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Cisplatin inhibits chromatin remodeling, transcription factor binding, and transcription from the mouse mammary tumor virus promoter *in vivo*

JOE S. MYMRYK, EVA ZANIEWSKI, AND TREVOR K. ARCHER

Departments of Obstetrics and Gynaecology, Biochemistry and Oncology, The University of Western Ontario, London, ON Canada, N6A 4L6

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ABSTRACT **The anticancer drug *cis*-diamminedichloroplatinum(II) (cisplatin) covalently modifies DNA, and these lesions are thought to lead to cell death by inhibiting DNA and RNA synthesis. By using *in vivo* analysis techniques, we have investigated the influence of cisplatin on hormone-induced transcription from the mouse mammary tumor virus (MMTV) promoter. Cisplatin substantially reduced glucocorticoid-induced expression from the MMTV promoter stably incorporated into mouse tumor cells. The glucocorticoid-receptor-dependent chromatin remodeling and loading of transcription factors that is a signature response of this promoter in the context of chromatin were significantly reduced by cisplatin but not by the clinically ineffective *trans*-isomer *trans*-diamminedichloroplatinum(II) (transplatin). Additional *in vivo* studies on transiently introduced nonchromatin MMTV templates demonstrated that cisplatin modification of DNA blocked binding of the transcription factor NF1. These results provide strong evidence that cisplatin influences transcription by interfering with the opening of repressive chromatin structures and by blocking transcription factor binding directly, each of which could contribute substantially to its toxicity.**

cis-Diamminedichloroplatinum(II) (cisplatin) and its various derivatives have become one of the most widely used class of anticancer drugs in the world today (1). The efficacy of cisplatin is such that complete remission is obtained in >85% of patients with testicular cancers. The drug is also effective for treatment of bladder and ovarian cancer, osteogenic sarcoma, non-small-cell lung cancer, and cancers of the head, neck, endometrium, and cervix (1).

Cisplatin binds to DNA *in vivo* to form monofunctional and bifunctional adducts at guanines and adenines (2, 3). The initial effect of the drug is an inhibition of DNA synthesis, which occurs rapidly and at low drug concentrations. *In vitro* experiments with both prokaryotic and eukaryotic DNA polymerases demonstrate that DNA synthesis is blocked at the site of adduct formation, predominantly at G^G intrastrand crosslinks, the major adduct formed by cisplatin (2, 3). These alterations in DNA synthesis occur prior to changes in bulk RNA or protein levels and are selectively responsive to low levels of drug (1). Furthermore, the activities of a variety of enzymes (e.g., alkaline phosphatase and Na^+/K^+ ATPase) show no significant changes upon cisplatin treatment (4).

Inhibition of *in vitro* synthesis of RNA by eukaryotic and prokaryotic RNA polymerases from a template modified by cisplatin occurs at G^G and A^G intrastrand crosslinks (5, 6). *In vitro* studies with simian virus 40 revealed cisplatin induced G^G crosslinks within regulatory sequences of the virus, and the high concentration of such lesions within the promoter raised the possibility that lesions occurring at sites bound by regulatory proteins may influence transcription (7). Further support for this idea came from the observation that cisplatin-resistant simian virus 40 mutants had portions of their regulatory regions deleted (5).

Intriguingly, transient transfection studies have revealed that transcription from strong promoters is preferentially reduced by cisplatin treatment (4), and this inhibition correlates with the presence of more potential cisplatin modification sites (i.e., G-rich regions) within these promoters. In addition, a direct role for cisplatin in the inhibition of loading of mammalian RNA polymerase was demonstrated by examining total mRNA synthesis in the presence of both α-amanitin and cisplatin (4). Rather surprisingly, cisplatin-mediated inhibition of promoter activity is selective, because the activity of some promoters is repressed (e.g., simian virus 40 promoter and adenovirus major late promoter) whereas the activity of others is enhanced (e.g., human immunodeficiency virus and human adenovirus E3 promoters) (4, 8). Thus, cisplatin-induced DNA damage elicits a variety of cellular responses (9). Indeed, cisplatin treatment has been linked to induction of c-*jun* (10), c-*fos* (11), and human immunodeficiency virus gene expression (8).

In an attempt to understand how cells detect and deal with cisplatin-induced lesions in DNA, a significant effort has been devoted to examining the ability of cellular proteins to bind cisplatin-damaged DNA (12–15). Two such proteins, high mobility group 1 (HMG-1) (16) and structure-specific recognition protein 1 (14, 17), have defined DNA-binding properties. Inspection of the sequence of structure-specific recognition protein 1 revealed the presence of a HMG-1 box, which is a sequence of ≈30 amino acids common to all HMG proteins (17, 18) that may confer the ability to selectively interact with cisplatin-modified DNA (16, 19).

HMG-1 is an abundant and strongly conserved component of mammalian chromatin whose physiological function remains elusive (18). There is considerable evidence that it plays an important role in DNA replication, nucleosome assembly, and transcription (18). The ability of HMG-1 to selectively identify DNA modified by cisplatin but not by the *trans*-isomer *trans*-diamminedichloroplatinum(II) (transplatin) *in vitro* (16) may explain why transplatin is of no therapeutic value (20). Other transcription factors also contain a HMG-1 box, including lymphocyte enhancer factor (21), the mammalian sex-determining region Y protein (22), and the human upstream binding factor (hUBF) (23). Recent studies have demonstrated a phenomenon of "transcription factor hijacking" in that cisplatin-modified DNA lacking a hUBF binding site is efficiently bound by hUBF *in vitro* (24). Thus by analogy, the

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Abbreviations: MMTV, mouse mammary tumor virus; GR, glucocorticoid receptor; HMG, high mobility group; CAT, chloramphenicol acetyltransferase; Dex, dexamethasone; Nuc-B, nucleosome B; LTR, long terminal repeat; hUBF, human upstream binding factor; LUC, luciferase.

transcriptional activity of an entire spectrum of proteins containing a HMG-1 box may be compromised after cisplatin modification of DNA.

In eukaryotic cells, DNA is highly packaged in a chromatin hierarchy that begins with the initial wrapping around core histones to form nucleosomes and ends with the fully condensed structure of chromosomes. This assembly of DNA as chromatin has an important role in modulating the access of transacting proteins and thus participates in the regulation of gene expression (25, 26). We have investigated the consequences of cisplatin treatment on transcription from a DNA template assembled as chromatin by using the mouse mammary tumor virus long terminal repeat (MMTV LTR) as our model system.

The MMTV system offers an excellent opportunity to address the effects of cisplatin-induced DNA damage on the complex interplay between transcription factors and chromatin because it adopts a highly reproducible chromatin structure *in vivo* and *in vitro* (27–29). Glucocorticoids, acting through the glucocorticoid receptor (GR) stimulate the rate the transcription initiation from the promoter (30). Coincident with this increase in transcription is the appearance of an extended hypersensitive region, encompassing the second nucleosome (Nuc-B) in the phased array (27, 31) and the hormone-dependent loading of a preinitiation complex containing transcription factor NF1 (32). Inspection of the MMTV promoter reveals that there are >50 potential modification sites for cisplatin within the first 250 bp of the proximal MMTV promoter/enhancer, with 5 of these being G^G dimers that occur within the binding sites for the GR and NF1 (Fig. 1). The presence of these potential crosslinks immediately suggested that cisplatin treatment could influence binding of transacting factors to this promoter. Our results reveal that cisplatin treatment results in the introduction of adducts at the promoter and these significantly reduce the hormone-receptor-dependent chromatin remodeling and loading of transcription factors that is a signature response of this promoter.

MATERIALS AND METHODS

Cell Line and Transfections. Cell line 1471.1 was derived from murine C127 cells by stable cotransfection of the chimeric bovine papilloma virus-based construct pm25, carrying the MMTV LTR attached to the bacterial chloramphenicol acetyltransferase (CAT) gene as described (28). Cells were grown at 37°C with 5% CO_2/95% air in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum. Transient transfection was performed by using the calcium phosphate procedure with 5 μg of pLTRLUC, containing the MMTV LTR driving expression of the luciferase (LUC) gene

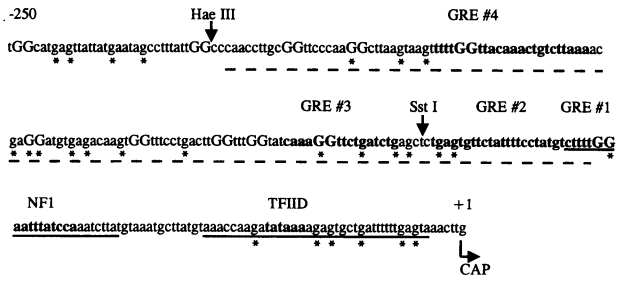

FIG. 1. Sequence of the coding strand of the 5′ proximal portion of the MMTV LTR from positions −250 to +1. Binding sites for the GR, NF1, and TFIID are in boldface type. Protective regions for partially purified TFIID and NF1 are underlined. Potential G^G crosslinks are in uppercase type and potential G^A or A^G crosslinks are indicated by an asterisk underneath the corresponding sequence. The position of Nuc-B is indicated by the dashed line and cleavage sites for *Hae* III and *Sst* I are also indicated.

(33). Cells were treated with cisplatin (30 μM) or transplatin (300 μM) for 16 h prior to analysis.

RNA Isolation and Primer-Extension Analysis. Total cytoplasmic RNA was prepared as described (34). Oligonucleotide primers for MMTV and actin (34) were labeled with [^{32}P]ATP by using T4 polynucleotide kinase and primer extension was performed as described (35).

***In Vivo* Chromatin Analysis and Transcription Factor Loading.** Cells were treated with dexamethasone (Dex; 0.1 μM) for 1 h. Nuclei were isolated, digested with restriction endonucleases, and subjected to exonuclease III footprinting analysis as described (29, 32). After purification of the genomic DNA, 10 μg of each sample was analyzed by using linear *Taq* polymerase amplification with ^{32}P-labeled single-stranded primers specific for the MMTV LTR (oligo-22), CAT (oligo-18), or LUC (oligo-205) genes as described (36). Purified-extension products were analyzed on 8% polyacrylamide denaturing gels and exposed to Kodak X-Omat AR film at −80°C. Quantitation was performed using a Molecular Dynamics PhosphorImager according to the manufacturer's instructions.

RESULTS

The structure of the MMTV promoter, its chromatin architecture, and the general and specific transactivating factors that regulate it are well characterized (37). As such, it represents an ideal system to examine the effects of cisplatin on chromatin structure and how this may affect gene expression. For our initial experiments, we took advantage of a mouse cell line, 1471.1, that contains the MMTV LTR attached to a CAT reporter gene. Cells were treated with 30 μM cisplatin for 16 h prior to treatment with Dex for an additional 4 h. RNA was isolated and primer-extension analysis was performed. Dex treatment alone induced MMTV expression (Fig. 2 *Upper*, compare lanes 1 and 2), and this was significantly inhibited by treatment with cisplatin (lane 4). No induction by cisplatin alone was observed relative to control samples (lanes 3 and 1, respectively); indeed, cisplatin reduced the basal expression in this experiment. Similarly, analysis of CAT enzymatic activity confirmed that cisplatin treatment reduced the induction of MMTV expression by Dex (data not shown). Over the same time period, no significant effects on the production of actin mRNA were observed (Fig. 2 *Lower*, compare lanes 1 and 3 with lanes 2 and 4). The reduction in MMTV expression observed was not simply a result of cell killing, as trypan blue

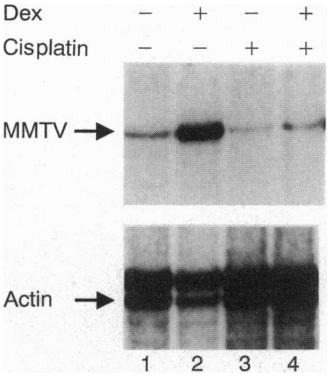

FIG. 2. Primer-extension analysis of mRNA induction in cells treated with cisplatin and Dex. A mouse C127 cell line containing a MMTV–bovine papilloma virus chimera driving the CAT gene, 1471.1, was incubated with 30 μM cisplatin for 16 h and then treated with Dex for 4 h. Total RNA was isolated and primer extension was performed with primers specific for the CAT and actin genes by using 20 μg and 10 μg of RNA, respectively. Purified extension products were analyzed on 8% denaturing sequencing gels and exposed to Kodak XAR film at −80°C.

exclusion assays indicated that >95% of the cells remained viable under this regimen of drug treatment (data not shown).

A signature response of the MMTV promoter to hormone stimulation is the appearance of a region of hypersensitivity to restriction enzymes in the proximal promoter (31). This region of hypersensitivity coincides with the position of the second nucleosome (Nuc-B) in a phased array assembled when the MMTV LTR is stably introduced into mouse cells (27). We have examined the effect of cisplatin treatment on the ability of the GR to remodel the chromatin architecture of the MMTV promoter by using an *in vivo* restriction access analysis (Fig. 3). Cells were treated with cisplatin for 16 h and exposed to Dex for 1 h. Nuclei were isolated and digested with *Sst* I, which cleaves within Nuc-B, or with *Hae* III, which cleaves on the Nuc-B boundary (Fig. 1). As expected, *in vivo* cleavage by *Sst* I was dramatically enhanced upon Dex treatment (Fig. 3, compare lanes 1 and 3). In cells treated with cisplatin, the ability of *Sst* I to cut the MMTV promoter in response to Dex induction was severely diminished (lane 4). As a control, we examined *in vivo* cleavage by *Hae* III. As demonstrated (29), the extent of cleavage by *Hae* III was not affected by hormone treatment (Fig. 3, compare lanes 5 and 7). Cisplatin treatment clearly did not affect the extent of cleavage (compare lanes 5 and 7 with lanes 6 and 8), although there is a potential G∧G crosslink within the *Hae* III recognition site (GG ↓ CC). These results argue *a priori* that cisplatin modification of DNA within the MMTV promoter compromises its ability to respond to hormonal induction.

The formation of a hypersensitive region in the proximal portion of the MMTV promoter is accompanied by the hormone-dependent loading of the preinitiation complex containing NF1, octamer proteins, and the TATA binding protein (34). In the next set of experiments, we used an *in vivo* footprinting approach to examine the effect of cisplatin on the hormone-dependent binding of NF1 to the MMTV promoter stably maintained as a chromatin template (Fig. 4). NF1 loading was not detected prior to Dex addition but was readily

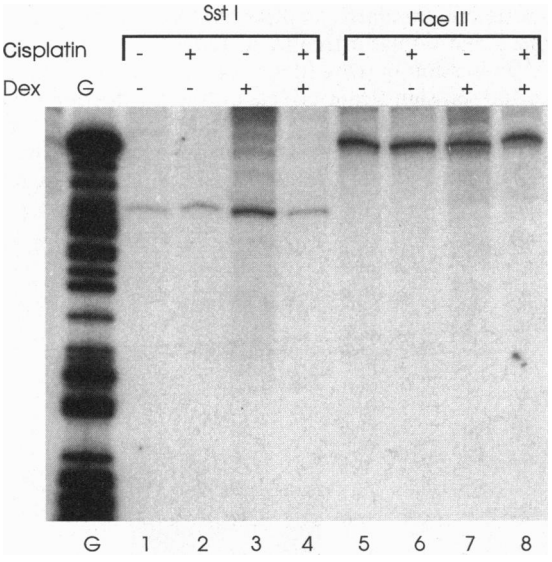

FIG. 3. *In vivo* restriction enzyme analysis of cells treated with cisplatin and Dex. Cells were incubated with 30 μM cisplatin for 16 h and then treated with Dex for 1 h. Nuclei were isolated and digested with the restriction enzyme *Sst* I, which cleaves within the Nuc-B region of the MMTV promoter, or *Hae* III, which cleaves on the boundary of Nuc-B. After purification, 10 μg of DNA was analyzed by linear *Taq* polymerase amplification with a ^{32}P-labeled single-stranded primer specific for the MMTV promoter (36). Specific-extension products were separated on an 8% denaturing polyacrylamide gel and autoradiographed by using Kodak XAR film. Lanes: G, G sequencing track; 1–4, *Sst* I; 5–8, *Hae* III.

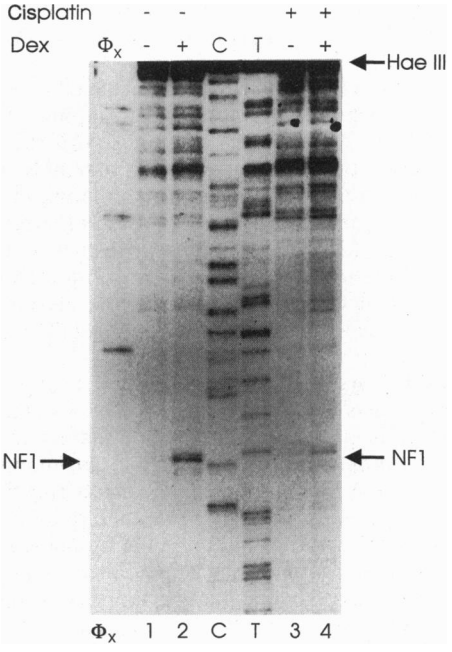

FIG. 4. *In vivo* detection of NF1 binding to the MMTV promoter in chromatin of cells treated with cisplatin and Dex. Drug and hormone treatments were as in Fig. 3. Nuclei were digested with *Hae* III (1000 units/ml) and exonuclease III (625 units/ml) to detect specific stops corresponding to the 5′ boundaries of bound factors (29). After purification of DNA and removal of single-stranded DNA by using mung bean nuclease, samples were analyzed by *Taq* polymerase primer-extension with a ^{32}P-labeled single-stranded primer specific for the MMTV promoter. Lanes: C, C sequencing track; T, T sequencing track; Φ_x, ϕX174 replicative form of DNA digested with *Hae* III. The positions of the *Hae* III parental band and the specific stop corresponding to NF1 are indicated by arrows.

observed after 1 h of Dex treatment (lanes 1 and 2, respectively). Treatment with cisplatin reduced the binding of NF1 to its cognate binding site after hormone stimulation (lane 4). This result was anticipated because NF1 binding is only detected as a result of the GR-mediated opening of Nuc-B, and as shown in Fig. 3, cisplatin treatment severely reduces the ability of the GR to stimulate hypersensitivity. The diminished loading of NF1 did not result from a lack of access by exonuclease III because the degree of cleavage by *Hae* III, which generates the entry site, was not affected by cisplatin (Fig. 3, lane 8). Although these *in vivo* footprinting experiments do not provide a direct measure of the effect of modification of the NF1 site, they do confirm the results of the RNA and CAT analyses (Fig. 2 and data not shown). This leaves open the question of whether cisplatin can actually inhibit NF1 binding by modifying the DNA composing its binding site.

To address this question, we took advantage of our recent observation that transiently introduced copies of the MMTV LTR are not assembled into a specific chromatin structure and thus display constitutive loading of NF1 onto the LTR (34, 36). Thus, analysis of the effect of cisplatin on NF1 loading on transient templates would directly measure the effect of cisplatin-induced DNA modification on NF1 binding. Cells were transfected with pLTRLUC, which like the stably maintained template contains the MMTV LTR but differs by containing the LUC gene rather than the CAT gene as a reporter. As before, cells were treated with cisplatin for 16 h and with Dex for 1 h. Nuclei were isolated and *in vivo* footprinting analysis was performed [note: by using a primer specific for the LUC gene, analysis of the transient template can be performed independently from that of the stable template (34, 36)] (Fig. 5). As reported (34, 36), strong NF1 binding to the transient

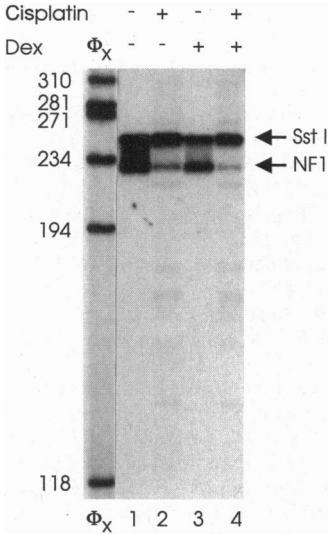

FIG. 5. *In vivo* detection of NF1 binding to transiently transfected MMTV promoter templates in cells treated with cisplatin and Dex. Drug and hormone treatments were as in Fig. 3. Nuclei were digested with *Sst* I and exonuclease III to detect specific stops corresponding to the 5′ boundaries of bound factors (29). After purification of DNA and removal of single-stranded DNA with mung bean nuclease, samples were analyzed by *Taq* polymerase primer extension with a ^{32}P-labeled single-stranded primer specific for the transiently introduced MMTV promoter. Lane Φ_x contains ϕX174 replicative form DNA cut with *Hae* III. The positions of the *Sst* I parental band and the specific stop corresponding to NF1 are indicated by arrows.

template was independent of the presence of Dex (lanes 1 and 3). Treatment of cells with cisplatin greatly reduced the amount of NF1 bound to the transiently transfected promoter in the presence or absence of Dex (lanes 2 and 4), suggesting that cisplatin modification of DNA can inhibit binding of at least some transcription factors.

The clinically inactive isomer of cisplatin transplatin forms 1,3- but not 1,2-intrastrand crosslinks (20). We compared the effects of transplatin treatment to those obtained with cisplatin by using *in vivo* restriction enzyme cleavage analysis of both stable and transiently introduced MMTV templates (Fig. 6). As shown in Fig. 3, Dex-induced cleavage of the stable template by *Sst* I was severely diminished by cisplatin treatment. In contrast, transplatin treatment had no effect on cleavage by *Sst* I (Fig. 6*A*). Analysis of CAT activities confirmed that transplatin had no effect on Dex-mediated induction of transcription from a stably maintained MMTV template (data not shown). Cleavage within the transiently introduced MMTV LTR by *Sst* I was unaffected by Dex or by treatment with either cisplatin or transplatin (Fig. 6*B*). This result confirms that the reduced cleavage observed for the cisplatin-treated stable template is not related to actual modification of the *Sst* I cleavage site but rather to a reduced ability of the GR to remodel chromatin.

DISCUSSION

There is evidence from a number of laboratories that cisplatin treatment can influence gene expression both positively and negatively (4, 8, 10, 11). To investigate directly the effects of cisplatin on transcription, we examined the effect of this drug on expression from the well-characterized MMTV promoter (28, 37). We have demonstrated that cisplatin treatment markedly reduced the levels of transcription from the hormone-inducible MMTV promoter stably maintained as chromatin (Fig. 2). As activation of the MMTV promoter by glucocorticoids occurs efficiently in the absence of replication (28), we can rule out cisplatin inhibition of DNA synthesis as

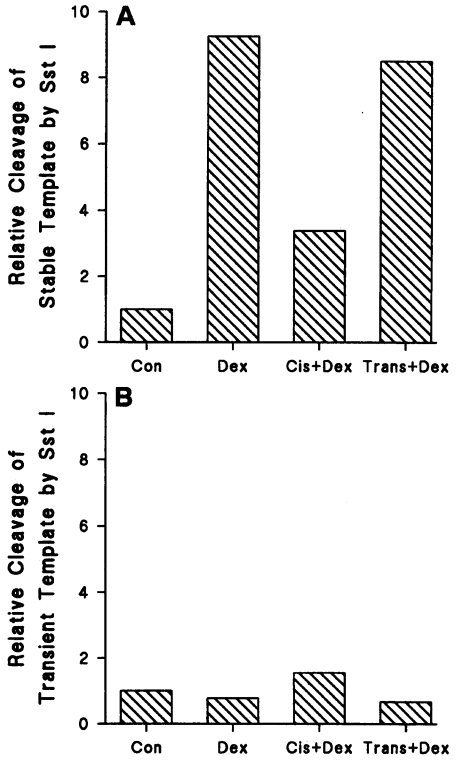

FIG. 6. *In vivo* restriction enzyme analysis of chromatin and transiently transfected MMTV templates in cells treated with cisplatin, transplatin, or Dex as indicated. Cells were treated with 30 μM cisplatin or 300 μM transplatin for 16 h and then treated with Dex for 1 h. *In vivo* restriction enzyme cleavage with *Sst* I was performed as indicated in Fig. 3. Analysis was performed by using *Taq* polymerase primer extension with a ^{32}P-labeled single-stranded primer specific for either the stable or transiently introduced MMTV promoter. Quantitation was performed with a Molecular Dynamics PhosphorImager. The cleavage by *Sst* I was normalized to *Hae* III cleavage for both transient and stable templates (data not shown). (*A*) Stable template. (*B*) Transiently introduced template. Con, control.

the cause of the effects observed here. Our *in vivo* analysis of the chromatin architecture of the MMTV LTR shows that cisplatin treatment severely reduced the restriction enzyme hypersensitivity characteristic of hormone stimulation of transcription from a stable MMTV template (Figs. 3 and 6*A*). In contrast, the clinically ineffective *trans*-isomer was unable to influence hormone-induced MMTV gene expression (data not shown) or restriction enzyme hypersensitivity (Fig. 6*A*). Cisplatin treatment also reduced the hormone-dependent loading of NF1 (Fig. 4) and presumably the rest of the transcription preinitiation complex.

The inhibition of NF1 binding to the chromatin template by cisplatin treatment may have resulted from direct or indirect mechanisms. Specifically, cisplatin modification of the DNA in the NF1 binding site could lead to a direct inhibition of NF1 binding. Alternatively, the failure of NF1 to bind may reflect the inhibition by cisplatin of the hormone-induced alterations in chromatin architecture that is required to permit NF1 binding (32) (Figs. 3 and 6*A*). Direct evidence that cisplatin modification of DNA was sufficient to block transcription factor binding was obtained by examining NF1 loading on a transiently introduced template that was not assembled into a specific chromatin structure (Fig. 5). The decreased binding of NF1 to the transiently introduced template in cisplatin-treated cells demonstrates that cisplatin modification of DNA can block binding of at least some transcription factors.

Inspection of the DNA sequence protected by partially purified NF1 reveals five potential crosslinking sites (Fig. 1).

Cisplatin modification of DNA could directly block NF1 binding by inducing distortions into the conformation of the binding site (38) or perhaps through steric interference by the platinum adduct. Alternatively, cisplatin could act indirectly to block NF1 binding by recruiting factor(s) that compete(s) for binding at the same or overlapping site. This would be analogous to mechanisms proposed with respect to cisplatin effects on repair and replication (19, 39) and is supported by evidence from a variety of cellular proteins including HMG-1 that selectively bind cisplatin-damaged DNA (12, 13, 39). Thus, in addition to the transcription factor hijacking model of cisplatin action proposed for HMG-1-box-containing proteins such as hUBF (24), inappropriate binding of such proteins to cisplatin-modified regulatory sites could effectively "shield" these sites from the appropriate transcription factor. As we did not detect new exonuclease III stops within the MMTV LTR in cells treated with cisplatin (Figs. 4 and 5), our data would clearly support a direct effect of cisplatin for the observed reduction in NF1 binding. Thus, these results suggest that the inhibition of transcription factor binding described here, combined with the sequestering of essential factors at inappropriate sites (24), could dramatically alter the regulation of critical genes leading to profound effects on cellular homeostasis.

Several findings in this work are significant with respect to the regulation of transcription by cisplatin. (*i*) Cisplatin modification may act to inhibit transcription in the context of chromatin by preventing changes in nucleosomal organization required for transcription factor access. (*ii*) Our studies on transiently introduced templates show that cisplatin modification of DNA can block binding by essential transcription factors. In summary, cisplatin but not transplatin inhibits hormonal activation of the MMTV promoter. This result mirrors their respective clinical efficacies, providing strong evidence that the effects of cisplatin on gene transcription may contribute substantially to its toxicity.

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