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Cisplatin inhibits synthesis of ribosomal RNA *in vivo*

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ABSTRACT

***Cis*-diammininedichloroplatinum(II) (cisplatin or *cis*-DDP) is a DNA-damaging agent that is widely used in cancer chemotherapy. Cisplatin crosslinks DNA and the resulting adducts interact with proteins that contain high-mobility-group (HMG) domains, such as UBF (upstream binding factor). UBF is a transcription factor that binds to the promoter of ribosomal RNA (rRNA) genes thereby supporting initiation of transcription by RNA polymerase I. Here we report that cisplatin causes a redistribution of UBF in the nucleolus of human cells, similar to that observed after inhibition of rRNA synthesis. A similar redistribution was observed for the major components of the rRNA transcription machinery, namely TBP, TAF_Is and RNA polymerase I. Furthermore, we provide for the first time direct *in vivo* evidence that cisplatin blocks synthesis of rRNA, while activity of RNA polymerase II continues to be detected throughout the nucleus. The clinically ineffective *trans* isomer (*trans*-DDP) does not alter the localization of either UBF or other components of the RNA polymerase I transcription machinery. These results suggest that disruption of rRNA synthesis, which is stimulated in proliferating cells, plays an important role in the clinical success of cisplatin.**

INTRODUCTION

The inorganic compound *cis*-diammininedichloroplatinum(II), commonly referred to as cisplatin or *cis*-DDP, is one of the most widely used anticancer drugs with well established effectiveness against a number of cancers, particularly metastatic testicular tumors (for recents reviews see 1,2). Although the mode of action of cisplatin has been under intensive study since its discovery more than 30 years ago (3), the molecular basis for the biological effects of this drug are not entirely clear.

Cisplatin forms covalent adducts with many biological molecules, but its principal target is DNA. The most prevalent DNA lesion caused by cisplatin is a 1,2-intrastrand crosslink, with the platinum covalently bound to the N^7 positions of adjacent purine bases. Other platinum–DNA adducts include 1,3-intrastrand, interstrand and protein–DNA crosslinks. The formation of the major 1,2-intrastrand cross-links is most probably responsible for the biological activity of cisplatin, since the stereoisomer *trans*-DDP cannot form this type of adduct and is clinically inactive (1,2,4).

Recently, much attention has been focused on high-mobility-group (HMG)-domain proteins, which bind specifically to cisplatin-damaged DNA and may therefore participate in the cytotoxic effects of the drug (4). The HMG domain is a DNA-binding motif of ~80 amino acid residues and HMG-domain proteins are architectural proteins that function to bend DNA (5). This class of proteins recognizes DNA structural elements present in linear, cruciform or cisplatin-modified DNA (6). Importantly, HMG-domain proteins bind to DNA lesions induced by cisplatin but not by *trans*-DDP, and the binding is specific for the major 1,2-intrastrand adducts. This strongly suggests that HMG-domain proteins are involved in cisplatin cytotoxicity, and several models have been proposed to explain their role. One possibility is that recruitment of HMG-domain proteins to cisplatin-lesioned DNA masks the DNA damage from being repaired. Another possibility is that the cisplatin–DNA adducts may titrate or 'hijack' HMG-domain proteins from their normal sites of action thereby disrupting normal gene expression. Alternatively, binding of HMG-domain proteins to cisplatin-lesioned DNA may displace certain tumor-specific regulatory DNA-binding proteins, resulting in tumor cell death. It is also possible that binding of HMG-domain proteins to unplatinated DNA may create a favorable DNA conformation for cisplatin to act upon (reviewed in 2).

The model that cisplatin-damaged DNA may serve as a molecular decoy was based on the observation that *in vitro*, human upstream binding factor (hUBF, a member of the HMG-domain protein family) binds with high avidity to the intrastrand crosslinks produced by the drug. As UBF is an essential transcription factor for RNA polymerase I, it was suggested that rRNA synthesis may be disrupted if the adducts hijack hUBF *in vivo* (7). Here, we have analysed the effects of cisplatin on the subnuclear distribution of UBF and other components of the machinery for RNA polymerase I transcription. The results show that cisplatin blocks synthesis of rRNA and causes a redistribution of UBF, RNA polymerase I, TATA-binding protein (TBP) and TBP-associated factors for RNA polymerase I (TAF_Is) to the periphery of the nucleolus, where they co-localize with inactive rDNA genes. In contrast, activity of RNA polymerase II continues to be detected throughout the nucleus. This suggests that disruption of rRNA

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synthesis, which is stimulated in proliferating cells, plays an important role in the clinical success of cisplatin.

MATERIALS AND METHODS

Cell culture and drug treatment

HeLa monolayer cultures were maintained mycoplasm-free in Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum.

Stock solutions of 10 mg/ml *cis*-DDP and *trans*-DDP (Sigma) were prepared in dimethylformamide and stored at –20°C. Alternatively, cisplatin was dissolved in sterile phosphate buffered saline (PBS) (1 mg/ml; 3.3 mM) and each stock was used for no longer than 3 days (cf. 8).

Immunofluorescence

For indirect immunofluorescence the cells were grown on 10 × 10 mm glass coverslips and harvested at 50–70% confluency. Coverslips with attached cells were washed twice in PBS, fixed with 3.7% formaldehyde in PBS for 10 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature. Alternatively, the cells were first permeabilized with 0.5% Triton X-100 in CSK buffer (9) containing 0.1 mM PMSF for 1 min on ice, and then fixed with 3.7% formaldehyde in CSK for 10 min at room temperature. Formaldehyde (7.4% stock solution) was prepared from paraformaldehyde and stored frozen until use.

After fixation and permeabilization, the cells were rinsed in PBS containing 0.05% Tween 20 (PBS-T), incubated for 30–60 min with primary antibodies diluted in PBS-T, washed, and incubated for 30 min with the appropriate secondary antibodies conjugated to either fluorescein or Texas Red (Vector Laboratories, UK). Finally, the coverslips were mounted in VectaShield (Vector Laboratories, UK) and sealed with nail polish.

The following antibodies were used: rabbit polyclonal antibody E29 raised against human UBF (10), auto-immune human anti-RNA polymerase I serum S18 (kindly provided by Dr U. Scheer), rabbit anti-hTBP KD 55/1 (11) and rabbit anti-hTAF_I63 sera (12).

Visualization of transcription sites

Visualization of transcription sites was performed essentially according to Jackson *et al.* (13). Briefly, cells grown as monolayers on coverslips were washed twice in PBS and incubated with 0.05% Triton X-100 in PB buffer for 2 min on ice, in order to permeabilize selectively the plasma membrane. Then, the cells were incubated with a transcription-mix containing bromo-UTP (Sigma) for 20 min at 33°C. Subsequently, the cells were further incubated in 0.2% Triton X-100 for 10 min, fixed with 3.7% formaldehyde for 10 min, and the incorporated bromo-uridine detected using a monoclonal antibody directed against bromodeoxyuridine (Boehringer Mannheim, Germany).

In order to visualize the sites of transcription by distinct RNA polymerases, the transcription assay was performed in the presence of selective inhibitors. The drugs were added for 10 min prior to, as well as during incubation with the modified nucleotide. In the presence of α-amanitin at a concentration of 100 µg/ml (to inhibit RNA polymerases II and III), the labeling was exclusively observed in the nucleolus. In the presence of actinomycin D at a concentration of 0.08 µg/ml (to inhibit transcription by polymerase I), the labeling was detected in the nucleoplasm but not in the nucleolus, and in the presence of 5 µg/ml actinomycin D (which inhibits transcription by all RNA polymerases), all labeling was abolished.

Visualization of DNA replication

For *in vivo* labeling of replication sites, cells were pulse-labeled in culture medium with 1 µM bromodeoxyuridine (BrdU; Boehringer Mannheim) for 30 min. For detection of incorporated BrdU the cells were washed with PBS, fixed in pre-cooled 70% ethanol, 50 mM glycine for 20 min at –20°C, rinsed in PBS, incubated with 4 N HCl for 8 min and rinsed again in PBS. The cells were then blocked with 5% fetal calf serum in PBS for 15 min and incubated with mouse monoclonal anti-bromodeoxyuridine antibody (Boehringer Mannheim) and an appropriate secondary antibody coupled to FITC. After washing the samples were mounted in VectaShield.

Microscopy

Samples were analysed using the laser scanning microscope Zeiss LSM410 equiped with an Argon Ion laser (488 nm) to excite FITC fluorescence and a Helium-Neon laser (543 nm) to excite Texas Red fluorescence. For double-labeling experiments, images from the same focal plane were sequentially recorded in different channels and superimposed. In order to obtain a precise alignment of superimposed images the equipment was calibrated using multicolor fluorescent beads (Molecular Probes, Eugene, USA), and a dual-band filter that allows simultaneous visualization of red and green fluorescence.

Run-on transcription assay

Cells were grown in 10 cm plates and scraped-off in PBS. To isolate nuclei, the cells were incubated on ice for 5 min in lysis buffer (10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40) and centrifuged at 500 *g*. Nuclei were resuspended in 120 µl glycerol buffer (50 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$, 40% glycerol) and mixed with 1 vol 2× reaction buffer (10 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 300 mM KCl, 1 mM of each GTP, ATP, CTP and 60 µCi [α-^{32}P]UTP) and incubated for 30 min at 30°C. Subsequently, DNA was digested for 30 min at 30°C by adding 360 µl DNase-buffer (10 mM Tris–HCl, pH 7.4, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 500 mM NaCl) containing 15 U of RNase-free DNase (Promega). Protein was then digested for 30 min at 42°C by adding 120 µl PK-buffer (500 mM Tris–HCl, pH 7.4, 125 mM EDTA, 5% SDS) containing 150 µg Proteinase K. RNA was isolated by phenol/chloroform extraction, precipitated with ethanol, dissolved in 200 µl TE and separated from unincorporated nucleotides by gel-filtration through a Sephadex G50 spin-column. The labeled, total RNA was then denatured for 10 min on ice by adding 15 µl 2 N NaOH, neutralized with 30 µl 1 N HCl and 12 µl 1 M Tris–HCl, pH 7.5, before hybridization. As target DNA, a plasmid containing 28S rDNA (pBS28S; 14) was linearised by digestion with *Eco*RI, then denatured for 30 min at room temperature with 0.1 vol of 0.1 N NaOH, neutralized with 10 vol of 6× SSC and dot blotted onto a nitrocellulose membrane. After prehybridisation of the membrane for 3 h at 42°C in hybridisation buffer (50% formamide, 6× SSC, 1× Denhardt's reagent, 100 µg/ml denatured tRNA), the labeled RNA was added to the mixture and hybridized for 3 days at 42°C. The membrane was then washed

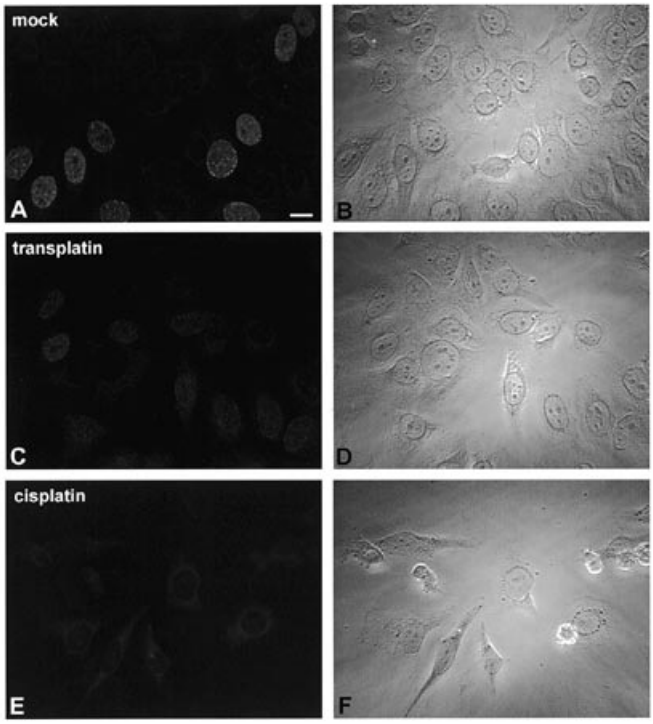

Figure 1. *In vivo* effects of *cis*- and *trans*-DDP on DNA replication. HeLa cells were treated for 20 h with either *cis*- (**E** and **F**) or *trans*-DDP (**C** and **D**) (final concentration 20 µg/ml, from 10 mg/ml stock solutions dissolved in dimethylformamide). Mock treated cells were incubated for the same time in the presence of 0.2% dimethylformamide (**A** and **B**). Following drug treatment, the cells were incubated with 1 µM BrdU for 30 min and the bromyl residues incorporated into newly synthesized DNA strands were visualized by indirect immunofluorescence. (A), (C) and (E) depict confocal fluorescent images recorded and printed using precisely the same settings in order to allow comparison of the signal intensities. (B), (D) and (F) depict the corresponding phase contrast images; note that cells were initially plated at similar densities. Bar, 10 µm.

once in 1× SSC, 0.1% SDS for 20 min at 42°C, three times in 0.2× SSC, 0.1% SDS for 20 min at 68°C, and finally exposed to a Kodak X-Omat film.

RESULTS

Cis- and *trans*-DDP affect DNA replication *in vivo*

Since it has been previously demonstrated that both *cis*- and *trans*-DDP block DNA replication *in vitro* (4), we analysed whether a similar effect occurs *in vivo*. Stock solutions of *cis*- and *trans*-DDP were prepared in dimethylformamide and added to the tissue culture medium at a final concentration of 20 µg/ml for 20 h. This corresponds roughly to the estimated blood concentration of cisplatin in a patient administered intravenously with the clinically used dosage of 50–120 mg/m^2 body surface area (8). Mock-treated cells were incubated for the same time in dimethylformamide (final concentration 0.2%).

After drug treatment, bromo-deoxyuridine (BrdU, 1 µM) was added to the culture medium and incubated for 30 min. Subsequently the cells were fixed and the incorporated bromyl residues were detected by indirect immunofluorescence. In mock-treated samples, ~28 % of the cells were brightly labeled (Fig. 1A and B), indicating that DNA synthesis was actively taking place. In samples treated with *trans*-DDP, the proportion of labeled cells decreased to ~15%; moreover, the fluorescence intensity in these cells was significantly lower than in controls (Fig. 1C and D), indicating a general reduction in DNA synthesis. Treatment with cisplatin completely abolished incorporation of BrdU indicating a complete block of DNA replication (Fig. 1E and F).

As cisplatin is commonly administered to patients diluted with physiologic saline (8), we next prepared fresh stocks of the drug in PBS and performed a time course analysis of its effect on DNA synthesis. A decrease in both the number and the intensity of BrdU-labeled cells was first noticed at 3 h, whereas by 5 h after drug treatment no labeling was observed (data not shown).

In conclusion, these results show that both *cis*- and *trans*-DDP affect DNA replication *in vivo*. However, the inhibitory effect of cisplatin is significantly higher than that of *trans*-DDP.

Cisplatin induces a redistribution of UBF and other major components of the RNA polymerase I transcription machinery

According to a currently proposed model, when HMG-domain proteins such as UBF bind cisplatin-damaged DNA, they can be displaced from their natural binding sites on the genome. To further address this view, we analysed the effect of cisplatin on the localization of UBF in the nucleus of human cells.

Initially, cells were either mock-treated with 0.2% dimethylformamide or treated with 20 µg/ml *cis*- or *trans*-DDP for 20 h. In both mock-treated cells and cells exposed to *trans*-DDP, UBF is localised in intranucleolar foci (Fig. 2A and B). A similar distribution pattern of UBF was previously described in non-treated cells and shown to correspond to sites of transcription by RNA polymerase I (15,16). Following exposure to cisplatin, UBF is detected in large aggregates which form ‘caps’ at the periphery of the nucleolus (Fig. 2C). This redistribution of UBF is detected as early as 5 h after treatment with a concentration of 20 µg/ml cisplatin. Decreasing the concentration of cisplatin to 10 µg/ml produces a similar effect within 8 h of treatment, whereas at 1 µg/ml the redistribution of UBF occurs after 20 h of exposure to the drug (Fig. 3A and B). No significant increase in the extra-nucleolar staining produced by anti-UBF antibody is observed (Fig. 3A and B), as recently reported by Chao *et al*. (17).

Previous studies from our and other laboratories have shown that UBF colocalizes with components of the nucleolar transcriptional machinery, namely TBP, TAF$_I$s and RNA polymerase I, when rRNA synthesis is either active or inactive (16,18). We therefore performed double-labeling experiments in cells treated with either cisplatin or *trans*-DDP. The results show that the redistribution of UBF induced by cisplatin is paralleled by a redistribution of TBP, TAF$_I$s and RNA polymerase I, which continue to co-localize with each other (Fig. 3C and D; and data not shown). In contrast, treatment with *trans*-DDP does not affect the distribution of any of these factors (data not shown).

Transcription of ribosomal RNA is blocked in the nuclei of cisplatin-treated cells

Since cisplatin causes a redistribution of the RNA polymerase I transcription machinery similar to that observed when rRNA synthesis is inhibited (16), we next studied the effects of the drug

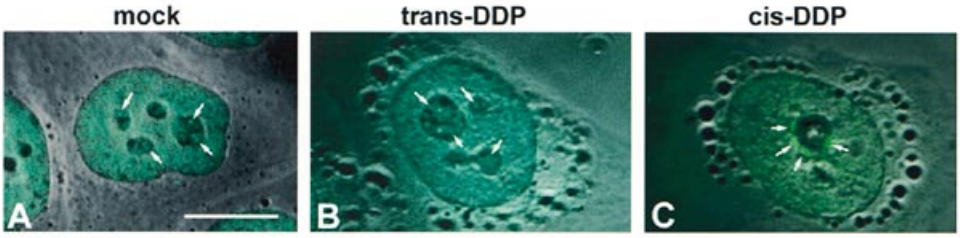

Figure 2. Cisplatin induces a redistribution of UBF in the nucleolus. HeLa cells were either mock-treated (**A**) or treated with *trans*-DDP (**B**) or cisplatin (**C**) for 20 h (final concentration 20 μg/ml). Following drug treatment, indirect immunofluorescence was performed using anti-UBF antibodies. The panels depict a superimposition of confocal fluorescent images with the corresponding phase contrast images. In mock-treated cells and in cells treated with *trans*-DDP, UBF is localized in discrete foci scattered within the nucleolus [(A) and (B), arrows]. In contrast, in cells treated with cisplatin, UBF is detected in large cap-like structures located at the outer surface of the nucleolus [(C), arrows]. Bar, 10 μm.

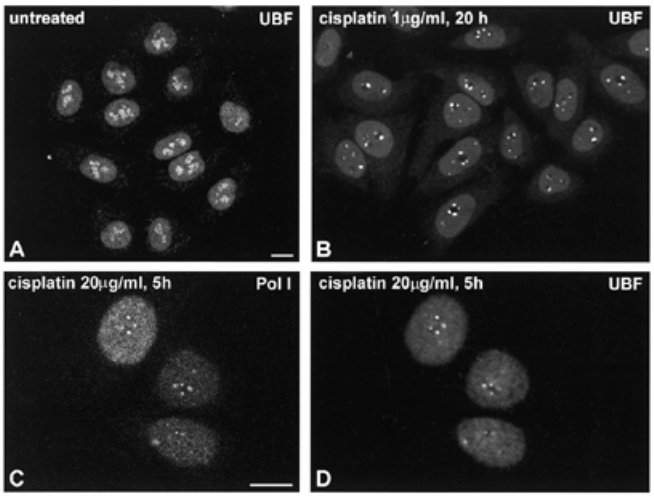

Figure 3. UBF co-localizes with RNA polymerase I in cisplatin-treated cells. Indirect immunofluorescence using anti-UBF antibodies was performed on HeLa cells that were either untreated (**A**) or treated for 20 h with 1 μg/ml cisplatin (from a fresh stock solution dissolved in PBS) (**B**). Note the redistribution of UBF similar to that observed in cells treated with higher concentration of the drug (cf. Fig. 2C). (**C** and **D**) Cells treated for 5 h with 20 μg/ml cisplatin (from a fresh stock solution dissolved in PBS) and double-labeled using anti-RNA polymerase I (C) and anti-UBF (D) antibodies. Bar, 10 μm.

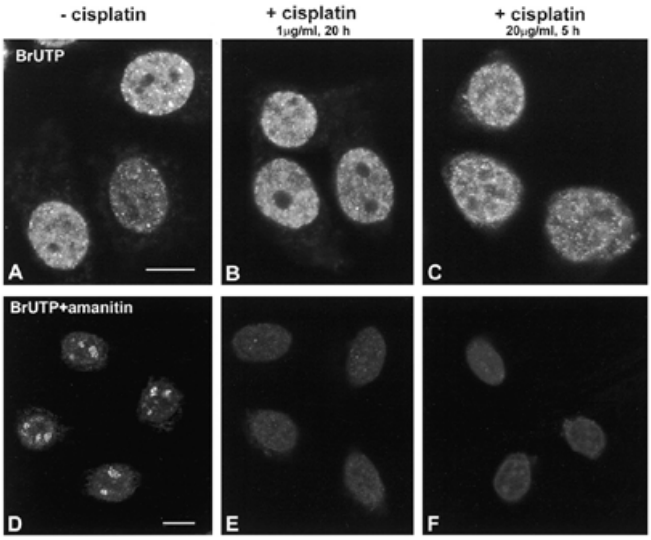

Figure 4. *In situ* labeling of transcription sites with BrUTP. HeLa cells were either untreated (**A** and **D**), or treated with cisplatin for the indicated time (**B**, **C**, **E** and **F**); stock solution of cisplatin prepared in PBS. Cells were incubated with BrUTP after a selective permeabilization of the plasma membrane, and the incorporated bromyl residues were visualized by immunofluorescence. (A–C) show that BrUTP is similarly incorporated in the nucleoplasm of treated and untreated cells. In contrast, when cells are incubated with BrUTP in the presence of 100 μg/ml amanitin (which inhibits RNA polymerases II and III), transcription sites are visualized in the nucleoli of untreated cells (D), but no signal is detected in cisplatin-treated cells (E and F). Bar, 10 μm.

on transcription. HeLa cells were mildly treated with Triton X-100 in order to selectively permeabilize the plasma membrane, and incubated with bromo-uridine 5′-triphosphate (Br-UTP). Subsequently, the incorporated Br-UTP was visualized by immunofluorescence using anti-bromyl antibodies. The results show several hundred fluorescent foci scattered throughout the nucleoplasm of untreated cells (Fig. 4A). Similar data has been previously reported and shown to correspond to sites of transcription by RNA polymerase II (13,19–22). Surprisingly, no significant changes in either labeling pattern or signal intensity are observed following exposure of cells to cisplatin (Fig. 4B and C), indicating that the drug does not block the activity of RNA polymerase II.

To specifically analyse transcription by RNA polymerase I, the cells were incubated with Br-UTP in the presence of 100 μg/ml α-amanitin to inhibit RNA polymerases II and III (13,16). Under these conditions, the labeling is exclusively observed in nucleolar foci of untreated cells (Fig. 4D), whereas no labeling is detected in cells treated with cisplatin (Fig. 4E and F). To further investigate this apparent inhibition of rRNA synthesis induced by the drug, a nuclear run-on transcription assay was performed. Nuclei from untreated and cisplatin-treated cells were isolated and incubated with [^{32}P]UTP. Total RNA was then isolated and hybridized to 28S rDNA immobilized on nitrocellulose. As depicted in Figure 5, cisplatin has a clear inhibitory effect on the synthesis of rRNA.

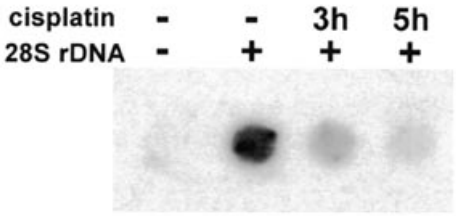

Figure 5. Run-on transcription assay. HeLa cells were either untreated or treated with 20 µg/ml cisplatin for 3 or 5 h. From these samples, nuclei were isolated and incubated with [α-^{32}P]UTP for 30 min. Subsequently, RNA was isolated and separated from unincorporated nucleotides by gel-filtration. The RNA was then denatured and hybridized to a nitrocellulose membrane. As target DNA, a plasmid containing 28S rDNA was linearized, denatured and dot blotted.

We therefore conclude that *in vivo* cisplatin affects preferentially the synthesis of rRNA.

DISCUSSION

Although it has been postulated that cisplatin has deleterious consequences for DNA replication and transcription, very few studies have addressed the direct effects of this drug on human cells. Here we describe that cisplatin causes a redistribution of the RNA polymerase I transcription machinery similar to that observed after inhibition of rRNA synthesis. Furthermore we show that cisplatin blocks preferentially the synthesis of rRNA *in vivo*.

Accurate and specific transcription of rRNA genes by RNA polymerase I requires at least two transcription factors, UBF and SL1 (promoter selectivity factor) (23,24). UBF is a DNA binding protein that binds to the upstream control element of the promoter; it consists of two alternatively spliced polypeptides of 97 and 94 kDa (UBF1 and UBF2, respectively) and was found to be highly conserved among various vertebrates (25,26). UBF interacts with DNA by way of multiple HMG-box domains and in doing so greatly enhances recruitment of SL1, which appears to mediate communication with RNA polymerase I (27,28). Thus, if UBF fails to bind to the promoter, RNA polymerase I cannot initiate transcription. SL1 is a multisubunit complex containing TBP and three TAF_Is of 110, 63 and 48 kDa, which are essential to activate rRNA transcription (12).

As the UBF–promoter interaction is highly sensitive to the antagonistic effects of cisplatin–DNA adducts (7,29), it was predicted that the drug could disrupt rRNA synthesis. Our results fully support this view. Importantly, our data show that the DNA adducts induced by the clinically ineffective *trans* isomer of cisplatin, which are not recognised by UBF (7), do not interfere with the localization of the RNA polymerase I transcriptional machinery. This strongly suggests that block of rRNA synthesis is involved in the cytotoxicity of cisplatin.

Previous work revealed that cisplatin–DNA adducts cause an elongation block during *in vitro* RNA synthesis by prokaryotic and eukaryotic RNA polymerases (30,31). Additionally, a 2–3-fold decrease in transcriptional level was observed when platinum-modified reporter genes were transfected into human or hamster cells (32). Cisplatin was also shown to substantially reduce transcription from the mouse mammary tumor virus promoter stably incorporated into mouse cells (33). Moreover, the basal transcription factor TBP was recently reported to bind selectively and competitively to cisplatin-damaged DNA, thereby inhibiting transcription from an independent and transcriptionally viable template (34). It is therefore surprising that cisplatin-treated HeLa cells continue to incorporate Br-UTP in the nucleoplasm (Fig. 3). However, it should be emphasized that, in contrast with the previous studies, we have analysed the effects of cisplatin on endogenous transcription levels of the cellular genome. One possibility to reconcile these data is that *in vivo* rDNA represents a preferential target for cisplatin damage. In fact, cisplatin does not remove UBF from the nucleolus, and the peripheral caps containing the rRNA transcriptional machinery in inactivated nucleoli have been previously shown to co-localize with rDNA (16). Thus, it seems unlikely that the block of rRNA synthesis is due to hijacking of UBF from the nucleolus to other sites in the nucleus. Alternatively, we favour the view that the high concentration of proteins that bind to cisplatin-damaged DNA (such as UBF and TBP) in the nucleolus is responsible for a higher incidence of cisplatin lesions in rDNA. In this regard it is noteworthy that a yeast strain deprived of Ixr1 (an HMG-domain protein that binds to platinated DNA) was reported to be half as sensitive to cisplatin and accumulated one-third as many platinum–DNA lesions as the wild-type strain (35). At least two models are consistent with these data. One is that UBF (and possibly also TBP, as part of SL1) shields rDNA from repair enzymes and therefore cisplatin lesions persist and accumulate in the nucleolus, while extra-nucleolar lesions are more efficiently repaired. The other possibility is that binding of UBF and TBP to rDNA facilitates the formation of cisplatin lesions in the nucleolus.

In conclusion, our data suggest that a preferential inhibition of rRNA synthesis is likely to be involved in the cytotoxicity of cisplatin. Because ribosomal transcription regulates ribosome production and, consequently, the translation potential of a cell, it is conceivable that deregulation of ribosomal transcription may, in the long term, be an important determinant in neoplastic transformation (36). By blocking ribosomal transcription, cisplatin may therefore be preferentially cytotoxic to rapidly proliferating transformed cells.

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