text{-}ras}$ oncoprotein includes stimulation of *jun* gene expression. *EMBO J* **8**: 815–822, 1989.
56. Pegg AE, Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res* **48**: 759–774, 1988.
57. Venturelli D, Lange B, Narni F, Selleri L, Mariano MT, Torelli U, Gewirtz AM and Calabretta B, Prognostic significance of "short-term" effects of chemotherapy on MYC and histone H3 mRNA levels in acute leukemia patients. *Proc Natl Acad Sci USA* **85**: 3590–3594, 1988.
58. Sheibani N and Eastman A, Analysis of various mRNA potentially involved in cisplatin resistance of murine leukemia L1210 cells. *Cancer Lett* **52**: 179–185, 1990.
59. Kashani-Sabet M, Wang W and Scanlon KJ, Cyclosporin A suppresses cisplatin-induced c-*fos* gene expression in ovarian carcinoma cells. *J Biol Chem* **265**: 11285–11288, 1990.
60. Hollander MC and Fornace AJ Jr, Induction of *fos* RNA by DNA-damaging agents. *Cancer Res* **49**: 1687–1692, 1989.
61. Barry MA, Behnke CA and Eastman A, Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol* **40**: 2353–2362, 1990.