THE ROLE OF HUMAN UPSTREAM BINDING FACTOR hUBF IN MEDIATING THE CYTOTOXICITY OF THE ANTICANCER DRUG CISPLATIN

by

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Submitted to the Division of Toxicology on September 3, 1996, in partial
fulfillment of the requirements for the Degree of Doctor of Philosophy

ABSTRACT

Recently a class of cellular proteins that contains a DNA-binding motif homologous to
nuclear high mobility group (HMG) proteins has been shown to specifically recognize DNA
lesions formed by cis-diamminedichloroplatinum(II) (cis-DDP). Models have been
introduced to describe possible roles for HMG box proteins in mediating the anticancer
activity of cis-DDP. One hypothesis suggests that HMG box proteins can, by preferentially
binding to cis-DDP damaged site, block the adduct from access by cellular repair machinery,
thereby sensitizing cells to cis-DDP treatment. Another hypothesis proposes that the
transcription factors among the HMG box proteins can be titrated from their original DNA
regulatory sites by cis-DDP adducts, resulting in a disruption of downstream gene regulation
that is critical for cell survival. The results presented in this dissertation identify the
ribosomal transcription factor hUBF as one of the HMG box proteins that specifically
recognize DNA modified by cis-DDP. Domains of hUBF that contribute to the recognition
of platinated DNA were mapped. The data showed that multiple HMG boxes contribute to
the binding in an additive way, which may explain the remarkable binding affinity of hUBF
for the cis-DDP adducts. Moreover, in a proof of concept experiment, in vitro rRNA
transcription in the presence of cis-DDP modified plasmid DNA was drastically inhibited in
a dose dependent manner. In contrast, the level of transcription is not perturbed by an
increased amount of control unmodified DNA or DNA modified by trans-DDP. The ratio of
cis-DDP adduct to rDNA promoter at which rRNA is completely inhibited correlates with
the relative binding affinity of hUBF for platinum adducts and rDNA promoters. These data
suggest that hUBF could be involved in mediating cis-DDP toxicity by a titration
mechanism. In addition, the relationship between the binding of hUBF to DNA modified by
other platinum compounds with respect to their stereochemical structures, and their clinical
effectiveness was investigated and discussed. Several preliminary experiments examining
the titration model in mammalian cells were carried out and the results are discussed.

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Title: Professor of Toxicology and Professor of Chemistry
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<tr>
<td>AA</td>
<td>atomic absorption spectroscopy</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPDE</td>
<td>benzo[a]pyrene diol epoxide</td>
</tr>
<tr>
<td>cis-DDP</td>
<td>cis-diamminedichloroplatinum(II)</td>
</tr>
<tr>
<td>DACH</td>
<td>diaminocyclohexane</td>
</tr>
<tr>
<td>DIEN</td>
<td>diethylenetriamine</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>DRP</td>
<td>damage recognition protein</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
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<td>ethylenediamine</td>
</tr>
<tr>
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<td>fetal calf serum</td>
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<td>hUBF</td>
<td>human upstream binding factor</td>
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<tr>
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<tr>
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<td>formal bound drug/nucleotide ratio</td>
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<td>TATA binding protein</td>
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<tr>
<td>trans-DDP</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>XP</td>
<td>Xeroderma Pigmentosum</td>
</tr>
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INTRODUCTION
The biological effect of the platinum coordination compound cisplatin (or cis-DDP) was first observed by Rosenberg in the study of the effects of electric fields on the growth of the bacterium *E. coli* (Rosenberg *et al.*, 1965). The filamentous structure formed by the bacteria under the experimental conditions was discovered to be the effect of the platinum amine chloride complexes generated by electrolysis at the platinum electrodes. One of the toxic compounds that were responsible for the growth inhibition was identified to be *cis*-diamminedichloroplatinum(II), also known as Peyone’s chloride, a classic platinum coordination complex whose structure and synthesis had been known for over a century. The antitumor effects of *cis*-DDP were demonstrated in rapidly growing sarcoma 180 and leukemia L1210 cell lines (Rosenberg *et al.*, 1969). In 1971, *cis*-DDP was introduced into clinical trials and was subsequently approved by the FDA for the treatment of several cancers in 1979.

The mechanism underlying the efficacy of *cis*-DDP has been under intensive study since the discovery of its anticancer activity. The following lines of evidence have led most workers to conclude that the critical molecular target of *cis*-DDP is DNA (for reviews, see Roberts and Thomson, 1979; Rosenberg, 1980). Inhibition of cell division and the induction of filamentous growth in bacteria by *cis*-DDP reported during initial research were traits characteristic of the DNA damaging agents. Induction of prophage lysis in bacteria was also a common effect of DNA damaging agents. Bacterial DNA synthesis was selectively inhibited by *cis*-DDP. Bacterial mutants, eukaryotic strains and human cells from patients defective in
DNA excision repair were most sensitive to *cis*-DDP. By contrast, *trans*-DDP, the stereoisomer of *cis*-DDP, was clinically ineffective in cancer treatment. The *cis*/*trans* stereochemical constraints in these compounds strongly influence their chemical reactivity and the structures of the DNA adducts they form. A comparison of the adduct spectra reveals that the 1,2-intrastrand crosslinks formed predominantly by *cis*-DDP at d(GpG) and d(ApG) sites, are absent in the population of *trans*-DDP adducts (Douple, 1982). Thus, it is speculated that the 1,2-intrastrand crosslinks are the lesions responsible for the antitumor activity of *cis*-DDP (Pinto and Lippard, 1985). Although adduct-induced blockage of DNA replication, transcription and repair have been shown to occur upon treatment of *cis*-DDP, the precise mechanism by which the formation of DNA adducts leads to cell death is still unclear (Chu, 1994; Eastman et al., 1993).

Recent studies have led to the discovery of cellular proteins with sequence homology to nuclear high mobility group proteins, HMG1/HMG2, that are able to bind *cis*-DDP-DNA adducts (Toney et al., 1989; Donahue et al., 1990; Bruhn et al., 1991; Pil and Lippard, 1992; Billings et al., 1992; Brown et al., 1993; Treiber et al., 1994). It was soon apparent that the HMG box proteins in general have distinct cellular functions, such as transcription regulation, DNA packaging and recombination. The observed binding to *cis*-DDP modified DNA is probably an inadvertent event. Two mutually non-exclusive models have been proposed to describe the possible mechanisms by which physical binding of the HMG box proteins to DNA adducts can mediate the cytotoxicity of *cis*-DDP. The “DNA repair
blocking" model suggests that the binding of HMG box proteins to cellular platinum adducts would shield the adducts from the cellular repair machinery, thus allowing adducts to persist and cause cell death. The "transcription titration" model proposes that the binding of the HMG box-containing transcription factors to the adducts would divert the transcription factors away from their original promoter binding sites and disrupt transcription of downstream genes that are important for survival.

In Southwestern experiments with the original goal being to accurately determine the molecular weight of the previously identified cis-DDP binding protein, SSRP1, a doublet of bands corresponding to molecular weights of 97 and 94 kDa were observed. These proteins recognized specifically DNA modified by cis-DDP. From available amino acid sequence data, a likely candidate was the human upstream binding factor (hUBF), an HMG box protein existing in HeLa cells as both 97 and 94 kDa species due to alternative splicing (Jantzen et al., 1990). The titration model was suggested after work done during a collaboration with my colleague Daniel Treiber. We determined that the ribosomal transcription factor hUBF has the ability to recognize platinated DNA with remarkable affinity (Treiber et al., 1994).

The main goal of my thesis research was to investigate the role of hUBF in mediating the cytotoxicity of cis-DDP. In the work described in this dissertation, the binding specificity of hUBF for DNA modified by several platinum compounds was examined, and domains of hUBF that participated in binding were mapped. In vitro
evidence supporting the titration model was generated. Finally, preliminary experiments to test the titration hypothesis *in vivo* were performed and the results discussed. The results of this work provide the first evidence in support of a “titration model” for the role of hUBF in mediating the cytotoxicity of *cis*-DDP. *cis*-DDP adducts may serve as molecular decoys for hUBF in cells treated with *cis*-DDP and contribute to anticancer efficacy by inhibiting ribosomal RNA transcription.
CHAPTER 1

Literature Survey
1. DNA lesions formed by platinum compounds

The kinetics of the reaction between cis-DDP and DNA has been extensively reviewed (Sherman and Lippard, 1985). In circulating plasma where the chloride ion concentration is as high as 100 mM, both cis-DDP and trans-DDP retain their chloride ligands. However, upon entering the cytoplasm, where the chloride ion concentration is only 4 mM, the chloride ligands become hydrolyzed. The labile chloride ligands are replaced by water molecules to generate a positively charged active species that can react at the N7 position of purines to form a variety of DNA adducts. The adduct spectrum in salmon sperm DNA treated with cis-DDP has been determined by nuclease digestion followed by chromatographic and NMR analysis (Fichtinger-Schepman et al., 1985). The major DNA adducts formed by cis-DDP include 1,2-intrastrand d(GpG) (65%) and d(ApG) (25%) crosslinks. Minor adducts include 1,3-intrastrand d(GpNpG) (<8%) crosslinks, monofunctional adducts and interstrand crosslinks (<1%) (Fichtinger-Schepman et al., 1985). The adduct spectrum formed by trans-DDP has not been definitively determined, but it is known that trans-DDP can form adducts occur in the G-rich regions with a greater variety and less apparent sequence specificity than that of cis-DDP; they include 1,3- and 1,4-intrastrand crosslinks, monofunctional adducts and a small fraction of interstrand crosslinks (Bruhn et al., 1990). Because of stereochemical constraints in trans configuration, trans-DDP is unable to form the 1,2-intrastrand crosslinks predominantly
formed by *cis*-DDP at d(GpG) and d(ApG) sites (Figure 1-1). Thus, it is speculated that the 1,2-intrastrand crosslinks are the lesions responsible for the antitumor activity of *cis*-DDP (Pinto and Lippard, 1985).

The DNA adduct spectra formed by platinum compounds in which the leaving groups are in the cis geometry, such as [Pt(en)Cl₂] (or EN) and [Pt(dach)Cl₂] (or DACH), bear close similarity to that determined for *cis*-DDP. The therapeutic effectiveness of this category of compounds is well known (Roberts and Thomson, 1979). Monoclonal antibodies raised against *cis*-DDP damaged DNA exhibit cross-reactivity toward adducts formed by these molecules, suggesting stereochemically similar structures on DNA (Sundquist et al., 1987). EN and the (R, R) and (S, S) isomers of DACH form nearly identical adduct profiles, 60-65% d(GpG) and 25% d(ApG) 1,2-intrastrand crosslinks, 7-10% monofunctional adducts and 6-10% 1,3-intrastrand crosslink and <2% interstrand crosslinks (Eastman et al., 1986; Jennerwein et al., 1989). A third (R, S) isomer of DACH forms 22, 16, 12, and 17% of the 1,2-d(GpG), 1,2-d(ApG), 1,3-d(GpTpG) and interstrand crosslinks, respectively. Interestingly, this is the least effective among the three isomers in cancer treatment, suggesting that specific types of adducts are involved in the cytotoxicity.

Platinum compounds with only one leaving group, such as [Pt(dien)Cl]⁺ (or DIEN) and [Pt(NH₃)₂(N₃-cytosine)] form monofunctional adducts mainly at N7-guanine in DNA (Hollis et al., 1989). DIEN possesses a tridentate ligand and is clinically ineffective.
[Pt(NH₃)₂(N₃-cytosine)], by contrast, is among a novel class of effective antitumor agents that contains three separate nitrogen donor ligands, cis-[Pt(NH₃)₂(N-donor)Cl]+ (donor = pyridine, pyrimidine, purine, piperidine, or aniline) (Hollis et al., 1989).

It should be noted that although platinum compounds with stereochemical structure similar to cis-DDP in general have high therapeutic indices, therapeutically active platinum compounds encompass various types of geometries (cis/trans) and coordinations (Pt(II)/Pt(IV)) and the mechanisms involved in the drug action may not be identical. For example, cis-[Pt(NH₃)₂(N-donor)Cl]+ forms exclusively monofunctional adducts and is active against tumor cells. Recently, a trans platinum complex, [PtCl₂(imino ether)₂], has been shown to possess higher antitumor activity than its cis congener (Coluccia et al., 1993). The drug mainly forms stable monofunctional adducts at guanine residues (Brabec et al., 1996). These drugs may utilize mechanisms different from the 1,2-intrastrand crosslinks formed by the cis-DDP category of compounds.

2. Structural features of platinum adducts

Electrophoretic studies of DNA modified by single platinum adducts situated at discrete positions have determined the effects of cis-DDP and trans-DDP adducts on DNA structure. With cis-DDP, both 1,2-intrastrand adducts of d(GpG) and d(ApG) bend DNA
34-40° towards the major groove and unwind DNA by 13°, whereas a 1,3-d(GpTpG) intrastrand adduct bends DNA by 35° and unwinds it by 23° (Rice et al., 1988; Bellon and Lippard, 1990; Bellon et al., 1991). The interstrand crosslink between two guanine residues in the base pairs of d(GpC) bends DNA by approximately 55° toward major groove with structural distortion localized at the two crosslinked base pairs (Sip et al., 1992). The 1,2-intrastrand d(GpG) crosslinks formed by the cis compounds EN and DACH also induce bending in DNA (Boudny et al., 1991).

By contrast, a multimer ligation experiment to determine the bending angle of a trans-1,3-intrastrand crosslink in DNA was inconclusive, presumably because the adduct induces in DNA a degree of hinge flexibility that disrupts the phase of the bending (Bellon et al., 1991); the unwinding was reported to be 6° -13° (Keck and Lippard, 1992). The major monofunctional adducts at dG sites formed by DIEN distort DNA in a sequence-dependent manner and decrease the thermal stability of the duplex (Brabec et al., 1992). Moreover, monofunctional adducts formed by DIEN and cis-[Pt(NH₃)₂(N3-cytosine)]⁺ do not bend DNA (Marrot and Leng, 1989; Bellon and Lippard, 1990).

The structure of the 1,2-intrastrand d(GpG) cis-DDP adduct in an oligonucleotide (octamer to dodecamer) has been solved by NMR as well as X-ray crystallography (Herman et al., 1990; Yang et al. 1995; Takahara et al. 1995). DNA bending (58°-60°) and unwinding (12°-21°) determined by gel electrophoresis has been confirmed by NMR. The
crystal structure of a 1,2-d(GpG) cis-DDP adduct in a dodecamer reveals that in addition to DNA bending (39° or 55° without disrupting neighboring base pairing) and unwinding (from 8° to 37°), the minor groove is enlarged at the lesion site and an abrupt transition from a B-type helix segment to an A-type helix segment occurs at the adduct site (Takahara et al., 1995). The intrastrand crosslink formation causes the platinum atom to be somewhat displaced from the two guanine planes. Recently, a novel class of DNA binding proteins with homology to the nuclear chromosomal protein HMG1/2 (HMG stands for high mobility group) has been found to bind specifically to DNA modified by cis-DDP. It has been proposed that the HMG box domain may recognize the A/B hybrid DNA conformation and that binding of the protein may relieve the strain on platinum coordination (Takahara et al., 1995).

3. Proteins that recognize cis-DDP adducts

Evidence suggests that the binding of cellular proteins to platinum adducts in DNA may play a role in the cytotoxicity and antitumor activity of cis-DDP. Proteins that bind specifically to DNA modified by cis-DDP have been observed in human cellular extracts using a gel mobility shift assay (Chu and Chang, 1988; Donahue et al., 1990). Overexpression or induction of certain platinum-DNA recognition proteins in cis-DDP
resistant cell lines has been reported in Southwestern experiments (Chao et al. 1991; Bissett et al., 1993; Mello et al., 1996).

In a gel mobility shift assay, the cis-DDP damage recognition protein (DRPs) in the HeLa extract bind only to the 1,2-d(GpG) and 1,2-d(ApG) intrastrand crosslinks of cis-DDP, but not to unmodified DNA or the 1,3-d(GpTpG) intrastrand crosslinked products of either cis-DDP or trans-DDP (Donahue et al., 1990). The proteins also bind to the adducts of two other platinum compounds, EN and DACH, which can also crosslink adjacent nucleotides in DNA. By contrast, the d(G)-N7 monofunctional adducts of DIEN and [Pt(NH₃)₂(N3-cytosine)]⁺ are not complexed by the DRPs. On the basis of these data, it was surmised that certain platinum adducts form structures that mimic certain natural DNA motifs recognized by cellular proteins. The 1,2-intrastrand d(GpG) and d(ApG) adducts formed by cis-DDP cause the DNA to bend by 32° toward the major groove (Rice et al., 1988, Bellon et al., 1990) and unwind by 13° (Bellon et al., 1991). It was suggested that the combined features of bending and local unwinding may be the motif needed for protein recognition. Bending alone is insufficient as UV irradiated DNA causes a similar degree of bending, but it does not compete with cis-DDP treated DNA for the binding of DRPs (Donahue et al., 1990; Bellon et al., 1990). Moreover, the cis-1,3- d(GpTpG) adduct, which bends DNA to a similar degree of 35° as 1,2-intrastrand d(GpG) and unwinds it by 23° (Bellon and Lippard, 1990; Bellon et al., 1991), is not recognized by HMG1 (Pil and Lippard, 1992), indicating HMG box proteins do not bind to all of the types of bent structures formed by cis-DDP.
An 81 kDa human protein that binds selectively to DNA modified by cis-DDP was cloned and named SSRP1 (structure-specific recognition protein) (Toney et al., 1989; Bruhn et al., 1992). The binding profile of histidine-tagged SSRP1 to DNA modified by various platinum compounds has been investigated by a gel mobility shift assay (Pil, 1993). SSRP1 recognizes only adducts formed by compounds with a cis geometry that are able to form coordinate bonds to two adjacent purines, which is consistent with a pattern of structure-specific recognition. Sequence analysis revealed a domain of 75 amino acids which is homologous to the chromosomal protein HMG1/2 and which is the DNA binding motif of a novel class of transcription factors and nuclear nonspecific DNA binding proteins (Laudet et al., 1993). No functional role for SSRP1 has been determined, although it has homology to a mouse protein identified in screens for recognition of the V(D)J recombination sequence (Shirakata et al., 1991), to a Drosophila single-stranded DNA/RNA binding factor identified in screens for chorion gene promoter binding and to a transcription factor that binds to enhancer elements upstream from the rat and chick collagen II gene (Wang et al., 1993). It is emphasized, however, that none of the studies assigned a functional role for the homologs of SSRP1.

The prototype of the HMG box proteins, the nuclear chromosomal protein HMG1, which contains two HMG boxes (designated A and B) without strong homology to each other, has also been identified as a cis-DDP-modified DNA binding protein (Pil and Lippard,
The protein binds to cis-1,2-intrastrand d(GpG) and d(ApG) but not to cis-1,3-d(GpTpG) crosslinked DNA. The dissociation constant of HMG1 to a single d(GpG) or d(ApG) intrastrand crosslink in a 100 bp duplex is reported to be $3.7 \times 10^{-7}$ M, which is approximately 100-fold tighter than binding to unmodified DNA. Recently, HMG1 has been shown to bind to a single d(GpC) interstrand crosslink formed by cis-DDP but not by trans-DDP (Kasparkoa and Brabec, 1995). The authors suggest that additional structural distortions such as local denaturation may also play a role. Repair of the cis-DDP 1,2-d(GpG) crosslink by human excision nuclease in a HeLa cell free extract is specifically inhibited by HMG1 and another HMG box protein, the mitochondrial transcription factor, mtTF1 (Huang et al., 1994), indicating that the “block of repair” model is feasible at least in vitro. More recently, the repair of cis-1,2-d(GpG) and -d(ApG) adducts in HeLa cell free extracts was shown to be inhibited by HMG box domains from rat HMG1 box B, mouse testis determining factor mSRY and mouse testis specific HMG protein (tsHMG), in addition to full length HMG1 (Zamble et al., 1996). The study of the role of HMG1 in mediating cis-DDP toxicity in vivo by down regulating its expression has proven difficult as HMG1 appears to be an essential cellular component (Treiber, 1993).

Ixrl, a yeast protein containing two repeated HMG-box motifs that binds DNA modified by cis-DDP, has been isolated and sequenced (Brown et al., 1993). It is a transcription repressor for the yeast cytochrome b oxidase subunit, Cox5b (Lambert et al., 1994). Disruption of the Ixr1 gene causes the yeast strain to become two-fold resistant to cis-
DDP as compared to the parental yeast strain. The Ixr1 inactivated strain also accumulates only one-third as many platinum-DNA adducts after treatment with cis-DDP. These observations are consistent with a model in which the Ixr1 protein plays a role in mediating cis-DDP cytotoxicity by shielding intrastrand crosslink adducts from the cellular repair machinery. It is also possible, however, that the gene alters the cellular uptake of platinum drugs. Subsequent work showed that the desensitization to cis-DDP treatment caused by an Ixr1 knockout can be as high as six-fold in other yeast strains. Moreover, resistance was nearly abolished when the effect of Ixr1 knockout was examined in several nucleotide excision repair mutant strains (McA’Nulty et al., 1996), further supporting the “repair blocking” model. By contrast, treatment with cis-DDP does not alleviate Ixr1 related transcription repression in the Cox5b gene present in genomic DNA or in a reporter gene controlled by the Cox5b promoter (McA’Nulty and Lippard, 1996), indicating that Ixr1 cannot be titrated away by cis-DDP-DNA adducts.

Figure 1-2 lists HMG boxes that have been tested so far for their ability to bind DNA modified by cis-DDP. The only known HMG box that does not bind to platinated DNA is the yeast HMG box protein, Rox1, which is also a Cox5b gene repressor. (Balasubramanian et al., 1993, Di Flumeri et al., 1996) Experiments have shown that the protein does not bind platinated DNA (McA’Nulty and Lippard, 1996). However, it is possible that this negative activity may be related to the unusual HMG box structure of the Rox1 protein. This HMG box possesses a five amino acid insertion not present in other HMG boxes.
Several cis-DDP DRPs identified so far do not contain HMG boxes. The *E. coli* UvrA and UvrB components of the UvrABC excision repair complex bind a *cis*-d(GpG) adduct in a 96 bp DNA fragment with an apparent *K*_d* on the order of 10^-9 M (Visse *et al.*, 1990 and 1992). The bacterial repair nuclease recognizes the *cis*-DDP damage site and cleaves the 8th or sometimes 15th phosphodiester bond 5’ to the lesion and the 4th phosphodiester bond 3’ to the lesion. Footprinting analysis suggests that UvrAB binds to the convex side of the kink caused by adduct formation. Human single strand binding protein (SSB), a protein known to be involved in mammalian excision repair, has been found to bind *cis*-DDP adducts in a gel mobility shift assay using a 54 bp platinated DNA as a probe (Hsu *et al.*, 1993). A protein that is absent from extracts prepared from the human UV repair deficient cell line *Xeroderma pigmentosum* complementation group E not only binds to UV-damaged DNA, but also to DNA modified by *cis*-DDP, although with much lower affinity (Chu and Chang, 1988). In cells made resistant to *cis*-DDP, this protein is found to be overexpressed. Interestingly, this cell line also exhibits slightly increased repair capacity for platinum-DNA adducts. The authors suggest that the protein may play a role in the nucleotide excision repair mechanism. Most recently, the human mismatch repair protein hMSH2, a protein involved in recognition of the insertion/deletion mispairs and single-base mismatches, and a risk marker in the development of human hereditary non-polyposis colorectal carcinomas, has been shown to recognize DNA adducts formed by *cis*-DDP and EN, but not by *trans*-DDP, DIEN or unmodified DNA (Mello *et al.*, 1996). Notably, hMSH2
protein is highly enriched in testicular and ovarian tissues, suggesting a possible role in the organotropism of the drug (cisplatin is primarily useful in treating tumors that arise in these organs). It is proposed that binding to cis-DDP adducts by hMSH2 may contribute to toxicity either by engaging the mismatch repair system in futile repair cycles or by blocking the repair machinery and allowing the persistence of the adducts, both ultimately resulting in cell death. Both repair shielding and abortive repair may work in concert to potentiate the cytotoxic effects of the drug.

4. HMG box proteins: structure specificity vs. sequence specificity

hUBF was the first protein identified to have homology with HMG1 (Jantzen et al., 1990). Since then, the number of HMG box proteins identified has grown to 123, and 14 different HMG box genes have been recognized in humans (Baxevanis and Landsman, 1995).

One prominent feature of all the HMG box proteins is the ability to bind pre-bent DNA structures as well as to bend DNA sequences upon binding (Lilly, 1992; Laudet et al., 1993). HMG box proteins can be divided into two categories. The first group includes HMG1/2, and other proteins such as hUBF and mtTF1, which contain multiple HMG domains but weak or no sequence specificity for DNA binding. The second group of
proteins, containing a single HMG box with substantial sequence specificity for DNA recognition, includes mammalian testis-determine region SRY, Sox (the SRY related series of proteins, lymphocyte enhancer factor LEF-1, yeast mating-type control protein Ste11, and SSRP1. The homology between these HMG boxes is loose, with average 25% sequence identity among different HMG boxes. It is due to this low level of homology that hUBF was originally reported to have only 4 HMG boxes when in fact six are present. NMR studies confirm that the basis for the homology is mainly structural.

The solution structures of several HMG box domains have been solved by NMR, including structural specific boxes from murine HMG1 B domain and Drosophila HMG-D, as well as a sequence specific HMG box from murine Sox-4 (Weir et al., 1993; Jones et al., 1994; Read et al., 1993; van Houte et al., 1995). The structure of the boxes are similar despite loose base homology. Three α-helical regions fold into an L-shaped structure and the angle between the two arms is 70-90°, defined by a group of conserved aromatic amino acid residues.

The requirement for structure specific HMG box proteins to bind bent DNA is remarkably low. Mutagenesis studies in domain A of HMG1 show that certain mutants cannot be produced in E. coli, which indicates grossly disrupted protein folding. However, whenever the HMG box can fold, it can interact specifically with four-way junction DNA (Falciola et al., 1994). In another study, mutation of a highly conserved tryptophan residue
in domain A of HMGI largely but not completely destroys the protein tertiary structure and abolishes its ability to supercoil DNA. The mutant has only 10% α-helix (originally 70%), and the structure does not refold in the presence of DNA. However, the binding to four-way junction DNA remains active. These observations suggest that the structure specific DNA binding might be a property of some primary structure element that forms a junction-binding-mode (Teo et al., 1995a).

The structure specific HMG boxes do differ among themselves (such as box A and B in HMGI) with regard to the relative affinities for four-way junction DNA and their abilities to bend DNA (Teo et al., 1995b). These differences in HMG boxes may be further enhanced in the presence of different neighboring residues and additional domains in the full length protein such as the C-terminal acidic domain of HMGI. This indicates the adaptation of HMG box proteins to different functions during evolution (Stros et al., 1994; Lnenicek-Allen et al., 1996).

By contrast, sequence specific DNA recognition requires the presence of specific amino acid residues that may not be required for the protein to bind bent DNA. A mutation in the human SRY protein, the factor that causes sex reversal (Ile168 to Tyr168), reduced sequence specific binding by over 50-fold but retained nearly wild-type binding affinity for a four-way DNA junction (Peter et al., 1995). An analysis of the amino acid residue in the equivalent position of Ile168 in other HMG box proteins reveals that only
Isoleucine, methionine and phenylalanine are present in sequence specific HMG boxes whereas a diverse group of residues are present in the same position in structure specific HMG boxes (King et al., 1993). The study also suggested that the insertion of Ile168 into the minor groove plays a critical role in sequence specific recognition. The above three amino acids in the sequence specific recognition group may be able to preserve the specific steric and polarity requirements of side-chain insertion into minor groove (King et al., 1993).

The structural components of sequence specific recognition can be separated from structure specific DNA binding by a mutational analysis. Five SRY mutations from patients with complete gonadal dysgenesis were analyzed for their abilities to bind sequence specific DNA and four-way DNA junctions and their abilities to bend DNA. Four-way junction DNA binding was fully retained in all the mutants. Two mutants bind and bend DNA almost normally, two bind inefficiently but bend DNA normally and one binds DNA with near normal affinity but produces a different bending angle. It appears therefore, that binding to DNA by SRY is not enough to direct potential transcription, and the exact angle induced by SRY may play a role in the spatial arrangement of the nucleoprotein complex important for sex determination (Pontiggia et al., 1994).

The molecular basis for sequence specific DNA recognition by HMG box domains of LEF-1 and SRY has been determined by NMR (Werner et al., 1995; Love et al., 1995).
The protein structures are similar to each other and to previously determined HMG boxes, possessing the characteristic L-shaped fold of three helices. The DNA is bound to the concave surface of the HMG box and contacts occur exclusively in the minor groove of DNA. The DNA is severely bent towards the major groove throughout the recognition region, causing an average $117^\circ \pm 10^\circ$ bend for the 15-mer LEF-1 sequence and a $70-80^\circ$ bend for the octamer SRY binding sequence. The DNA helix is also unwound by $19-34^\circ$. Among several other amino acid residues that contact and widen the minor groove are the isoleucine in the SRY, and the methionine in LEF-1 as predicted by mutation studies.

Although the structure of a cis-DDP adduct/HMG box complex has not yet been solved, the structure of a dodecamer containing a cis-1,2-intrastrand d(GpG) adduct closely resembles the DNA structures in the SRY/DNA and LEF-1/DNA complexes: The DNA is bent severely toward a narrowed major groove and unwound, while the minor groove is shallow and expanded (Takahara et al., 1995). Therefore, it appears that the specific structural distortion by the adduct mimics the bent structure of sequence specific protein-DNA interaction. This may serve as the motif for recognition by HMG boxes (Figure 1-3).

5. cis-DDP treatment and altered gene expression
The analysis of cell death induced by *cis*-DDP reveals a pattern of DNA fragmentation into multimers of approximately 180 base pairs that is consistent with internucleosomal cleavage of chromatin by an endonuclease followed by loss of membrane integrity and cell shrinkage. Cells treated with *cis*-DDP progress through the S phase of the cell cycle and arrest at G$_2$ for several days where the cells either recover or die. It was proposed that apoptosis (programmed cell death), may be the mechanism mediating the antitumor activity of the drug (Sorensen *et al.*, 1988 and 1990; Eastman, 1990). The process of apoptosis may explain the earlier observation that DNA repair-deficient Chinese hamster ovary cells die at a *cis*-DDP concentration that does not inhibit DNA synthesis (Howle and Gale, 1970). This process is an active process, since inhibition of protein synthesis inhibits the internucleosome cleavage (Eastman, 1990).

Alteration of cellular gene expression is one of the hallmarks of tumorigenesis. Patterns of gene expression associated with *cis*-DDP treatment have been investigated. Studies have shown that the wild-type tumor suppressor p53 levels increase upon treatment of cells with DNA damaging agents, including *cis*-DDP. These treatments also cause cells to arrest at the G$_1$ phase of the cell cycle followed by induction of apoptosis. Cells with mutant p53, however, failed to arrest at the G$_1$ phase and the cells exhibited an enhanced survival upon treatment of the drug (Kastan *et al.*, 1991; Lowe *et al.*, 1993). Interestingly, wild-type p53 was found in all testicular tumors (Peng *et al.*, 1993), and thus the presence of the wild type protein may correlate with the high success rate of *cis*-DDP treatment for this specific
type of cancer. The mRNA levels of Rb, another tumor suppresser gene, were found to be lowered in testis and small-cell lung cancer cell lines, which are treatable by *cis*-DDP (Sakesela *et al.*, 1989). Rb has recently been shown to translocate into nucleoli and bind hUBF in monocyte-like U937 cells upon induction of differentiation by a phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). This process is correlated with a dramatic decrease in the rate of rRNA synthesis (Cavanaugh *et al.*, 1995). Therefore, Rb is able to inhibit cell growth by regulating hUBF mediated rRNA synthesis, and the reduced Rb expression in tumor cells may be associated with a reduced inhibition of rRNA synthesis resulting in increased growth in tumor cells.

Alternation in many other cellular genes can also modulate the toxicity of *cis*-DDP. Mouse NIH 3T3 cells exhibit an 8-fold increase in *cis*-DDP resistance upon transfection of an inducible viral promoter linked to an activated c-Ha-*ras* gene. Induction of the *ras* oncogene reduced *cis*-DDP accumulation by 40% and intrastrand adduct formation by 17% (Isonishi *et al.*, 1991) without altering the cellular glutathione content or the activity of glutathione-S-transferase, although the metallothionein content was increased by 1.6 to 3.0 fold. In another study, the effect of *cis*-DDP on gene expression was investigated by transient transfections in HeLa cells using four different viral promoters (Evens and Gralla, 1992). HIV or adenovirus E3 promoter are induced by low doses of *cis*-DDP whereas monkey virus SVER and adenovirus MLP promoters are inhibited. The authors suggest that
a drug-induced imbalance in gene expression may contribute to the antitumor properties of

cis-DDP.

6. RNA polymerase I transcription machinery

The human rDNA region that controls rRNA synthesis is contained close to the
sequence upstream of the transcription initiation site (+1) from position -158 to +18 (Learned
et al., 1983). A detailed analysis identifies two distinct elements that control the efficiency of
transcription, a core element from -45 to +18, and an upstream core element (UCE) located
between -156 to -107. The UCE can function in only one orientation and its position relative
to the core element is constrained. These observations suggest a requirement for a specific
spatial arrangement during the promoter interaction (Haltiner et al., 1986).

DNA promoter sequences from different species share little homology (except closely
related species such as rat and mouse). Although rRNA structure is highly conserved among
eukaryotes, their rRNA synthesis is species specific; i.e. the rDNA promoter of one species
cannot direct rRNA synthesis in extracts from another species or in a reconstituted system
using transcription factors from another species (Bell et al., 1990). The transcription is
modulated by the coordinate binding of two factors to the promoter. The upstream binding
factor (hUBF), is involved in initial promoter recognition, whereas the second factor, SL1,
confers species specificity. Paradoxically, UBFs can bind to promoters from different species and produce an identical footprinting pattern on the same promoter, indicating perhaps that UBF is recognizing a common structure (Bell et al., 1990). In support of this notion, evidence suggests that mutants in the promoter region that completely abolish transcription consist of stretches of deletions or clustered mutations, whereas single base pair substitutions only reduce transcription (Haltiner et al., 1986; Sollner-Webb and Tower, 1986).

The species specific transcription factor SL1 cannot bind to DNA alone. Instead, it interacts with promoter-bound UBF extending the footprinting region on the DNA and promotes species specific transcription (Learned et al., 1986; Bell et al., 1989 and 1990). The complete components of the human RNA polymerase I transcription machinery (shown in Figure 1-5) have been cloned and reconstituted in vitro and transcription using these purified proteins has been demonstrated (Jantzen et al., 1990; Comai et al., 1992 and 1994; Zomerdijk et al., 1994; Beckmann et al., 1995). The SL1 complex consists of the TATA binding factor (TBP) as well as three other species specific subunits of 110, 63, and 48 kDa called TBP associated factors for RNA polymerase I (TAF1s) (Comai et al., 1992). The TATA binding factor, originally found in the RNA polymerase II system, binds the TATA box sequences upstream of most genes and stimulates RNA polymerase II transcription. Interestingly, the ribosomal DNA promoter does not contain the TATA box sequence and TBP alone cannot assist rRNA transcription. It was shown that TBP binds exclusively to two different sets of subunits that form SL1 and TFIID (RNA polymerase II transcription
factor) and directs the formation of promoter selective and RNA polymerase selective TBP-
TAF complexes (Comai et al., 1994). During rRNA transcription initiation, both subunits
TAF\textsubscript{110} and TAF\textsubscript{63} contact the promoter, whereas TAF\textsubscript{48} binds TBP rendering it
unavailable for RNA polymerase II transcription. In addition, TAF\textsubscript{48} binds hUBF and may
serve as a species specific promoter selectivity factor (Beckmann et al., 1995).

The domains of hUBF that mediate RNA polymerase I transcription have been
mapped and are shown in Figure 1-6 (Jantzen et al., 1992). hUBF contains an N-terminal
dimerization domain, six tandem HMG box repeats, and a C-terminal acidic domain. A 94
kDa variant of hUBF containing a partial deletion in HMG box2 exists (37 amino acids
deletion toward the N-terminus of amino acid residue 221, see Chan et al., 1991); and in
mouse cells, this species is associated with reduced transcription activity. The dimerization
domain and HMG box1 are required for sequence specific interaction with the promoter,
whereas the rest of the HMG boxes participate in nonspecific DNA binding. Domains for
transactivation, nonspecific DNA binding as well as interaction with SL1 overlap in the
second half of the protein (Jantzen et al., 1992).

7. Regulation of ribosomal RNA synthesis
Of the three RNA polymerase transcription systems that exist in cells, RNA polymerase I directs exclusively the 18S and 28S ribosomal RNA synthesis (the polymerase II controls mRNA synthesis and polymerase III participates in 5S rRNA and tRNA synthesis, respectively). Ribosomal RNA synthesis constitutes 45% of total RNA synthesis, but rRNA makes up over 80% of total existing RNA due to differences in half-life (Marzluff and Huang, 1985).

Eukaryotic 18S and 28S rRNA are transcribed from a single cistron as a 45S precursor. Following methylation and splicing, the mature 18S and 28S rRNA are subsequently incorporated into ribosomes. The rRNA genes are organized as head-to-tail repeats clustered on a number of chromosomes and form densely staining structures in the nucleus called nucleoli, or nucleolar organization regions (NORs). The RNA polymerase I transcribes at ~30 nucleotide/sec and each molecular is spaced at ~ 100 bp intervals on a single rDNA cistron (Long and Dawid, 1980). Multiple copies of rDNA genes are present in all species. For example, 560 copies exist in HeLa cells (Attardi and Amaldi, 1970), and one hundred to several hundred copies per haploid genome in eukaryotic cells are not uncommon. The redundancy of genetic information for rRNA has evolved to satisfy a quantitative requirement. Deletion of over half of the rDNA genes (with wild-type copy number 260 per diploid cell) is characterized by short bristles, slow development, low viability and fecundity in the Drosophila bobbed phenotype, indicating a general defect of the protein-synthesizing machinery. The rRNA levels in the less severe mutants formed by
deletion of less than half of the rRNA gene copies eventually reach a normal level with cellular feedback regulation possibly being involved in dose compensation (Long and Dawid, 1980).

Proliferating cells have high levels of rRNA synthesis to satisfy a high demand for protein synthesis (Sirlin, 1972). Quiescent cells, upon hormonal stimulation, exhibit a massive increase in the rate of rRNA synthesis before an overall acceleration of protein synthesis (Tata, 1968). During serum conversion of mouse 3T6 cells from resting to growing states, mRNA, rRNA and tRNA accumulate long before the beginning of DNA synthesis. Cells that have undergone neoplastic transformation also contain higher levels of RNA polymerase I activity (Chesterton and Humphrey, 1972; Babcock and Rich, 1973; Schwartz et al., 1974). Phorbol ester TPA induces differentiation of human monocyte-like U937 cells and drastically decreases the rate of rRNA synthesis (Cavanaugh et al., 1995). As mentioned in Section 5, the reduced rRNA synthesis may correlate with the binding of the tumor suppressor Rb gene product to the rRNA transcription factor hUBF.

The correlation of altered rRNA synthesis and changes in mammalian UBF appears to occur at two levels, depending on cell line and species. The first is at the level of transcription. In rat muscle cell differentiation induced by phorbol ester TPA, there is a coordinated decrease in the UBF mRNA level and subsequent rRNA synthesis (Larson et al., 1993). In rat neonatal cardiomyocytes, norepinephrine-induced hypertrophy is associated
with increased rRNA and protein synthesis. The drug increases the UBF mRNA levels and protein levels without changing the RNA polymerase I level. In mouse NIH 3T3 fibroblast cells, studies of transcription repression by serum starvation and transcription stimulation following refeeding demonstrate that there is a correlation between the UBF protein levels, mRNA levels and rRNA transcription rates in quiescent and serum stimulated cells. Furthermore, the time course of the changing levels of UBF mRNA during serum stimulation exhibits characteristics similar to those of c-myc and serum responsive factor (SRF), suggesting that UBF is the product of a primary response gene (Glibetic et al., 1995). Sequences upstream of the mouse UBF promoter region (-1183 to -343) contain multiple cAMP response elements and serum responsive elements. In a transfection assay of pUBF-CAT reporter gene expression, this region has been shown to direct high levels of CAT expression upon serum stimulation compared to a pUBF-CAT construct without the additional cAMP responsive upstream sequences (Nishimura et al., 1994).

The second level of mammalian UBF regulation involves post-translational modification. When serum-starved Chinese hamster ovary cells are refed, the increased rRNA synthesis correlates with an enhanced UBF activity by phosphorylation, whereas UBF protein levels do not vary (O'Mahony et al., 1992). In angiotensin II induced hypertrophy of rat vascular smooth muscle cells, rRNA is increased by 5-fold within 6 h of stimulation. Angiotensin II also causes a rapid increase in rat UBF phosphorylation on serine residues, as well as nucleolar localization, but does not change the amount of UBF (Hershey et al., 1995).
Therefore, it is possible that different cell systems utilize multiple, non-exclusive pathways for regulating the activity of UBF, thereby controlling rRNA synthesis in a sophisticated way in response to various external stimuli.

The nucleolus is the interphase nuclear subcompartment where rRNA is synthesized and processed and where ribosomes are packaged (Busch et al., 1972). During mitosis, it disintegrates into distinctive nucleolar organizing regions (NORs). hUBF is tightly associated with the NORs during mitosis even though at this stage, rRNA synthesis is believed to be at a minimum due to the condensed packing of the chromosomes (Chan et al., 1991; Roussel et al., 1993). This effect may be partially due to the extraordinarily tight binding of hUBF with the rDNA promoter. Interestingly, cancer cells generally have abnormal nucleoli, suggesting abnormal rRNA synthesis. In recent years, enlarged or abnormally shaped NORs have become hallmarks of certain cancers in pathological studies (Egan and Crocker, 1991). For example, in neuroblastoma cell lines, studies show the amount of silver-stained interphase NORs are strictly proportional to the proliferative activity of cells (i.e., doubling time) (Trere et al., 1989).

8. Inhibition of rRNA synthesis by cis-DDP

Early research on cis-DDP demonstrated that cis-DDP causes severe inhibition of total RNA synthesis, in addition to inhibition of DNA and protein synthesis (Harder and
Based on the fact that DNA synthesis inhibition was more severe and persistent at certain doses, the authors concluded that inhibition of DNA synthesis triggers cell death. However, later experiments comparing the effect of cis-DDP and trans-DDP at equitoxic doses and equal r_b ratios suggested that quantitative inhibition of DNA synthesis does not correlate with antitumor activity (Salles et al., 1983; Ciccarelli et al., 1985). In the previously mentioned study, Ciccarelli proposed that the differential accumulation of cis-DDP and trans-DDP DNA adducts observed may be due to the preferential repair of the DNA adducts formed by the latter isomer. The observation of differential isomer accumulation is controversial (Roberts and Friedlos, 1987). More recently, Eastman and coworkers report that cells treated with cis-DDP progress through the S phase of the cell cycle and arrest at G_2 for several days whereupon the cells either recover or die (Sorensen, et al., 1988 and 1990; Eastman, 1990). They observed significant inhibition of DNA, total RNA, mRNA and protein synthesis even at very low doses, and the trend of inhibition correlates with the G2 arrest. Therefore, although the cause-effect relationship is not clear among the many cellular changes upon treatment with cis-DDP, rRNA synthesis is indeed inhibited by the drug at various doses. Studies on the renal and neuronal toxicity of cis-DDP indicate that among the early observed morphological changes are nucleolar segregation (Müller et al., 1992) and ribosome dispersion (Jones et al., 1985). Therefore, it is possible that a reduction in rRNA to a significant degree may contribute to growth arrest and eventually leading to cell death.
9. Small molecules as molecular decoys

Molecular mimicry has been exploited in natural biological systems and drug design. Gene expression can be regulated by introducing oligonucleotides such as double-stranded phosphorothioate oligonucleotides, hairpin, and dumbbell DNA sequences that harbor gene regulatory sequences and serve as DNA decoys for RNA II transcription factors. (Bielinska et al., 1990; Chu and Orgel, 1993). In human T cells, overexpression of the transactivation response element (TAR) provides an RNA decoy that sequesters the HIV transactivation protein complex Tat (Sullenger et al., 1990 and 1991).

Recently, it has been shown that DNA modified by the carcinogenic compound benzo[a]pyrene diol epoxide (BPDE) attracts Sp1, a ubiquitous transcription factor involved in transcription of many housekeeping genes (MacLoed et al., 1995). Sp1 binds to specific GC-box sequences with three zinc-finger motifs near the carboxyl terminus. Globally modified BPDE-DNA competes with the GC-box binding of Sp1 as demonstrated by a gel mobility shift assay; furthermore, the footprinting pattern of the Sp1/GC-box complex was abolished. Sp1 has been shown to bend DNA by approximately 60°, whereas electrophoretic studies suggest that BPDE adducts either form a static bend or induce DNA flexibility. It is therefore proposed that Sp1 may recognize the enhanced bend induced by the adduct. The binding constant of the Sp1-BPDE adduct interaction is unknown; the apparent affinity of the complex depends on a high level of DNA-BPDE modification and
the effect on RNA polymerase II transcription has not yet been demonstrated. Therefore, while of interest, the biological relevance of these observations cannot be ascertained immediately. However, this is an interesting example of transcription factor hijacking by another DNA adduct in the general scheme of RNA polymerase II transcription machinery.
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Figure 1-1. Comparison of adduct spectra of cis-DDP and trans-DDP. Note the 1,2-intrastrand crosslinks formed by cis-DDP are absent in the population of adducts formed by trans-DDP.
Comparison of Adduct Spectra of cis-DDP and trans-DDP

**cis-DDP**

**EFFECTIVE**

**trans-DDP**

**INEFFECTIVE**

### Adduct Profiles

**DNA-Protein**
- Yes
- Yes

**DNA-DNA**
- Interstrand: Yes
- Yes

**Intrastrand**
- **GXG**
  - Yes
  - Yes

<table>
<thead>
<tr>
<th>Adduct</th>
<th>Frequency</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GG</strong></td>
<td>65%</td>
<td>No</td>
</tr>
<tr>
<td><strong>AG</strong></td>
<td>25%</td>
<td>No</td>
</tr>
</tbody>
</table>

**Monofunctional**
- Yes
- Yes
Figure 1-2. Summary of the HMG box proteins examined to this date for their abilities to bind DNA modified by *cis*-DDP.
# HMG Proteins Recognize Specifically DNA modified by cis-DDP

<table>
<thead>
<tr>
<th>Full length</th>
<th>MW (kDa)</th>
<th>Species</th>
<th>#HMG boxes</th>
<th>Kd</th>
<th>single adduct DNA</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSSRP 1</td>
<td>81</td>
<td>human</td>
<td>1</td>
<td>n.d.</td>
<td></td>
<td>Unknown, mammalian homologs bind a V(D)J recombination sequence or a collagen promoter sequence. Drosophila homolog binds chorion promoter sequence and single-stranded DNA/RNA</td>
</tr>
<tr>
<td>HMG1</td>
<td>28</td>
<td>rat, calf</td>
<td>2</td>
<td>3.7x10^-7 M</td>
<td>100 bp</td>
<td>Unknown, is a chromosomal protein, may regulate transcription and chromosomal packaging</td>
</tr>
<tr>
<td>HMG2</td>
<td>26.5</td>
<td>calf</td>
<td>2</td>
<td>n.d.</td>
<td></td>
<td>Unknown, chromosomal protein</td>
</tr>
<tr>
<td>lex1</td>
<td>80</td>
<td>yeast</td>
<td>2</td>
<td>2.5x10^-7 M</td>
<td>92 bp</td>
<td>Transcription repressor of cytochrome c oxidase Cox5b</td>
</tr>
<tr>
<td>hUBF</td>
<td>97/94</td>
<td>human</td>
<td>6</td>
<td>6x10^-11 M</td>
<td>100 bp</td>
<td>Ribosomal RNA transcription factor</td>
</tr>
<tr>
<td>mtTFA</td>
<td>24</td>
<td>human</td>
<td>2</td>
<td>~10^-7 M</td>
<td>92 bp</td>
<td>Mitochondrial transcription factor</td>
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<tr>
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<td>23.3</td>
<td>mouse</td>
<td>2</td>
<td>n.d.</td>
<td></td>
<td>Unknown, is testis specific, binds promoter of another testic specific gene, PGK-2</td>
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<td>human</td>
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<td>n.d.</td>
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<td>ARS binding factor, binds chromosomal origin of DNA replication</td>
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## HMG box only

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## Exception

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<td>1</td>
<td>NEGATIVE</td>
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<td>Another transcription repressor of cytochrome c oxidase Cox5b</td>
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* E. Trimmer, personal communication
Figure 1-3. Schematic drawing comparing the sequence specific recognition by HMG box proteins to their cognate DNA regulatory elements and the structure specific recognition to bent DNA. The 1,2-intrastrand d(GpG) and d(ApG) adducts in cis bifunctional coordination by cis-DDP, EN and DACH may form structures that mimic the natural DNA motifs recognized by the HMG box proteins. The binding is favorable perhaps because the strains in the platinum coordination plane may be relieved by an induced-fit following initial binding.
Structure Specific and Sequence Specific Recognition by an HMG Box

Promoter

Sequence Specific

Structure specific

HMG box

Induced fit

Facilitates formation of transcription initiation complex

Relieves strain induced by platinum coordination

cis-DDP Adduct
Figure 1-4. Schematic diagram illustrating the RNA polymerase I transcription initiation complex, adapted from Goodrich and Tjian (1994) *Curr. Opin. Cell Biol.* 6, 403-9. hUBF is involved in the initial recognition of the rDNA promoter whereas SL1 regulates species specificity. SL1 consists of the universal transcription factor, the TATA binding factor (TBP) and another three species specific subunits, the TBP associated factors for polymerase I, TAF148, TAF110, and TAF63.
RNA Polymerase I Transcription
Initiation Complex

RNA Pol I

hUBF, TBP, TAFi110, TAFi48, TAFi63

rRNA Transcription
Figure 1-5. Schematic diagram illustrating the functional domains of hUBF, adapted from Jantzen et al. (1992) Genes & Dev. 6, 1950-1963. HMG box 1 is necessary and sufficient for sequence specific recognition whereas the rest of HMG boxes are involved in weak- and non-specific DNA binding. Regions that are involved in nonspecific DNA binding, transcription activation and interaction with SL1 overlap at the carboxyl-terminus.
Functional Domains of hUBF

Interaction with SL1

Transcription Activation

Dimerization Domain

HMG box1

HMG box2

HMG box3

HMG box4

HMG box5

HMG box6

Acidic Domain

Dimerization

Sequence Recognition

Weak and Non-Specific DNA Binding
CHAPTER 2

Identification of Human Upstream Binding Factor hUBF

as a cis-DDP Damage Recognition Protein
INTRODUCTION

Cisplatin (cis-diamminedichloroplatinum II or cis-DDP) is a widely used anticancer drug that is particularly effective as a cure for testicular tumors (Loehrer and Einhorn, 1984). Cytotoxicity is believed to be mediated by cis-DDP-DNA adducts, which include mainly 1,2-intrastrand d(GpG) (65%) and d(ApG) (25%) crosslinks as well as 1,3-d(GpNpG) intrastrand crosslinks (6%) and interstrand crosslinks (<2%) (Bruhn et al., 1990). The cis-DDP-DNA adducts have been demonstrated in vitro and in vivo to block both DNA and RNA synthesis and to induce apoptosis (Chu, 1994; Eastman et al., 1993). In addition, the major 1,2-intrastrand d(GpG) and d(ApG) adducts are poorly repaired compared with the minor cis-DDP 1,3-d(GpTpG) adduct (Szymkowski et al., 1992; Zamble et al., 1996), suggesting that cisplatin toxicity may be mediated through the persistence of its major adducts. However, the precise mechanism by which cis-DDP exerts its anticancer effect still remains elusive.

Of possible importance to the mechanism of cis-DDP is a family of cis-DDP adduct-binding proteins that contain a DNA binding motif called high mobility group that is homologous to chromosomal protein HMG1 (Bruhn et al., 1992; Pil and Lippard, 1992; Billings et al., 1992). The HMG box is an 80-amino acid region that has conserved basic and aromatic residues. One prominent feature of all the HMG box proteins is their ability to bind to DNA with bent structures, such as four-way junction DNA, without apparent
sequence specificity (Bianchi et al., 1989; Ferrari et al., 1992). Moreover, the proteins themselves are capable of causing a remarkable degree of DNA bending and looping (Lilley, 1992). It was soon apparent that the HMG box proteins in general have distinct cellular functions, such as transcription regulation (Giese et al., 1991; Harley et al., 1992; van de Wetering et al., 1993), DNA packaging (Paull et al., 1993) and recombination (Shirakata et al., 1991). HMG1 binds selectively to 1,2-d(GpG) and 1,2-d(ApG) cisplatin crosslinks but not to 1,3-d(GpNpG) crosslinks nor to trans-DDP-DNA adducts, indicating that HMG1 does not bind to all DNA structures bent by platinum coordinates and that this selective affinity for clinically important DNA adducts may contribute to the anticancer effect of the drug.

A mechanism heretofore not addressed is that cis-DDP-DNA adducts may titrate essential transcription factors away from their natural sites of action. The work described in this chapter provides evidence that human upstream binding factor (hUBF), a nucleolar HMG box protein, is a cis-DDP damage recognition protein. The work of Daniel Treiber in the laboratory demonstrated that hUBF binds with striking affinity (K_{d(app)} ~ 60 pM) to the 1,2-d(GpG) cis-DDP adduct; this affinity differs by only 3-fold from that of hUBF for the ribosomal DNA promoter (K_{d(app)} ~ 18 pM). Furthermore, the hUBF-promoter interaction can be competed away by cis-DDP-DNA adducts. These results, when taken together,
suggested that *cis*-DDP might disrupt rRNA synthesis, which is essential for stimulated cell growth, by a transcription factor hijacking mechanism.
MATERIALS AND METHODS

A. Materials

Restriction endonucleases, DNA ligase, T4 polynucleotide kinase, E. coli Klenow fragment of DNA polymerase I and Hind III DNA molecular weight markers were purchased from New England Biolabs. The synthetic poly(dI-dC)-poly(dI-dC) was obtained from Pharmacia. The transcription-cap m$^7$GpppG for transcription reactions was obtained from Boehringer Manheim. RNase-free DNase was from Worthington Enzymes. Rabbit reticulocyte lysate for translation reactions were purchased from Promega. The platinum compounds EN, DACH, and DIEN were kindly provided by S.J. Lippard’s group. cis-DDP and trans-DDP were originally provided by S.J. Lippard’s group and were later purchased from Sigma. $^{32}$P-labeled nucleotides were purchased from Dupont/NEN. $^{35}$S and $^{14}$C radioactive materials were obtained from Amersham. Plasmid pTβGUBF1 was provided by H.-M. Jantzen of R. Tjian’s laboratory at the University of California, Berkeley. Anti-NOR90, the human antiserum against hUBF from autoimmune patient JO, was kindly provided by E. Chan at Scripps Institute. Goat anti human IgG antibody conjugated to alkaline phosphatase was purchased from BIO-RAD. HeLa S3 cells were obtained from J. Mello at MIT. HeLa extracts prepared from cis-DDP resistant cell lines were from D. Treiber. Extracts prepared from murine L1210 cis-DDP resistant cell lines and human XPG83 lymphocytes were from R. Wood at Imperial Cancer Research, UK.
B. Methods

1. Cellular extracts preparation

HeLa nuclear extracts were prepared following a published procedure (Dignam et al., 1983). Whole cell extracts for the Southwestern analysis were prepared by sonication according to published procedures (Samson et al., 1986). Protein concentrations were determined by the method of Bradford (Bradford, 1976) using reagents from BIO-RAD. Extracts were stored at -80 °C.

2. Preparation of DNA modified by platinum compounds

The DNA probes used in the Southwestern blot experiments were from either a 422 bp Ava I fragment excised from the replicative form of M13mp18 or a 272 bp Clal-Smal fragment from plasmid pStr3. The platination reactions with cis-DDP, trans-DDP, EN, DACH, DIEN and [Pt(NH$_3$)$_2$(N3-cytosine)]$^{2+}$ were carried out as described (Donahue et al., 1988; Toney et al., 1990) with minor modifications. The 422 bp fragment, together with the remaining fragments of the M13mp18 genome included as carrier DNA, were incubated in 1 mM sodium phosphate, 3 mM NaCl (pH 7.4) and the respective platinum complex at an appropriate drug/nucleotide ratio ($r_b$) at 37°C for 16 h. Unreacted platinum
complex was removed by overnight dialysis against TE. The $r_b$ of a platinated DNA was determined by atomic and UV absorption spectroscopy. The probes were labeled by Klenow fragment of *E. coli* DNA polymerase with [$\alpha^{32}\text{P}$]-dCTP, and were isolated on a 5% PAGE gel followed by electroelution and ethanol precipitation.

3. *In vitro* transcription and translation of hUBF

The template plasmid pTβGUBF1 (Jantzen *et al.*, 1990) was linearized after the stop codon by enzymatic digestion with EcoR I and purified by phenol-chloroform extraction followed by ethanol precipitation. *In vitro* transcription of hUBF from the template vector was performed by using a Stratagene T7 RNA transcription kit according to the manufacturer's recommendations with minor modifications. *In vitro* transcription reactions were performed in 75 μl containing an RNase concentration of 0.67 U/μl and an $m^7\text{GpppG}$ to rGTP ratio of 2:1. An Ambion T7 Maxiscript kit was later used according to the manufacturer's recommendations in order to obtain a high yield of wt hUBF transcript. *In vitro* translation reactions contained 1.0-1.5 μg RNA transcript, 4 μl $^{35}\text{S}$-methionine (1200 Ci/mmol, Amersham) and 33 μl wheat germ extract or nuclease-treated rabbit reticulocyte lysate (Promega) in a total volume of 50 μl. For quantification, 2-4 μl of the lysate was resolved on a 5-15% mini-gradient polyacrylamide gel and transferred onto nitrocellulose membrane using a BIO-RAD mini-gel electrophoresis and transfer system. Quantification of hUBF was based on $^{35}\text{S}$-methionine incorporation and measured by
PhosphorImager analysis (Molecular Dynamics) of hUBF bands compared with $^{35}$S-methionine standards spotted on the same membrane.

4. Gradient SDS polyacrylamide gel electrophoresis

The protocol for gradient SDS polyacrylamide gel electrophoresis was based upon a published procedure with minor modifications (Ausubel et al., 1993). The lower separating gel was prepared using a 50 ml gradient maker (Hoefer Scientific). The first gradient solution contained 15% sucrose and 15% or 20% acrylamide (29:1) in 1X Tris-SDS lower separation buffer; the second gradient solution contained 5% acrylamide in the same buffer as above. For a 14x17 cm gel, 50 µl of 10% ammonium persulfate and 4.6 µl TEMED were added into 14 ml of each above solution to catalyze the polymerization. Gravity controlled flow produced a polyacrylamide gradient that could successfully separate the 97/94 kDa proteins of hUBF. Gels were electrophoresed at 10-12 mA for 12 h. For the preparation of mini-gradient gels with the BIO-RAD mini-gel system, 10 µl of 10% ammonium persulfate and 1.5 µl of TEMED were added to 2.1 ml of each solution. The mini-gels were electrophoresed at 200V for 30-40 min. until the bromophenol blue dye reached the bottom of the gel. Protein molecular weights were determined using protein standards (Amersham rainbow markers) that were loaded in parallel with samples during electrophoresis. In experiments where protein samples were $^{35}$S-labeled, $^{14}$C-labeled rainbow markers (Amersham) were used for visualization by PhosphorImager
analysis. Molecular weights of the species were determined from a standard curve derived from the log of molecular weight plotted against the migrating distance.

5. Southwestern analysis

Southwestern analysis was carried out as previously described (Toney et al., 1989) with minor modifications. Proteins were resolved on 5-15% or 5-20% gradient SDS/polyacrylamide gels and transferred to nitrocellulose membranes (Hoefer Scientific) for 2-4 h at 0.5-1 A and air dried. The membranes were then carried through blocking/denaturing/renaturing steps as previously described, except that the TNE50 washes were reduced to 8 min each, the denaturing step to 30 min, and the third renaturing step to 2-4 h. The binding solution contained radiolabeled DNA at 1-5 x 10^4 cpm/ml and nonspecific competitor poly(dI-dC)-poly(dI-dC) at 5 µg/ml. Results were analyzed by PhosphorImager analysis or autoradiography. During autoradiography, a 0.254-mm copper sheet was used to selectively block 35S emissions from the in vitro translated proteins.

6. Western analysis

Western analysis was carried out using a BIO-RAD chemiluminescent immunoassay kit. Protein samples (75-100 µg protein per lane was loaded for a 14x17 cm gel and 10-20 µg per lane for a mini-gel) were resolved using 5-15% gradient SDS/PAGE.
and transferred onto Immun-Light nylon membrane (BIO-RAD). The membrane was carried through blocking/washing steps according to the manufacturer’s recommendations. hUBF was detected with either a human anti-NOR90 serum present at 1:250 dilution, or a rabbit anti-recombinant hUBF serum at present at a 1:100 dilution. Due to the limited availability of anti-NOR90 antiserum, this primary antibody incubation was performed in a total volume of 1.5 ml in a 20 ml glass scintillation vial that was in constant rotation (Labquaker rotator); nylon mesh was used to separate layers of overlapping membrane. Antigen-specific signals were visualized by chemiluminescence following exposure to an X-ray film for 3-5 min when anti-NOR90 was used and 15 min when rabbit anti-recombinant hUBF serum was used.
RESULTS

1. Development of an improved Southwestern procedure and detection of multiple protein species in HeLa extracts that recognize DNA modified by cis-DDP

The modified Southwestern protocol developed in this work employed a gradient SDS gel electrophoresis system that gave greater resolution of HMG box proteins than has been previously obtained. Using molecular weight markers, a standard curve generated from the log of molecular weight vs. migration distance yielded a best fit line with an $r^2 = 0.980-0.999$, compared to $r^2 = 0.866$ obtained with an 8% uniform gel (Figure 2-1). By using this modified SDS/PAGE gradient Southwestern analysis, multiple cellular proteins that bind specifically to DNA modified by cis-DDP were detected both in HeLa nuclear and whole cell extracts (Figure 2-2). Previously, two species of approximately 93 kDa and 30 kDa had been observed to bind cis-DDP modified DNA when an 8% uniform separating gel was employed (Toney et al., 1989). The molecular weight of the cis-DDP damage recognition proteins found by my modified Southwestern procedure were approximately 115, 105, 97/94 and 28 kDa (p115, p105, p97, p94 and p28, respectively). The calculated molecular weight corresponding to the various Southwestern bands would vary by ~5 kDa from one experiment to the next due to measuring error or gel conditions. Some of the bands were inconsistently detected owing to variations in extracts and experimental
conditions. However, p97 was the most easily identified species due to its perfect alignment with the 97 kDa marker. Identical blots probed with control DNA that was either unmodified or modified by trans-DDP revealed weaker signals corresponding to the p115 and p104 kDa species, and no signal was detected for p97/p94 or p28. Furthermore, in a Southwestern experiment that detected the p115, p105 and p97 species by using a radiolabeled 272 bp (r_b=0.031) DNA modified by cis-DDP, p97 was increasingly competed away by 6- and 1200- fold molar excesses of cis-DDP adducts present on unlabeled calf-thymus DNA (r_b=0.085), whereas the p115 and p105 signals were not competed away. These results indicated that the binding by p97 was highly specific (data not shown).

2. hUBF binds specifically to DNA modified by cis-DDP

The identities of the cis-DDP damage recognition proteins revealed by Southwestern analysis were the next focus of research. The 28 kDa species had been identified as the chromosomal nonhistone protein HMG1 (Pil and Lippard, 1992; Billings et al., 1992). Since the HMG box is a unifying feature of many cis-DDP damage recognition proteins, it was reasoned that the 97- and 94- kDa species may possess this DNA-binding domain as well. Moreover, the RNA polymerase I transcription factor hUBF was known to contain at least 4 regions of homology to HMG1 and to exist as both 97- and 94- kDa species due to an alternative splicing event. On account of this similarity, a direct
examination of hUBF for its ability to bind platinated DNA was conducted. pTβGUBF1, a Bluescript based vector (Stratagene) containing the human upstream binding factor (Jantzen et al., 1990), was linearized and run-off transcripts were synthesized using E. coli phage T7 RNA polymerase. Translation of this transcript was performed by using a rabbit reticulocyte lysate (Promega) in the presence of 35S-methionine for identification and quantification purposes. The in vitro translated hUBF was then tested by Southwestern analysis for binding to cis-DDP modified DNA (Figure 2-3). The use of a 0.254-mm copper sheet enabled the 35S signal to be blocked, thereby allowing only 32P to be exposed. Consecutive lanes containing increasing amounts of hUBF exhibited a linear increase in binding to cis-DDP modified probe; DNA modified by trans-DDP or unmodified control exhibited no binding activity. Further, a mock translation containing no RNA transcript did not exhibit any binding activity (data not shown). These observations suggested that hUBF recognizes and binds to cis-DDP modified DNA.

Experiments were next carried out in order to determine whether the p97/p94 platinum binding species observed in HeLa extract was hUBF. A Southwestern experiment was performed in which in vitro synthesized hUBF was run in parallel with HeLa whole cell extracts and the resulting blot probed with cis-DDP modified DNA (Figure 2-4). The binding activity exhibited by hUBF was found to co-migrate closely with the HeLa p97 band (Figure 2-4A). In addition, a corresponding western blot probed
with hUBF antiserum showed a doublet that resembled the bands detected by Southwestern analysis (Figure 2-4A and 2-4D). These results strongly suggested that the p97/p94 cis-DDP adduct binding species observed in HeLa extract was in fact the two isoforms of human upstream binding factor hUBF.

3. Levels of hUBF in mammalian repair deficient cell lines and cis-DDP resistant cell lines.

The titration model predicts that increased hUBF expression would render cells resistant to cis-DDP treatment while a repair blocking model suggests that this would sensitize cells to cis-DDP treatment. Two cis-DDP resistant cell lines, R1 and R3, originally developed from HeLa cells by G. Chu at Stanford University, were examined for the levels of the 97 kDa species in an effort to establish whether hUBF is involved in the observed resistance. Western analysis showed that R1 and R3 exhibited similar level of hUBF expression compared with the HeLa parental cell lines. The levels of mouse UBF in extracts from the mouse cis-DDP resistant cell line L1210/10 and its parental cell line L1210/0 were also examined by using anti-NOR90. Mouse UBF tested positive using this hUBF antisera, but no difference in UBF levels was found in these two cell lines.
Extracts from the human excision repair deficient lymphoblast cell line XPG83 and from normal lymphoblasts were also examined for hUBF expression. XPG83 exhibited a decrease in hUBF level compared to the normal cell line by Western analysis. Southwestern analysis confirmed this 2-3-fold difference in hUBF expression. No strong conclusions, however, can be made from this observation. If a titration model is operative, a decrease in hUBF expression would reduce the growth potential of the cells and render cells more susceptible to DNA damage. If the repair blocking mechanism is operative, a decrease in hUBF expression would make cells more resistant to DNA damage. Future experiments should be directed at regulating the level of hUBF in the same DNA repair deficiency background, and examining how hUBF modulates the LD₅₀ upon cis-DDP treatment.
DISCUSSION

1. Methods of detecting cis-DDP damage recognition

Southwestern analysis, as an alternative to either gel mobility shift or footprinting assays, is a useful tool for the study of protein-DNA interactions. Relative to gel mobility shift assays, Southwestern analysis has the advantage of first separating the proteins by electrophoresis, thereby providing information about the sizes of the proteins. Furthermore, the proteins are transferred onto a solid phase, allowing high local concentration during probing. Interestingly, preliminary gel mobility shift experiments with purified hUBF and a cis-DDP modified DNA probe gave no bandshift signal (Treiber, personal communication). This result is not surprising, given that hUBF also did not shift a DNA probe containing its natural cellular substrate - the ribosomal DNA promoter (Jantzen, personal communication). The primary disadvantage of Southwestern analysis is that protein immobilized on a membrane produces an artificial equilibrium during the probing process that may not correlate with the equilibrium one would observe in real solution; thus the binding constant cannot be calculated. Further quantitative biochemical studies of the interaction between hUBF and a single cis-DDP G^G adduct were carried out by Daniel Treiber by using a footprinting assay.
2. The identification of hUBF as a cis-DDP adduct binding protein

Previous work (Donahue et al., 1989; Bruhn et al., 1991) in identifying cis-DDP damage recognition proteins led to the cloning of an 86 kDa protein, SSRP1, which contains an 80 amino acid DNA binding domain homologous to the human nonhistone chromosomal protein HMG1. Other studies had shown that HMG boxes were able to recognize either specific DNA sequences or a specific DNA structure. Thus it had been hypothesized that the HMG box may be responsible for the recognition of cisplatin adducts. The ubiquitous ribosomal RNA transcription factor hUBF was known to contain at least 4 HMG boxes (Jantzen, 1990) and, interestingly, was known to exit in HeLa cells as a doublet of 97/94 kDa owing to an alternative splicing event. These pieces of information enabled us to associate the p97/p94 species observed in our Southwestern experiments with the human upstream binding factor hUBF.

Increasing amounts of in vitro transcribed and translated hUBF bound in a linear fashion to cis-DDP modified DNA, but showed no binding activity for trans-DDP modified or unmodified DNA, indicating that the interaction was highly specific. Several lines of evidence indicated that the identity of the p97/p94 Southwestern bands was the human upstream binding factor hUBF. The hUBF translate was examined in parallel with
HeLa extracts by both Southwestern and Western blot analysis, and two co-migration patterns emerged. First, in a Southwestern blot probed with a 422 bp DNA modified by \textit{cis}-DDP, the signal from the hUBF translate co-migrated with the 97 kDa band observed in HeLa extracts. Second, using the same HeLa extract, the p97/p94 kDa bands observed by Southwestern analysis co-migrated with an hUBF-specific doublet observed by Western analysis. Moreover, since the amount of hUBF translates could be calculated from the incorporation of $^{35}$S-methionine, the abundance of the p97/p94 kDa species in the HeLa extract could be approximated by comparing that with the intensity of $^{32}$P signal in hUBF translates on the same blot and assuming identical binding strength. The number of combined p97/p94 species was estimated to be between 0.2-1 x 10$^5$ copies per cell; this number correlates well with the estimated 5x10$^4$ copies of hUBF in HeLa cells (Jantzen, personal communication). Finally, the binding profiles of translated hUBF for DNA modified by another series of platinum compounds (discussed in Chapter 3) were identical to that of HeLa whole cell extract (data not shown). This evidence strongly suggests, though it does not conclusively prove, that the p97/p94 kDa species observed in our initial Southwestern blots was actually the two hUBF species. These results guided our research efforts towards directly studying the role of hUBF in mediating the cytotoxicity of \textit{cis}-DDP.
CONCLUSIONS

Using a modified Southwestern blot analysis, HeLa cell extract was shown to contain a doublet of 97 and 94 kDa proteins that selectively binds to DNA modified by cis-DDP. This pattern of two proteins at 97/94 kDa was reminiscent of an HMG-box containing protein, the human upstream binding factor, hUBF, which exists as the two different molecular weight species due to an alternative splicing event. Western blot analysis of HeLa cell extract using antiserum against hUBF derived from autoimmune patients (obtained from Dr. E. Chang at the Scripps Institute) revealed a very similar doublet pattern to that observed by Southwestern analysis. In vitro translated hUBF synthesized from a plasmid containing a full length hUBF cDNA driven by a T7 promoter (obtained from Dr. M. Jantzen of Dr. R. Tijan's laboratory at U.C. Berkeley) was recognized by DNA modified by cis-DDP, but not by DNA modified by trans-DDP or unmodified control DNA. These results directly confirmed that hUBF is a cisplatin DRP. Further work establishing the binding constant for the hUBF-cisplatin adduct interaction was completed by Daniel Treiber (Treiber et al., 1994).
REFERENCES


Figure 2-1. Calibration of a gradient SDS PAGE gel. The migration of the presumed hUBF and HMG1 bands in a Southwestern gel align well with their published molecular weights on the standard curve.
Calibration of the Gradient SDS PAGE Gel

![Graph showing calibration of gradient SDS PAGE gel with the equation $y = -0.1485x + 2.3963$ and $R^2 = 0.9801$. The graph includes data points for Protein Standards and HMGbox proteins.](image-url)
Figure 2-2. A Southwestern blot revealing multiple species that recognize specifically DNA modified by *cis*-DDP in a HeLa extract. These species have molecular weights of approximately 115, 105, 97/94 and 28 kDa. The probes used in this experiment were a 422 bp DNA unmodified (Un-422) or modified by *cis*-DDP at $r_0=0.04$ (*cis*-Pt-422) in separate blots.
Multiple Proteins in HeLa Extract Recognize DNA Modified by cis-DDP
Figure 2-3. Human upstream binding factor hUBF binds specifically to cis-DDP DNA adducts in a Southwestern analysis. The observed binding using a 422 bp DNA modified by cis-DDP ($r_b=0.04$) increased linearly with increased amount of *in vitro* translated hUBF. No binding was observed in identical blots probed either with DNA modified by trans-DDP ($r_b=0.04$) or unmodified 422 bp DNA.
hUBF Binds Specifically to DNA Modified by \textit{cis}-DDP

\begin{tabular}{c|c|c|c|c|c|c}
 & \textit{cis}-Pt-422 & \textit{trans}-Pt-422 & Un-422 \\
\hline
\text{hUBF (ng)} & 5 & 10 & 15 & 5 & 10 & 15 & 5 & 10 & 15 \\
\end{tabular}

97kDa
**Figure 2-4.** The association between hUBF and the 97/94 kDa *cis*-DDP adduct binding species observed in a HeLa extract. *In vitro* synthesized hUBF and HeLa whole cell extracts were examined in parallel with *cis*-DDP modified DNA in a Southwestern analysis (A to C) and with anti-NOR90, a antiserum against hUBF in a Western analysis (D). The binding activity exhibited by *in vitro* translated hUBF co-migrates closely with the HeLa p97 band (Figure 2-4A) both in Southwestern and Western analysis, suggesting strongly that the p97/p94 *cis*-DDP adduct binding species observed in HeLa extract was in fact the two isoforms of human upstream binding factor hUBF.
hUBF Binds Selectively to cis-DDP Modified DNA: Southwestern\textsuperscript{(A,B,C)} and Western Analysis\textsuperscript{(D)}

- **A**: Probe: cis-Pt-422
  - lane 1: WCE, hUBF
  - lane 2: P\textsubscript{105}, P\textsubscript{97}, P\textsubscript{94}, P\textsubscript{28}

- **B**: Un-422
  - lane 1: WCE, hUBF
  - lane 2: P\textsubscript{105}, P\textsubscript{97}, P\textsubscript{94}, P\textsubscript{28}

- **C**: trans-Pt-422
  - lane 1: WCE, hUBF
  - lane 2: P\textsubscript{105}, P\textsubscript{97}, P\textsubscript{94}, P\textsubscript{28}

- **D**: Anti-NOR-90
  - lane 1: WCE, hUBF
  - lane 2: KDa markers: 200, 97, 69, 46, 30
CHAPTER 3

Cisplatin-DNA Adducts Inhibit Ribosomal RNA Synthesis

*in vitro* by Hijacking the Transcription Factor hUBF
INTRODUCTION

The anticancer drug cisplatin (cis-diamminedichloroplatinum(II) or cis-DDP; Fig. 3-1) has shown outstanding success in clinical regimens that produce nearly complete remission of testicular cancer (Loehrer and Einhorn, 1984). cis-DDP also is frequently used in combination with other drugs to treat a variety of other cancers (Blaneke and Johnson, 1995; Reed et al., 1995). The mechanism(s) underlying the efficacy of cis-DDP have been under intensive study since the discovery of its anticancer activity. DNA damage is generally accepted to be the main trigger for cell death in cis-DDP treated cells (Roberts and Thomson, 1979). The major DNA adducts formed by cis-DDP have been characterized as the 1,2-intrastrand d(GpG) (65%), d(ApG) (25%) and 1,3-intrastrand d(GpNpG) (6%) crosslinks (Fichtinger-Schepman et al., 1985). By contrast, the clinically ineffective trans isomer of cis-DDP forms 1,3- and 1,4-intrastrand crosslinks, monofunctional adducts and, at a low frequency, interstrand crosslinks (Bruhn et al., 1990). trans-DDP is unable, however, to form crosslinks at adjacent DNA bases. On that basis it was proposed that the 1,2-intrastrand crosslinks represent the DNA lesions responsible for the antitumor activity of cis-DDP (Pinto and Lippard, 1985). While there is ample evidence that DNA damage is a necessary prelude to cell death, the biological events subsequent to the chemical event of DNA adduct formation remain ill defined. Blockage of DNA replication and RNA transcription, inefficient or abortive damage repair,
and cellular apoptosis have all been shown to occur upon treatment with *cis*-DDP in mammalian cells (Chu, 1994; Eastman *et al.*, 1993; Mello *et al.*, 1996).

Recently a class of cellular proteins that contain a DNA-binding motif homologous to nuclear HMG proteins has been shown to recognize specifically DNA lesions formed by *cis*-DDP (Bruhn *et al.*, 1992; Pil and Lippard, 1992; Brown *et al.*, 1993; Treiber *et al.*, 1994). The consensus HMG box consists of approximately 80 amino acid residues with only 25% average sequence identity. The HMG box proteins can be divided into two subfamilies: One group consists of sequence specific DNA binding proteins with a single HMG box, and the other includes sequence tolerant DNA binding proteins with multiple HMG boxes (Laudet *et al.*, 1993). One characteristic of HMG box proteins shared by both subfamilies is their ability to bind to DNA with bent structures such as four-way junction DNA, without apparent sequence specificity (Bianchi *et al.*, 1989; Ferrari *et al.*, 1992). Moreover, the proteins themselves are capable of causing a remarkable degree of DNA bending and looping (Lilley, 1992). The ability to approach and modulate specific DNA structures and to interact with other nuclear structural or transcriptional components indicates an architectural role for HMG box proteins. They may provide a favorable DNA scaffolding for assembly of nucleoprotein complexes in both higher order DNA packaging as well as in downstream productive biochemical reactions such as RNA transcription and DNA recombination (Paull *et al.*, 1993; Wolffe, 1994).
An excess of 120 HMG box proteins are now known and there are at least 14
different HMG boxes genes in humans (Baxevanis and Landsman, 1995). Among the
known HMG proteins is the ribosomal transcription factor hUBF (Jantzen et al., 1990).
Interestingly, almost all the HMG box proteins investigated so far, including hUBF
(Treiber et al., 1994), recognize DNA modified by cis-DDP, suggesting a structure-specific
interaction similar to that exhibited in the binding of four-way junction DNA. Other cis-
DDP adduct binding HMG box proteins include rat nuclear high mobility group protein
HMG1 (Pil and Lippard, 1992), calf HMG1/2 (Billings et al., 1992), human structure
specific recognition protein SSRP1 (Bruhn et al., 1992), yeast mitochondrial cytochrome c
oxidase transcription repressor Ixr1 (Brown et al., 1993, McA’Nulty et al., 1996), human
mitochondrial transcription factor mtTFA (Chow et al., 1994), mouse testis-specific HMG-
domain protein (tsHMG) (Zamble et al., 1996) and human sex determination factor hSRY
(E. Trimmer, personal communication). Individual HMG domains from HMG1, mouse
SRY and LEF-1 (mouse lymphocyte enhancer-binding factor) have also been examined,
and all have comparable affinities for platinated DNA (Chow et al., 1994). In addition to
the HMG proteins, a second class of DNA binding molecules that recognize cis-DDP
adducts has recently been discovered. Mello et al. (1996) and Duckett et al. (1996)
showed that components of the mismatch DNA repair system exhibit a similar specificity.

Several models have been introduced to describe possible roles of hUBF and other
HMG box proteins in mediating the anticancer activity of cis-DDP (Donahue et al., 1990,
Treiber et al., 1994; Zamble and Lippard, 1995). One hypothesis suggests that HMG box proteins can, by preferentially binding to a cis-DDP damaged site, block access to the adduct by the cellular DNA repair machinery, thereby promoting adduct longevity and sensitizing cells to cis-DDP treatment. Another hypothesis proposes that the transcription factors with HMG boxes could be titrated by cis-DDP adducts away from their original DNA regulatory sites, resulting in disrupted regulation of genes that is critical for cell survival. We have previously shown that the ribosomal RNA transcription factor hUBF binds to DNA containing the intrastrand crosslink, cis-[Pt(NH\textsubscript{3})\textsubscript{2}]\textsuperscript{2+}-d(GpG), the major adduct formed by cis-DDP, with a striking affinity (K\textsubscript{d(app)} \sim 60 \text{ pM}) that is comparable to that measured for its cognate rDNA promoter (K\textsubscript{d(app)} \sim 18 \text{ pM}) (Treiber et al., 1994). Based on these results, we have proposed that transcription factor hijacking could occur upon cis-DDP treatment, resulting in both reduction in ribosomal RNA synthesis and resistance to DNA repair. Interestingly, a similar model has been proposed to describe some of the biological effects of DNA adducts reduced by an electrophilic derivative of benzo[a]pyrene, which hijacks the RNA polymerase II transcription factor Sp1 (MacLoed et al., 1995). Here we present data demonstrating that hUBF recognizes platinum compounds with similar stereochemistry to cis-DDP and that the high affinity of hUBF for cis-DDP adducts is likely the result of additive contribution from multiple HMG boxes participating in the binding. Furthermore, we demonstrate that functional titration of hUBF away from its cellular cognate DNA regulatory element occurs in a reconstituted transcription system.
MATERIALS AND METHODS

1. Preparation of DNA modified by platinum compounds.

   The DNA probe used in the Southwestern blot experiments was a 422 bp Ava I fragment excised from M13mp18 replicative form DNA. The platination reactions with *cis*-DDP, *trans*-DDP, EN, DACH and DIEN (Fig. 3-1) were carried out as described (Toney *et al.*, 1989). Platinum compounds were the generous gift of Professor S.J. Lippard. The drug-to-nucleotide ratio (rb) of the platinated DNA fragment was determined by atomic absorption and UV absorption spectroscopy (Toney *et al.*, 1989). The platinated DNA decoys for evaluating the transcription factor titration hypothesis were made from plasmid pBR322 modified by *cis*-DDP and *trans*-DDP at a wide range of rb following the same reaction procedures.

2. *In vitro* synthesis of hUBF wild type and deletion mutants.

   The N-terminal deletion mutants 84N, 192N, 284N, 491N and internal deletion mutants db1, db2, db3, db4, dbx and two of the C-terminal deletion mutants, 154C and 670C were synthesized from linearized vectors that have been previously described (Jantzen *et al.*, 1992). The wild type hUBF and other C-terminal deletion mutants were synthesized from one vector, pTβGUBF1 (Jantzen *et al.*, 1990), which was linearized with
different restriction enzymes. Specifically, WT, 489C, 408C, 282C, and 204C were synthesized from pTβGUBF1, which was digested with EcoRI, BglII, BglI, StyI and PvulII, respectively. The linearized templates were transcribed in vitro by using a Stratagene T7 RNA transcription kit. For in vitro translation, 1.0-1.5 μg RNA was used in a 50 μl reaction containing 4 μl 35S-methionine (1200 Ci/mmol, Amersham) and 33 μl nuclease treated rabbit reticulocyte lysate (Promega). Following in vitro translation, 2-4 μl of the mixture was separated on a 5-15% mini-gradient SDS polyacrylamide gel and transferred to nitrocellulose membrane (BIO-RAD minigel electrophoresis and transfer system). Quantification of hUBF was based on the incorporation of 35S-methionine into translation products as determined by PhosphorImager analysis (Molecular Dynamics) of hUBF bands compared with 35S-methionine standards spotted on the same membrane.

The number associated with each N-terminal or C-terminal deletion mutant corresponded to the position in the amino acid sequence at which the deletion was made. The internal deletion mutants db1 to db4 correspond to the deletion of HMG boxes 1 through box 4, respectively; dbx represents a deletion of HMG box 5 and 6. The exact internal deletion positions of these five mutants were amino acids 101-181, 205-284, 284-371, 371-491, 492-670 for db1, db2, db3, db4 and dbx, respectively. The boundaries of the HMG boxes in hUBF were defined as reported (Jantzen et al., 1992; Laudet et al., 1993)

3. Southwestern analysis.
Southwestern analysis was carried out as previously described (Toney et al., 1989) with minor modifications. Proteins were resolved on 5-15% or 5-20% gradient SDS/polyacrylamide gels and transferred to nitrocellulose membranes (Hoefer Scientific) for 2-4 h at 0.5-1 A and air dried. The membranes were then carried through blocking/denaturing/renaturing steps as described, except that the TNE50 washes were reduced to 8 min each, the denaturing step to 30 min, and the third renaturing step to 2-4 h. The binding solution contained radiolabeled DNA at 1-5 x 10^4 cpm/ml and nonspecific competitor poly(dI-dC)-poly(dI-dC) at 5 µg/ml. Since the ability of hUBF mutants to adhere to the nitrocellulose membrane throughout the Southwestern procedure differed according to their sizes (data not shown), proportionally adjusted amounts of translation mixtures were loaded such that the molar level of mutants remaining on the membranes at the end of the procedure was similar for each mutant (the molar equivalent of 8 ng of full length hUBF was loaded). In addition, for each experiment, an identical gel served as a control and underwent the same procedure except that the probing solution contained no ^32P-labeled DNA. Computation of binding strength was further corrected by determining the actual molar amount of mutants present on the membrane by ^35S-methionine quantification. During ^32P autoradiography, a 0.254-mm-thick copper sheet was used to block selectively ^35S emissions from in vitro translated proteins.
4. Ribosomal RNA transcription *in vitro* in a reconstituted system and nuclease S1 mapping.

Transcription reactions using recombinant hUBF, partially purified SL1 and highly purified RNA polymerase I as well as the detection of transcripts by nuclease S1 analysis were performed essentially as described (Beckmann *et al.*, 1995) with minor modifications on account of the presence of platinum modified DNA. For the cis-DDP series, 3 ng of pBR322 modified to rb values of 0, 0.0017, 0.0086, 0.019, 0.039 and 0.078 were used in each reaction; for the trans-DDP series, 10 ng of pBR322 with rb values of 0, 0.0018, 0.0090, 0.019, 0.042, and 0.071 were present. *In vitro* transcription reactions contained recombinant hUBF, the linearized transcription vector (100 ng of a 1500 bp plasmid containing one rDNA promoter upstream of a truncated rDNA gene) and pBR322 that was either unmodified or modified by *cis*-DDP or *trans*-DDP. The mixture was incubated for 5 min at 30 °C prior to the addition of SL1, followed by a 20 min incubation on ice. RNA polymerase I and nucleotide triphosphate-mix were subsequently added to allow transcription; the system was incubated for 30 min at 30 °C in a final reaction volume of 25 μl. The probe for S1 analysis was a 60 base ³²P-labeled oligonucleotide complementary to the region between -20 and +40 in the promoter and rDNA gene. After hybridization with synthesized RNA transcripts and digestion with S1 nuclease, the probe was visualized as a 40 base radiolabeled oligonucleotide on a 8% denaturing DNA sequencing gel.
RESULTS

1. hUBF binds selectively to DNA modified by cis-DDP and two other therapeutically active platinum drugs.

In order to explore the specificity of hUBF in binding to platinum adducts of different structures, DNA fragments modified by five different platinum compounds were used to probe identical blots of in vitro translated hUBF. The structures of platinum complexes are presented in Figure 3-1. cis-DDP, EN and DACH are either useful in cancer chemotherapy or show positive preclinical indications whereas trans-DDP and DIEN are therapeutically inactive (Roberts and Thomson, 1979). The 422 bp DNA fragments contained multiple platinum adducts in order to obtain a satisfactory binding signal. No background binding was detected when a blot of the reticulocyte lysate from a sham translation reaction was probed with cis-DDP modified DNA (data not shown). Our Southwestern results revealed that hUBF selectively recognized DNA modified by therapeutically active cis-DDP, EN and DACH, but not unmodified control DNA nor DNA modified by trans-DDP or DIEN, both of which are therapeutically inactive (Roberts and Thomson, 1979).
The DNA adduct spectra formed by platinum compounds in which the chemical reaction leaving groups are in the cis geometry, such as EN and DACH bear close similarity to those determined for cis-DDP, with 90% of adducts as 1,2-intrastrand d(GpG) and d(ApG) crosslinks (Eastman et al., 1986; Jennerwein et al., 1989). Monoclonal antibodies raised against cis-DDP damaged DNA exhibit cross-reactivity toward adducts formed by these molecules, suggesting that these adducts adopt similar stereochemical conformations (Sundquist et al., 1987). The high therapeutic indices of this category of compounds is well documented (Roberts and Thomson, 1979). Our data show that, at similar levels of platination (r_b), hUBF binds DNA modified by EN and DACH with reduced avidity compared to DNA modified by cis-DDP, reflecting less favorable adduct-DNA structures for binding. *trans*-DDP and DIEN generate adduct spectra that notably lack 1,2-intrastrand crosslinks. DIEN can only form monofunctional adducts owing to its single chloride leaving group. Interestingly, DNA modified by *trans*-DDP or DIEN was not recognized by hUBF, confirming the profoundly different structural nature of their adducts. Thus, the efficacy of platinum drugs correlates well with the ability to form a specific DNA-adduct structure or conformation that is recognizable by hUBF.

It should be noted that the 422 bp DNA fragment with r_b~0.038 used in this study contained an average of 32 adducts per DNA fragment (one adduct per 13 bp) and that an HMG box makes contact with at least 14-15 bp around a cis-1,2-intrastrand d(GpG) adduct (Treiber et al., 1994; Locker et al., 1995). Thus, it is highly likely that hUBF would
encounter multiple platinum adducts on a single DNA fragment. We do not know whether multiple HMG boxes within a single hUBF molecule can recognize multiple cis-DDP adducts simultaneously and, moreover, whether the hUBF homodimer or hUBF individual HMG boxes can recognize cis-DDP adducts situated in different DNA fragments at the same time. Future investigations with DNA fragments containing specific single or multiple adducts at known sites would be useful to address these issues. However, given the non-cooperative nature of binding between hUBF and a single platinum adduct (Treiber et al., 1994) and the randomness in distribution of the adducts on our probe, we believe that our observations derived from a globally platinated probe can be qualitatively applied to the situation of single adduct binding.

2. Multiple HMG boxes participate in binding of hUBF.

   The 97 kDa human upstream binding factor, hUBF, is involved in initial promoter recognition during ribosomal RNA transcription (Jantzen et al., 1992). Its 764 amino acid sequence contains an N-terminal dimerization domain, six tandem repeats of HMG boxes for sequence specific and nonspecific DNA binding, and a C-terminal acidic domain for interacting with SL1, a multi-component complex containing the TATA binding protein (TBP) and three other TBP associated factors (TAFs) that are responsible for promoter selectivity (Jantzen et al., 1990; 1992; Laudet et al., 1993). The functional domains for SL1 interaction, transcription activation and nonspecific DNA binding overlap at the C-
terminal half of the protein. The upstream binding factors are unique among HMG box
proteins for their abundance of HMG boxes; there are six HMG boxes in hUBF and other
mammalian homologs, and five in *Xenopus* UBF (xUBF). Due to the characteristically
low sequence identity among HMG boxes, only four boxes were originally recognized in
hUBF and the exact boundaries of each box have not been unequivocally defined. Most of
the HMG box deletions used in this study, however, closely encompassed the areas defined
by the original analysis (Jantzen *et al.*, 1992).

A panel of hUBF deletion mutants, including successive deletions from the N-
terminus, C-terminus as well as internal deletions of individual HMG boxes, were
synthesized by *in vitro* transcription and translation from previously constructed plasmids
containing wild type or various mutant genes (Jantzen *et al.*, 1992). These translation
products were analyzed by Southwestern blot analysis, using a 422 bp DNA probe globally
modified by *cis*-DDP. In duplicate blots, $^{35}$S labeled protein levels and $^{32}$P-labeled *cis-
DDP binding signals are shown in Figure 3-3A and 3.3B, respectively. Approximately
equimolar levels of hUBF mutant proteins were present in these blots. In a separate blot,
the proteins were probed by $^{32}$P-labeled unmodified 422 bp DNA; none of the mutants
exhibited any affinity for the control DNA (data not shown). The binding of each deletion
mutant to platinated DNA was quantified and normalized against that of wild type
(represented as 100% binding) and these results are summarized in Figure 3-4. For
comparison, the abilities of these hUBF mutants to generate a sequence specific footprinting pattern on a rDNA promoter (Jantzen et al., 1992) are listed in parallel.

Several conclusions can be derived from our results. First, the six HMG boxes of hUBF contributed to the binding of cis-DDP modified DNA in an additive way. Successive deletions of HMG boxes from either the N-terminus or C-terminus decreased binding activity, shown in Figure 3-3B, 670C to 491N. Due to the loading limit for the amount of reticulocyte lysate on a PAGE gel, the lower boundary for detection was three HMG boxes. Internal HMG box deletions (db1 to dbx) generally reduced binding, indicating that each HMG box made a contribution to the overall binding. A curious exception among the internal deletion mutants was the HMG box 2 deletion (db2), which exhibited an increased affinity for platinated DNA. However, removal of HMG box 2 from the N-terminus significantly reduced binding (from 84N to 182N) indicating that this HMG box was capable of interacting with platinated DNA. Since this deletion was made inside the N-terminal boundary of the HMG domains, it could therefore be the position of this particular deletion that rendered a slightly favorable binding.

Our deletion study did not locate a dominant HMG box in hUBF that was essential for the structure specific binding of cis-DDP modified DNA. Deletion of any of the individual boxes of hUBF did not prevent the remaining boxes from binding to platinated DNA. This was consistent with the structure specific nature of HMG box-platinum adduct
interactions; each HMG box alone was capable of binding to platinated DNA. By contrast, a study of the sequence specific interaction between hUBF and the rDNA promoter showed that the deletion of HMG box 1, but not other HMG boxes, abolished the footprinting pattern (Fig. 3-4; Jantzen et al., 1992). Our results did not directly suggest, however, that the full length hUBF was without preference for any given HMG box during its binding to a single platinum adduct. It is possible that in deletion mutants the neighboring HMG box is shifted to a similar position relative to the dimerization or acidic regions, for instance, and in so doing may be able to assume a new preferential domain for platinum binding. In such a scenario, the reduced binding in successive deletion mutants may reflect a loss of nonspecific binding that accompanied the loss of an additional HMG box.

Finally, other domains in hUBF modulated binding to cis-DDP modified DNA. Removal of the acidic area increased binding, perhaps by reducing electrostatic repulsion by negatively charged residues that could interfere with binding to DNA. A similar observation in Xenopus demonstrated that deletion of the acidic tail increased the affinity between xUBF and the rDNA enhancer (Hu et al., 1994). Deletion of the N-terminal dimerization domain substantially affected the binding, suggesting that a homodimer form of hUBF was in a more favorable conformation to interact with platinated DNA than a single hUBF molecule (although the proteins were initially separated under denaturing conditions in gel electrophoresis, a renaturing procedure was carried out before probing). In contrast to the cooperative nature of the hUBF ↔ rDNA interaction, however, binding
to a single 1,2-d(GpG) cis-DDP adduct was not cooperative. It is thus possible that
dimerization facilitated binding to cis-DDP adducts by bringing the hUBF monomers in
closer vicinity, thereby generating an even higher local HMG box concentration.

3. cis-DDP modified DNA inhibits ribosomal RNA synthesis in a reconstituted system.

   A previous competition study showed that a single 1,2-d(GpG) cis-DDP adduct
situated in a 100 bp DNA probe can efficiently inhibit the formation of the hUBF/rDNA
promoter complex in a footprinting assay (Treiber et al., 1994). This result suggested that
cis-DDP adduct recognition by hUBF may negatively affect rRNA transcription by
titration of the transcription factor away from its normal site of action in addition to the
“DNA repair blocking” mechanism previously suggested and demonstrated in vitro for
other HMG box proteins (Huang et al., 1994). A demonstration of functional inhibition of
ribosomal RNA synthesis, however, is necessary to establish biological feasibility of the
“titration” mechanism. We therefore investigated the effect of platinated DNA on in vitro
ribosomal RNA transcription. Reconstituted transcription reactions containing
recombinant hUBF, partially purified SL1 and highly purified RNA polymerase were
carried out in the presence of samples of bacterial plasmid pBR322 that were modified by
cis-DDP or trans-DDP to r_b levels ranging from 0.0017- 0.078. Transcripts were detected
by S1 nuclease analysis and results are presented in Figure 3-5.
Under standard conditions, hUBF was able to stimulate transcription of an rDNA minigene by four-fold above background. An increase in the cis-DDP adduct/promoter ratio greatly decreased hUBF-activated transcription in a dose-dependent manner (Fig. 3-5, panel A). The in vitro rRNA transcription returned to basal level when cis-DDP adducts were present at 2.6-5.8 fold above that of rDNA promoters. By contrast, the addition of 3 ng or 10 ng of unmodified pBR322 caused a similar initial decrease in transcription activity (as shown Fig. 3-5, panels A and B, respectively), and the rRNA transcription level remained more or less constant upon subsequent successive increases in the amount of trans-DDP adducts, from a trans-DDP adduct/promoter ratio of 1.8 to as high as 73 (Fig. 3-5, panel C). In addition, 100 ng of unmodified plasmid pBluescript caused no further inhibition in rRNA transcription beyond the initial inhibition observed with 1-10 ng (data not shown). Finally, increasing the level of hUBF in the transcription reaction reversed the inhibitory effect of the cis-DDP adducts and stimulated transcription. These data demonstrated that cis-DDP adducts are highly specific and effective in inhibiting the function of hUBF as a ribosomal RNA transcription activator in a well defined in vitro system. The observation that an average of four cis-DDP adducts were needed to inhibit transcription from one rDNA template correlated very well with our previous data indicating that hUBF binds with one third the avidity to a 1,2-d(GpG) cis-DDP adduct ($K_{d(app)} \sim 60 \text{ pM}$) as compared to an rDNA promoter ($K_{d(app)} \sim 18 \text{ pM}$). It is worthy of note that SL1 was not present in the previous footprinting experiments with hUBF and a 1,2-d(GpG) cis-DDP adduct. Thus, although the interaction between SL1 and hUBF extends
the protected region on the rDNA promoter caused by hUBF alone (Beckmann et al., 1995), our present results suggest that interaction of hUBF with SL1 does not enhance its affinity for the rDNA promoter relative to that for the cis-DDP DNA adducts.
DISCUSSION

We have demonstrated that human upstream binding factor recognizes the DNA adducts formed by a set of therapeutically effective platinum compounds that share stereochemical features and adduct profiles, even though the ligands themselves differ significantly in size and structure. A similar structure specific recognition pattern has previously been demonstrated for two other HMG box proteins, SSRP1 and HMG1 (Toney et al., 1989; Pil, 1993), by using platinum compounds overlapping those used in this study. In a separate experiment, we observed that hUBF did not bind to DNA modified by the platinum compound cis-[Pt(NH₃)₂(N3-cytosine)]⁺, which is therapeutically active but forms only monofunctional adducts (data not shown), a result that is also in agreement with that found for SSRP1. Therefore, the exclusive recognition of DNA modified by platinum compounds that can coordinate cis bifunctional binding to purines is likely to represent the characteristic pattern of binding for all HMG box proteins. Interestingly, HMG1 has recently been shown to bind a cis-DDP interstrand GC(CG) adduct equally as well as an intrastrand 1,2-d(GpG) cis-DDP adduct, while no binding is observed for the interstrand crosslink adduct formed by trans-DDP (Kasparkoa and Brabec, 1995). Thus binding of HMG1 to platinum interstrand adducts is also dictated by the cis/trans configuration with the cis geometry. It is not yet known whether hUBF recognizes the interstrand adduct.
The differential recognition of hUBF and other HMG box proteins to DNA adducts formed by platinum compounds with cis leaving groups is based on the distinct stereochemical structure of these DNA adducts. DNA bending and unwinding caused by cis-DDP have been proposed to be the features important for recognition by HMG box proteins (Rice et al., 1988; Bellon and Lippard, 1990; Bellon et al., 1991; Pil et al., 1992). While EN and DACH 1,2-d(GpG) intrastrand adducts have also been shown to bend DNA (Boudny et al., 1991), monoadduct-forming compounds, DIEN and cis-[Pt(NH$_3$)$_2$(N3-cytosine)]$^+$ do not (Marrot and Leng, 1989; Bellon and Lippard, 1990). The crystal structure of a 1,2-d(GpG) cis-DDP adduct in a dodecamer DNA provides the most informative structural analysis to date (Takahara et al., 1995). In addition to DNA bending and unwinding, the X-ray structure reveals an enlarged minor groove and an abrupt transition from a B-type helix segment to an A-type helix at the lesion region. Moreover, the intrastrand crosslink causes the platinum atom to be displaced from the two guanine planes. Thus it is proposed that the HMG box domain may recognize the A/B hybrid DNA conformation and that the binding of the protein may relieve the strain on platinum coordination (Takahara et al., 1995).

Our data indicated that DNA adducts of EN and DACH interacted with hUBF with less efficiency compared to those of cis-DDP, despite the similar $r_b$ and similar adduct profiles. One possible explanation for this result is that the bulky ligands of these compounds may directly block access of the HMG box to the DNA adduct. However, a
survey of the structural information regarding HMG box/DNA interactions suggests that this may not be the case. Although the structure of a cis-DDP adduct/HMG box complex has not been solved, the molecular basis for sequence specific DNA recognition by the HMG box domains of LEF-1 and SRY has been determined by NMR (Werner et al., 1995; Love et al., 1995). The DNA double helix conformation in both of these complexes resembles closely the structure of DNA containing a cis-DDP 1,2-d(GpG) cis-DDP adduct: the DNA is unwound and bent severely toward a narrowed major groove, and the minor groove is shallow and expanded. The sequence specific interaction between DNA and the HMG box of LEF-1 or SRY occurs exclusively in the minor groove. Evidence in support of upstream binding factor binding to DNA in the minor groove comes from the observations that (1) minor groove specific drugs compete against xUBF for enhancer binding and (2) that major groove modification by methylation does not interfere with binding (Copenhaver et al., 1994). Based on this structural information, the major adducts formed by cis-DDP, EN and DACH, which crosslink adjacent guanines in the major groove, may be situated on the opposite side of helix from the side that is in contact with the HMG box. The similar DNA distortions induced by the cis geometrical constraint that is common in these compounds may therefore serve as a signal for HMG box binding from the minor groove side, and the protein may not make direct contact with the diverse ligands of the platinum compounds in the major groove. Hence, the reduced affinity of hUBF for DNA modified by EN and DACH is unlikely due to obstructed HMG box access by the bulkier ligands. The observed reduction in binding could be due either to the increased
rigidity of the bidentate ligand structures or the bulky ligands in the major groove, which may permit a poor “induced-fit” compared to cis-DDP adducts upon binding by an HMG box.

The results of our hUBF deletion studies may lend insight into the unusually high binding affinity of the hUBF ↔ cis-DDP adduct interaction. DNase I footprinting studies with a single 1,2-d(GpG) cis-DDP adduct have revealed that full length hUBF and Ixrl, as well as two individual HMG boxes of HMG1, give nearly identical cleavage patterns, including a 14-15 bp protected region centered around the adduct and a DNase I sensitive site immediately 5’ to the adduct (Treiber et al., 1994; McA’Nulty et al., 1996; Locker et al., 1995). Thus, the actual contact with a single adduct by the multiple HMG box proteins hUBF and Ixrl seems to be mediated by a single HMG box of the protein. However, the affinity of a single HMG box for a 1,2-d(GpG) cis-DDP adduct (10^{-6}-10^{-7} M) is much lower than that of hUBF (6 \times 10^{-11} M), indicating that other factors must contribute to the interaction (Chow et al., 1994; Treiber et al., 1994). Indeed, our results revealed that multiple HMG boxes in hUBF contribute to the structure specific binding to platinated DNA in an additive way. An observation made in the Xenopus indicates that beyond a minimal requirement of a 60 bp enhancer sequence, the binding affinity of xUBF increases stepwise as the length of the DNA was extended from the enhancer element (Pikaard et al., 1990). This observation suggests an interaction between xUBF and additional DNA fragments, possibly through the additional HMG boxes. Furthermore, UBF has been
shown to bend severely and wrap DNA (Neil et al., 1996). Similarly, a single HMG box induces a sharp bend after binding to a cis-DDP adduct (Chow et al., 1994). These observations, when taken together, imply that the cis-DDP adduct may initially provide a pre-bent structure which, by reducing initial energy barriers, attracts recognition by an HMG box. Following this initial interaction, the hUBF dimer may accumulate extensive nonspecific DNA contacts with the remainder of its HMG boxes (up to 11) and may bend DNA further. In this way, the cumulative specific and nonspecific interactions afforded by the multiple HMG boxes may determine the high affinity interaction of full length hUBF with platinated DNA.

Finally, our data with the reconstituted rRNA transcription system demonstrated that the model for transcription factor titration is viable in vitro. Previous work with other HMG box proteins has focused on a repair-blocking hypothesis. For example, DNA repair in vitro of the cis-DDP 1,2-intrastrand d(GpG) crosslink by the human DNA repair excision nuclease is specifically inhibited by HMG1, mtTF1, and tsHMG (Huang et al., 1994; Zamble et al., 1996). A yeast strain deficient in HMG box protein Ixr1 exhibits a cis-DDP resistant phenotype (Brown et al., 1993) and such desensitization is nearly abolished by additional mutations in several nucleotide excision repair genes (McA’Nulty et al., 1996). Although it is possible that hUBF contributes to cytotoxicity by this same mechanism, our previous biochemical studies suggested that cis-DDP adducts may serve as highly effective decoys for hUBF, thereby disrupting in vitro rRNA transcription. A
critical parameter determining which of the two mechanisms may be operative for a particular HMG box protein may be the relative affinity of the protein for its cognate DNA sequence versus cis-DDP adducts. In the case of hUBF, this difference in affinities is a mere three-fold, rendering hUBF a good candidate for playing a role in the “titration” model (Treiber et al., 1994). In the present study, we found that the ratio of adduct/promoter at which transcription is abolished correlated well with the respective difference in binding constants. This ratio is also consistent with our previous prediction, based on the cooperative nature of hUBF-promoter binding, that a small decrease in the hUBF concentration may give a disproportionately large reduction in promoter occupancy, thereby disrupting rRNA transcription. To our knowledge this is the first evidence for a transcription factor hijacking model by a DNA damaging agent.

Seemingly in conflict with our present findings, yeast studies show that treatment with cis-DDP does not alleviate Ixr1-related transcription repression in the Cox5b gene present in genomic DNA nor in a reporter gene controlled by the Cox5b promoter (McA’Nulty and Lippard, 1996). One possible explanation for this observation is that the modest affinity of Ixr1 for cis-DDP adducts (K_d ~ 2.5x10^{-7} M) is insufficient to compete with the strong sequence-specific interaction between this transcription factor and its DNA regulatory element. The affinity of Ixr1 for its cognate DNA regulatory element has not been determined. It is known, however, that the HMG box domain from LEF-1 binds a T-cell antigen receptor (TCR) α enhancer motif with an affinity (K_d ~ 10^{-9} M), which is two
orders of magnitude higher than was determined for a single cis-DDP 1,2-d(GpG) adduct (K_d ~ 10^{-7} M) (Giese et al., 1991; Chow et al., 1994). Another SRY-like HMG box protein, Sox-4, binds this same DNA element with even higher affinity (K_d ~ 3x10^{-11} M) (van de Wetering et al., 1993). Thus, the striking disparity in binding strengths of many HMG box proteins for their DNA regulatory elements and cis-DDP 1,2-d(GpG) adducts predicts that the transcription factor-regulatory element complexes would unlikely be disrupted by cis-DDP adducts in these specific cases.

Enhanced ribosomal RNA synthesis is an important requirement for cell proliferation and tumor growth (Tata, 1968; Chesterton and Humphrey, 1972; Babcock and Rich, 1973; Schwartz et al., 1974). A rough quantitative comparison of the major components involved in the transcription factor hijacking model suggests that the level of rRNA synthesis could be inhibited significantly by cis-DDP adducts. For example, the number of hUBF molecules present in HeLa cells is approximately 50,000 (H.-M. Jantzen, personal communication), a number that is similar to the 10,000-100,000 adducts per cell found in cancer patients during treatment with cis-DDP (Reed et al., 1993). Considering that ~560 copies of rDNA upstream promoters are present in HeLa cells (Attardi and Amaldi, 1970), the ratio of platinum adducts/promoter present in cells is at least an order of magnitude higher than the level at which complete transcriptional inhibition is achieved in vitro. Moreover, the fact that the presence of other transcription components, such as SL1, did not inhibit the hijacking of hUBF by cis-DDP adducts in vitro further supports the
possibility that the titration model may be operative in vivo. The two models put forth to describe how the interaction between HMG box proteins and cis-DDP adducts contributes to cis-DDP toxicity, the titration and DNA repair shielding models, are not mutually exclusive. However, an operative titration mechanism is probably limited to a subset of multiple-HMG box transcription factors that possess an affinity for cis-DDP adducts that is comparable to that for the cognate promoter.
REFERENCES


Figure 3-1. Platinum compounds. Antitumor compounds cis-DDP, [Pt(en)Cl₂], 
[Pt(dach)Cl₂] and inactive compounds trans-DDP and [Pt(dien)Cl]⁺.
cis-DDP

$\text{[Pt(en)Cl$_2$]}$

(EN)

$\text{[Pt(dach)Cl$_2$]}$

(DACH)

trans-DDP

$\text{[Pt(dien)Cl]}^+$

(DIEN)
**Figure 3-2.** hUBF selectively binds to DNA modified by *cis*-DDP and other antitumor platinum compounds that can form 1,2-intrastrand crosslinks. Parallel blots of *in vitro* translated hUBF (40 ng) were probed in a Southwestern analysis with a $^{32}$P-labeled 422 bp Ava I fragment from M13mp18 (Donahue et al., 1990) that was unmodified or modified by different platinum compounds. The drug-to-nucleotide ratios ($r_b$) for modification by *cis*-DDP, *trans*-DDP, EN, DACH and DIEN were 0, 0.041, 0.039, 0.041, 0.034 and 0.039, respectively. hUBF recognized DNA lesions formed by therapeutically effective platinum compounds that can coordinate cis bifunctional binding to adjacent d(GpG) and d(ApG) sites.
hUBF Binds Clinically Effective Platinum Compounds That Can Form Bifunctional \textit{cis} Adducts on DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>rb</th>
<th>cis-DDP</th>
<th>EN</th>
<th>DACH</th>
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<tr>
<td></td>
<td></td>
<td>0.041</td>
<td>0.041</td>
<td>0.034</td>
</tr>
<tr>
<td>97 kDa—</td>
<td>•</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Unmodified</td>
<td></td>
<td>0.039</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>97 kDa—</td>
<td></td>
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<td></td>
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</tbody>
</table>
Figure 3-3. Multiple HMG boxes participate in binding with hUBF. *In vitro* translated hUBF mutants were analyzed by Southwestern blots in order to assess the contribution of various domains of hUBF in the interaction with a 422 bp platinated DNA ($r_5^*\sim0.038$). HMG boxes 5 and 6 were tested as a combined region. A.) A control membrane that was not probed with $^{32}$P-labeled probe demonstrated that approximately equal molar levels of $^{35}$S-labeled hUBF deletion mutants were present on the blots. B.) The $^{32}$P-labeled *cis*-DDP modified probe bound hUBF mutants with different affinities. The data indicated that multiple HMG boxes contributed to the binding additively.
Multiple HMG Boxes Participate in Binding with hUBF

A.

B.
Figure 3-4. Summary of the Southwestern analysis of binding to *cis*-DDP modified DNA by hUBF deletion mutants. The $^{32}$P signal was quantified and compared to that of wild type (normalized as 100% binding). The numbers represented the average of three individual experiments. For comparison, we also show in the table the capacity of these mutants to generate a footprinting pattern on the rDNA promoter (Jantzen *et al.*, 1992).
### Comparison of hUBF Binding to cis-DDP Adducts and to the rRNA Promoter Constructs

<table>
<thead>
<tr>
<th>Constructs*</th>
<th>UBF Footprinting</th>
<th>cis-DDP Binding**</th>
</tr>
</thead>
<tbody>
<tr>
<td>dimerization domain</td>
<td>HMG box1 box2 box3 box4 box5 box6 acidic tail</td>
<td>++ + + 100</td>
</tr>
<tr>
<td>WT</td>
<td>670C</td>
<td>++ + + 174</td>
</tr>
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<td>670C</td>
<td>489C</td>
<td>++ + + 18</td>
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<tr>
<td>489C</td>
<td>408C</td>
<td>++ + + 7</td>
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<tr>
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</tr>
<tr>
<td>db4</td>
<td>dbx</td>
<td>++ + --</td>
</tr>
</tbody>
</table>


**Binding normalized to the extent of interaction of wild type hUBF to platinated DNA (100).
Figure 3-5. *cis*-DDP DNA inhibits ribosomal RNA synthesis in an *in vitro* reconstituted system. Purified recombinant hUBF was incubated with the template rDNA gene in the presence of pBR322 that was modified by *cis*-DDP (A), or *trans*-DDP (B) prior to the addition of both SL1 and RNA polymerase I in order to initialize *in vitro* ribosomal RNA transcription. The resulting transcripts were detected by S1 analysis. Constant amounts of pBR322 with different r_b levels were used in the reactions. DNA modified by *cis*-DDP inhibited *in vitro* rRNA synthesis in a dose-dependent manner. By contrast, a high level of *trans*-DDP modified DNA and unmodified pBR322 had no significant effect on transcription. The transcription inhibition can be relieved by increasing the level of hUBF (C).
cis-DDP-DNA Adducts Inhibit rRNA Synthesis *in vitro*

| Ratio:          | Pt adducts | 0|6822 | 0.5|22 | 22 | 82 | 24 | 0|6922 | 12 | 9 | 9 | 3 | 13 | 5.8| 5.8| 5.8 |
|-----------------|------------|---|-----|----|----|----|----|----|---|----|----|----|----|----|----|----|----|
|                 | hUBF       | - | +   | +  | +  | +  | +  | +  | + | +  | +  | +  | +  | +  | +  | +  | +  |
|                 | cis-DDP    |   |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                 | trans-DDP  |   |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                 | cis-DDP    |   |     |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

* cis-DDP-DNA Adducts Inhibit rRNA Synthesis *in vitro*
Figure 3-6. Transcription factor hijacking: schematic diagram illustrating possible mechanisms for the involvement of hUBF in cis-DDP toxicity. The comparable avidity with which hUBF binds the rDNA promoter and a cis-DDP adduct renders hUBF a unique candidate for playing roles both in titrating rRNA synthesis and blocking DNA repair.
Inhibition of rRNA Synthesis *in vitro*

<table>
<thead>
<tr>
<th>Copies/Cell</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>hUBF</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td>rDNA Promoter</td>
<td>400</td>
</tr>
<tr>
<td>cis-DDP Adducts</td>
<td>$10^4 - 10^5$</td>
</tr>
</tbody>
</table>
CHAPTER 4

Investigation of the Possibility that cis-DDP Adducts Titrates hUBF away from the rDNA Promoter in vivo
INTRODUCTION

It is well documented that proliferating cells require high levels of rRNA synthesis (Sirlin, 1970). Quiescent cells, upon hormonal stimulation, exhibit a massive increase in the rate of rRNA synthesis followed by an overall acceleration of protein synthesis (Tata, 1968). Tumor cells also exhibit increased levels of rRNA synthesis (Babcock and Rich, 1973; Chesterton et al., 1972; Schwartz et al., 1974). Given the importance of hUBF in rRNA synthesis, and its tight binding to platinum adducts, we proposed a transcription factor hijacking mechanism to explain the role of hUBF in mediating the cytotoxicity of cis-DDP (Treiber et al., 1994).

One premise of the hijacking model is that hUBF would be titrated away from rDNA promoters. Subsequent reduction of ribosomal RNA synthesis would then lead to inhibition of cell growth. Ribosomal RNA synthesis is responsible for 45% of total cellular RNA transcription while the stationary rRNA content is 80% (Marzluff and Huang, 1985). Early research on cis-DDP did report a severe inhibition of total RNA synthesis in addition to inhibition of DNA and protein synthesis (Harder and Rosenberg, 1970; Howle and Gale, 1970). Recently, Eastman and coworkers discovered that cells treated with cis-DDP progress through the S phase of the cell cycle and arrest at G2 for several days, whereupon the cells either recovered or died. They proposed that apoptosis (programmed cell death) may be the mechanism mediating the antitumor activity of the drug (Sorensen, et al., 1988; 1990;
They observed a significant decrease in DNA, total RNA, mRNA and protein synthesis even at very low doses of cis-DDP, which correlated with G2 arrest. Therefore, although the causal relationship is not clear among the many cellular changes upon treatment with cis-DDP, rRNA synthesis inhibition is indeed one of the phenomena observed, but it is impossible to tell at this point whether cis-DDP selectively inhibits this event.

The nucleolus is the interphase nuclear subcompartment where rRNA is synthesized and processed and where ribosomes are packaged (Busch et al., 1972). During mitosis, it disintegrates into distinctive nucleolar organizer regions (NORs). hUBF has been reported to be tightly associated with the NORs during mitosis, even though at this stage rRNA synthesis is believed to be at a minimum due to the condensed packing of the chromosomes (Chan et al., 1991; Roussel et al., 1993). This effect may be partially attributable to hUBF's extraordinarily tight binding to the rDNA promoter. The nucleolus reforms at the early G1 period as a result of cumulative rRNA synthesis, which continues throughout the cycle and drops at the end of G2. The rate of rRNA synthesis in eukaryotes is directly influenced by the rate of protein synthesis, i.e., the number of functional polysomes. New ribosomes have to be made as the rate of protein synthesis increases. Therefore, rRNA regulation appears to be at the level of transcription and not by the activation of a large pool of pre-rRNA species (Sollner-Webb and Tower, 1986).
Studies on the renal and neural toxicity of cis-DDP show that among the early morphological changes are nucleolar segregation (Müller et al., 1992) and ribosome dispersion (Jones et al., 1985). A reduction of rRNA to a significant degree may trigger growth arrest, eventually leading to cell death. Interestingly, cancer cells generally have abnormal nucleoli. Indeed, enlarged or abnormally shaped NOR have become hallmarks of certain cancers (Egan and Crocker, 1991). In neuroblastoma cell lines, for example, studies show the amount of silver-stained interphase NORs are directly proportional to the proliferative activity of cells (i.e., doubling time) (Trere et al., 1989).

Multiple copies of rDNA genes are present in all species, ~100 to several hundred copies per haploid genome in eukaryotic cells (for example, 560 copies in HeLa cells). The redundancy of genetic information for rRNA has evolved to satisfy a quantitative requirement. Deletion of over half of the rDNA genes (wild type copy number 260 per diploid cell) is characterized by short bristles, slow development, low viability, and fecundity in *Drosophila* bobbed phenotype, indicating a general defect of protein-synthesizing machinery. The rRNA levels in the less severe type mutants (deletion of less than half of the rRNA gene copies) eventually reach normal level and cellular feedback regulation may be involved in dose compensation (Long and Dawid, 1980). These observations suggest that severe inhibition of rRNA synthesis may be necessary to diminish cell viability. On the other hand, it is known that ribosomal RNA synthesis occurs in a simultaneous and coordinated manner, with each cistron loaded with up to 100 molecules of polymerase (Sirlin, 1970).
high hUBF local concentration around the enhancer/promoter region is probably necessary to ensure continuous loading.

hUBF is present in HeLa cells to a level of approximately 50,000 molecules per cell (H.-M. Jantzen, personal communication), which is comparable to the 10,000-100,000 Pt adducts per cell found in cancer patients during treatment with cis-DDP (Reed et al., 1993). In a proof of concept experiment described in the previous chapter, it was demonstrated that in vitro ribosomal RNA synthesis was completely abolished at a platinum/promoter ratio of approximately 4:1 and this ratio had to be at least an order of magnitude higher to abolish transcription in a mammalian cell. Consistent with our proposal for an in vivo role, the presence of other transcription components did not inhibit hijacking. Thus, the presence of a large number of cis-DDP adducts in the nucleus may be able to divert a sufficient fraction of hUBF from nucleolar rDNA promoters to inhibit ribosomal RNA synthesis.

The following experiments are mainly aimed at studying the titration model in vivo, i.e., the effect of cis-DDP adducts on rRNA synthesis via diverting hUBF. Such knowledge could serve both to provide an understanding of the efficacy of cis-DDP as well as suggest new strategies for drug design. The results of preliminary data are presented and the implications discussed.
MATERIALS AND METHODS

A. Materials

1. Enzymes and chemicals

   Restriction endonucleases, DNA ligase, T4 polynucleotide kinase, *E. coli* DNA
   Klenow fragment of polymerase I and I Hind III DNA molecular weight markers were
   purchased from New England Biolabs. The synthetic poly (dI-dC) Poly (dI-dC) was from
   Pharmacia. Calf intestinal alkaline phosphatase (CIP) and m\(^7\)GpppG were obtained from
   Boehringer Manheim. RNase-free DNase was from Worthington Enzymes.
   Lipofectamine, G418, 100 bp ladder DNA markers, and cell culture media were from
   GIBCO/BRL. EN, DACH, and DIEN were kindly provided by Professor S.J. Lippard’s
   group. *cis*-DDP and *trans*-DDP were originally from Lippard’s group and were later
   purchased from Sigma. DAPI was purchased from Sigma. The vinol/antifading agent for
   mounting slides in the immunoflorescent labeling experiment was a gift from Scott Lowe
   at MIT. \(^{32}\)P and \(^{3}\)H radioactive nucleotides and nucleosides were purchased from
   Dupont/NEN. \(^{35}\)S and \(^{14}\)C radioactive materials were from Amersham.

2. Cell lines
HeLa S3 cells were obtained from J. Mellow and AD293 cells from M. Juedes at MIT. Both cell lines were originally from ATCC. Bacterial strain MC1061/P3 was from D. Moshinsky at MIT.

3. Plasmids

pTβGUBF1 and linearized vectors used for in vitro transcription and translation were provided by H.-M. Jantzen of R. Tjian’s laboratory at the University of California, Berkeley. pFLAGUBF for hUBF cloning was obtained from H. Beckmann of U.C. Berkeley. pHENA was a gift of B. McStay at University of Dundee, Scotland. pcDNA1Neo1, pSV2neo and pCMV-βgal, pStr3 and pGEM-3Zf(-) were from D. Treiber, J. Zhuang, J. Mello, E. Trimmer, and K. Yarema at MIT, respectively. pHrl0SK and pHFBa-1 were generous gifts from E. Maden from University of Liverpool and L. Kedes from University of Southern California, respectively.

4. Proteins and Antisera

Anti-NOR90, the human antiserum against hUBF from autoimmune patient JO was kindly provided by E. Chan at the Scripps Institute. Rabbit antisera raised against recombinant hUBF was from H. Beckmann. Fluorescence-conjugated immunoprotein
against human IgG was from Pierce. Goat anti human IgG antibody conjugated to alkaline phosphatase was from BIO-RAD.

B. Methods

1. Cell culture

HeLa S3 cells, when used for preparation of cell extract and intact nuclei (in a run-off assay) were grown in suspension in S-MEM with 5% fetal calf serum (FCS) maintaining a density between 0.5 and 5x10^5 cells/ml. When used for DNA transformation and cis-DDP treatment, the cells were grown attached to polystyrene tissue culture plates in D-MEM with 10% FCS. AD293 cells are grown attached in D-MEM with 10% FCS. All cultures contained 100 U/ml penicillin, 100 μg/ml streptomycin and were supplemented with 2 mM L-glutamine.

2. Cellular extracts preparation

HeLa nuclear extracts were prepared following a published procedure (Dignam et al., 1983). Whole cell extracts for the Southwestern analysis were prepared by sonication (Samson et al., 1986). For Western analysis of hUBF overexpression clones and assay for β-galactosidase activity, a freeze-thaw method was employed (Ausubel et al., 1993). Less
than 10^6 cells were trypsinized, washed with cold phosphate buffered saline and 
resuspended in 100 μl 0.25 M Tris-HCl buffer, pH 7.8) in a microcentrifuge tube. The 
cells were quickly frozen in dry ice and subsequently thawed at room temperature. After 
repeating the procedure twice, the thawed mixture was centrifuged at 4 °C at 14,000 x g for 
5 min and the supernatant was collected. Protein concentrations were determined by 
Bradford assay (Bradford, 1976) by using reagents from BIO-RAD. Extracts were stored 
at -80 °C.

3. Oligonucleotide preparation

Oligonucleotides were synthesized on an Applied Biosystems 381-A DNA 
synthesizer. They were purified by denaturing (7 M urea) polyacrylamide gel 
electrophoresis. Appropriated bands were recovered after identification by UV shadowing, 
by either a crush-and-soak procedure (Sambrook et al., 1989) or electroelution, followed 
by extensive washing and concentration using Centricon 3 or 10 microconcentrators 
(Amicon).

4. Preparation of DNA modified by platinum compounds

The DNA probes used in the Southwestern blot experiments are from either a 422 
bp Ava I fragment excised from the replicative form of M13mp18 or a 272 bp Clal-Smal
fragment from plasmid pStr3. The platination reactions with *cis*-DDP, *trans*-DDP, EN, DACH, DIEN and \([\text{Pt(NH}_3\text{)}_2(\text{N}_3\text{-cytosine})]^{2+}\) were carried out as described (Donahue *et al.*, 1988; Toney *et al.*, 1990) with minor modifications. The 422 bp fragment, along with the rest of the M13mp18 genome as carrier DNA, was incubated with 1 mM sodium phosphate/3 mM NaCl (pH 7.4) and the respective platinum complex at an appropriate drug/nucleotide ratio \((r_b)\) at 37 °C for 16 h. Unreacted platinum complex was removed by overnight dialysis against TE. The \(r_b\) of a platinated DNA was determined by atomic and UV absorption spectroscopy. The probes were labeled by Klenow fragment of *E. coli* DNA polymerase with \([\alpha^{32}\text{P}]\)-dCTP and isolated on a 5% PAGE gel followed by electroelution and ethanol precipitation. The Pt-DNA decoys for examining the transcription factor hijacking hypothesis were made from plasmid pBR322 modified by *cis*-DDP and *trans*-DDP to a wide range of \(r_b\) values following the same reaction procedure.

5. *In vitro* transcription and translation of hUBF and various mutants

*In vitro* transcription of wild type (wt) hUBF and deletion mutants from linearized template vectors was performed using a Stratagene T7 RNA transcription kit with minor modifications. Transcription was performed in 75 μl, at an RNase concentration of 0.67 U/μl and an m\(^7\)GpppG to rGTP ratio (2:1). An Ambion T7 Maxiscript kit was later used to provide a high yield of wt hUBF transcript. In this case, transcription was carried out in 20
µl reaction using an m\(^7\)GpppG:rGTP molar ratio of 1:1. The N-terminal deletion mutants (84N, 192N, 284N, 491N), internal deletion mutants (db1, db2, db3, db4 and dbx) and two of the C-terminal deletion mutants (154C and 670C), were synthesized from linearized vectors directly from H.-M. Jantzen (Jantzen et al. 1992). The wild type hUBF and other C-terminal deletion mutants are synthesized from pTβGUBF1 (Jantzen et al., 1990) that was linearized at various positions by different restriction enzymes. In particular, wt, 489C, 408C, 282C and 204C were digested with EcoRI, BglII, BglI, StyI, PvuII and purified by phenol-chloroform extraction and ethanol precipitation.

For in vitro translation, 1.0-1.5 µg RNA transcripts were used in 50 µl reaction with 4 µl \(^{35}\)S-methionine (1200 Ci/mmol, Amersham) and 33 µl nuclease treated rabbit reticulocyte lysate (Promega). For quantification, 2-4 µl of the lysate was resolved on a 5-15% mini-gradient polyacrylamide gel and transferred onto a nitrocellulose membrane using a BIO-RAD mini-gel electrophoresis and transfer system. Quantification was based on the incorporation of \(^{35}\)S-methionine as detected by PhosphorImager analysis (Molecular Dynamics) of hUBF bands against \(^{35}\)S-methionine standards (Amersham) spotted on the same membrane.

6. Gradient SDS polyacrylamide gel electrophoresis
The protocol was modified based upon a published procedure (Ausubel et al., 1993). The lower separating gel was prepared using a 50 ml gradient maker (Hoefer Scientific). The high gradient solution contained 15% sucrose and 15% or 20% acrylamide (29:1) in 1xTris SDS separation buffer (0.375 M Tris-Cl, pH 8.8, 0.1% SDS) whereas the low gradient solution contained the 5% acrylamide in the same buffer as above. For a 14x17 cm gel, 50 µl of 10% ammonium persulfate and 4.6 µl TEMED were added into 14 ml of each above solution to catalyze the polymerization. Gravity controlled flow gave a gradient that could separate the 97/94 kDa proteins. Gels were electrophoresed at 10-12 mA for 12 h. For preparing a mini-gradient gel with a BIO-RAD mini-gel system, 10 µl of 10% ammonium persulfate and 1.5 µl of TEMED were added to 2.1 ml of each solutions. The mini-gels were electrophoresed at 200V for 30-40 min until the bromophenol blue dye reached the bottom of the gel.

The protein molecular weight was determined using protein standards (Amersham rainbow markers) that were loaded in parallel with samples during electrophoresis. In experiments with 35S-labeled protein samples, 14C-labeled rainbow markers were used for PhosphorImager analysis. Molecular weights of the species were determined from a standard curve in which the log of molecular weight was plotted against the migrating distance.

7. Southwestern blot
The Southwestern experiment was carried out as previously described (Toney et al., 1989) with minor modifications. Proteins were resolved on 5-15% or 5-20% gradient SDS/polyacrylamide gels and transferred to nitrocellulose membranes (Hoefer Scientific) for 2-4 h at 0.5-1 A and air dried. The membranes were then carried through blocking/denaturing/renaturing steps as described in the protocol, except that the TNE50 washes were reduced to 8 min each, the denaturing step to 30 min, and the third renaturing step to 2-4 h. The binding solution contained radiolabeled DNA at $1-5 \times 10^4$ cpm/ml and nonspecific competitor poly(dI-dC)-poly(dI-dC) at 5 μg/ml. Results were analyzed by PhosphorImager system or autoradiography. During autoradiography, a 0.254-mm-thick copper sheet was used to selectively block $^{35}$S emissions from the in vitro translated proteins.

During the experiments with the hUBF deletion mutants, it was noted that the ability of hUBF mutants to adhere to the nitrocellulose membrane throughout the Southwestern procedure differed according to their size. A proportionally adjusted amount of translated material was therefore loaded such that the molar level of mutants remaining on the membranes at the end of the procedure was similar (molar equivalent of approximately 8 ng, or 0.01 pmol of full length hUBF was loaded). In addition, for each experiment, an identical gel served as a control and underwent the same procedure except that the probing solution contained no $^{32}$P-labeled DNA. Computation of binding strength
was further corrected by determining the actual molar amount of mutants on the membrane by \(^{35}\)S-methionine quantification.

8. Western blot analysis

The experiments were carried out using a BIO-RAD chemiluminescent immunoassay kit. Protein samples (75-100 \(\mu\)g per lane for a 14x17 cm gel and 10-20 \(\mu\)g for a mini-gel) were resolved using 5-15% gradient SDS/PAGE and transferred onto a Immun-Light blotting membrane (BIO-RAD). The membrane was carried through blocking/washing steps according to manufacturer’s manual. hUBF was detected with either a human anti-NOR90 serum at 1:250 dilution, or a rabbit anti-recombinant hUBF at 1:100 dilution. Due to the limited availability of anti-NOR90 antiserum, the primary antibody incubation was performed with 1.5 ml antibody buffer in a 20 ml glass scintillation vial in constant rotation (Labquaker rotator) and nylon mesh was used to separate layers of overlapping membrane rolled inside the vial. The chemiluminescence developed membrane was exposed to an X-ray film for 3-5 min when anti-NOR90 was used and 15 min when rabbit serum against recombinant UBF was used.

9. *In vitro* reconstituted ribosomal transcription system and S1 mapping
Transcription reactions containing recombinant hUBF, partially purified SL1 and highly purified RNA polymerase I and the detection of transcripts by S1 analysis were performed essentially as described in published procedures (Beckmann et al., 1995) with minor modifications with on account of the presence of platinum modified DNA. For the cis-DDP series, 3 ng of pBR322 modified to an $r_b$ of 0, 0.0017, 0.0086, 0.019, 0.039 or 0.078 were used in each reaction; for the trans-DDP series, 10 ng of pBR322 with an $r_b$ of 0, 0.0018, 0.0090, 0.019, 0.042, or 0.071 were present. The linearized transcription vector contains one rDNA promoter upstream of a truncated rDNA gene. Recombinant hUBF, the promoter DNA (100 ng) and pBR322 modified by either cis-DDP, trans-DDP or control unmodified pBR322 were incubated for 5 min at 30 °C prior to the addition of SL1 and a 20 min incubation on ice. RNA polymerase I and NTPs- were subsequently added to allow transcription for 30 min at 30 °C in a final reaction volume of 25 μl. The probe for S1 analysis was a 60 base $^{32}$P-labeled oligonucleotide complementary to the region between -20 and +40 in the promoter and rDNA gene. After hybridization with synthesized RNA transcript and S1 nuclease digestion, the probe was visualized as a 40 base $^{32}$P-labeled oligonucleotide on an 8% denaturing PAGE gel.

10. Plasmid construction

pcDNAUBF was constructed by subcloning a 2.2 kb Nde I-BamH I fragment containing hUBF gene, including the ATG transcription initiation codon from the baculovirus
expression vector pFLAGUBF (provided by H. Beckmann) into the pcDNA1Neo vector (Invitrogen) (Fig. 4-1). A 16/14-mer duplex adapter harboring Hind III and Nde I sites at two ends was used to connect incompatible sites. The sequence of the 16/14-mers were 5'-AGCTTGAACCCCTCA-3' and 5'-TATGAAGGGTCA-3'; and the duplex contained a single Xmn I single restriction site that can serve diagnostic purpose after ligation. The 14-mer was 5'-phosphorylated with T4 polynucleotide kinase and then annealed with the 16-mer. pcDNA1Neo was digested with Hind III and BamH I, dephosphorylated by calf intestinal alkaline phosphatase, and ethanol precipitated to remove the small cleaved fragment. After the three piece ligation between equal molar amounts of the 2.2 kb hUBF fragment, the linearized, dephosphorylated pcDNA1Neo vector and an excess of the duplex adapter, the resulting ligation product went through ethanol precipitation in order to separate the vector from most of the free dimer and monomer adapters. Since the adapter was phosphorylated only on one strand at the Nde I end, it can only self-ligate at Nde I site into a dimer, or ligate once at an Nde I site with hUBF fragment, which ligated with the pcDNA1Neo on the other end at BamH I site. Accordingly, the major ligation product would be the three piece ligated vector with free Hind III ends.

The ligation mixture was then ethanol precipitated, phosphorylated, and ligated to form a circular vector. The final ligation mixture was chemically transformed into bacterial MC1061/P3 strain (Invitrogen) made competent through a published procedure (Sambrook et al., 1989). The bacteria was originally grown in the presence of 40 µg/ml kanamycin.
Transformants were selected on SOB plates with 25 μg/ml ampicillin and 7.5 μg/ml tetracycline. DNA from plasmids of positive clones was extracted using a Promega wizard mini-prep kit and their structures were confirmed by restriction digestion and by \textit{in vitro} transcription/translation.

11. Immunofluorescent staining of HeLa nucleoli

HeLa cell nucleoli were visualized by immunofluorescent staining according to a protocol provided by E. Chan of the Scripps Institute. Cells were grown on coverslips that were placed in 6-well tissue culture plates (Falcon). During staining, the coverslips were washed once with PBS in the wells and fixed for 2 min on ice using a 3:1 mixture of acetone : methanol chilled to -20 °C. After a 3 min wash with PBS, the coverslips were incubated with primary antibody solution containing anti-NOR90 diluted to 1: 200 with cold PBS (40-50 μl of the solution was necessary to cover the surface). The 6-well plate was incubated in a moist chamber at room temperature for 30 min and washed with PBS for 5 min. Secondary antibody solution was applied, which contained fluorescein-conjugated goat anti-human IgG (1:100 dilution) and DAPI (final concentration 0.005%) in cold PBS. After a 30 min incubation at room temperature, the coverslips were washed once with PBS, dipped in deionized water for one second to remove the salt, and mounted with vinol/antifading agent, a gift from S. Lowe at MIT.
12. Transforming DNA into mammalian cells by lipofection

Lipofection was performed using a lipofectamine system and Optimem medium from BRL Life Sciences. HeLa cells were seeded at $1.5 \times 10^5$ cells/well in a 6-well tissue culture plate overnight. In two separate polystyrene tubes, $2.5 \mu g$ DNA ($0.4-0.5 \mu g/\mu l$) was diluted in $100 \mu l$ Optimem and $7 \mu l$ of lipofectamine was diluted in $100 \mu l$ Optimem, respectively. These two components were then mixed well together and incubated for 15-20 min at room temperature to allow lipid-DNA complex formation. Following the incubation, another 0.8 ml of Optimem was mixed in. Cells were rinsed once with Optimem and overlaid with the 1 ml lipofection mixture mentioned above. After 5 h in tissue culture incubator, 1 ml of warm D-MEM with 20% FCS was added. When the experiments required expression longer than 24 h from the beginning of transformation, the medium was replaced at 18-24 h with fresh complete D-MEM.

13. Stable transformation of the hUBF expression vector into HeLa cells

The expression vector pcDNAUBF, which contains the hUBF gene under the control of CMV promoter, was transformed into HeLa cells by lipofection. Twenty-four hours after the beginning of transformation, the cells were replenished with fresh D-MEM with 10% FCS. Two days later, cells were trypsinized and diluted 1:5, 1:10, 1:50 into 10 cm plates in D-MEM. G418 (BRL Life Sciences) at 400 \mu g/ml was added from
following day on and resistant clones were picked 2-3 weeks later. Individual clones were expanded and cellular extracts prepared by a freeze-thaw method.

14. Liquid scintillation counting using a dual label dpm program

A dual label dpm (disintegration per minute) program provided in the Beckman LS1801 liquid scintillation system was used to separate counts from a sample containing both $^{32}$P and $^3$H radioactivities. The quench curve was set up using the Oxi-test $^3$H and $^{14}$C lipophilic internal standards from Radiomatic Instruments & Chemical (RIC) and CH$_3$NO$_2$ as the quenching agent following published procedures (Beckmann liquid scintillation system manual; Amersham Cooperation, 1977).

15. Dual label experiment to detect de novo total RNA synthesis in vivo.

Human AD293 cells were seeded overnight in 15 cm tissue culture plates in D-MEM with 10% FCS. $^3$H-Thymidine (90 Ci/mmol, Dupont/NEN) was added at 0.5-12.5 µCi/ml and incubated for 18 h to label the nuclear DNA. Cells were then harvested, counted and resuspended in D-MEM at 1.3x10$^7$ cells/ml. Three to four hundred µl of cell suspension corresponding to 4-5 x10$^6$ cells were mixed with 50 µl of pBR322 DNA at 1 µg/µl, unmodified or modified with cis-DDP or trans-DDP. Electroporation was carried out at 170 V, 24 Ω, 950 mF in a 0.4 cm electroporation cuvette (BIO-RAD), a condition
that had been optimized by transformation of pCMV-βgal vector followed by an assay for β-gal activity in the extracts (Dustice et al., 1991). Cells in the cuvettes were allowed to sit at room temperature for 10 min before being transferred into 10 ml of warm D-MEM/10% FCS and distributed in aliquots into 6-well tissue culture plates. At various time points after electroporation, a set of samples was pulse labeled for 1 h with 14C-uridine (491 mCi/mmol, Amersham) at 0.5 μCi/ml by directly mixing into the medium. At the end of labeling, cells were trypsinized and resuspended in 100-200 μl PBS. One hundred μl of the cells in each sample were spotted onto a 2.5 cm Whitmann GF/C filter that had been presoaked with 10% TCA and air dried. After the filters spotted with the cell mixture were air dried again, they were placed in 6 well plates, each occupying one well, and washed three times with 5 ml of 10% trichloroacetic acid (TCA) per well for 5 min on a platform shaker (Hoefer Scientific). The filters were then rinsed with 96% ethanol and air dried before counting in liquid scintillation fluid.

16. Nuclear run-off experiment and slot blot analysis

The nuclear run-off assay was performed using a published protocol with several modifications (Aubudul, 1993) with regard to the presence of platinated DNA, the inclusion of tritiated marker RNA, and the RNA resuspension buffer. AD293 cells grown attached, or HeLa cells grown in suspension were harvested and lysed with hypotonic buffer. Nuclei were resuspended in glycerol storage buffer and aliquots of 2-5 x10^7 nuclei
in 100-200 μl were stored in liquid nitrogen. A Stratagene cloning vector, pGEM-3Zf(-), which contained a T7 promoter, was used as a template to synthesize tritiated RNA marker to monitor recovery of run-off transcripts. In vitro RNA synthesis was carried out using an Ambion T7 Maxiscript kit in the presence of 100 μCi ³H-UTP (38.6 Ci/mmol). The specific activity of the marker was 1.4 x 10⁵ cpm/μg. During run-off transcription, 100-200 μl nuclei were thawed out and an equal volume of 2 x reaction buffer with NTPs, 50-100 μCi [α-³²P]UTP at 800 Ci/mmol and pBR322 DNA unmodified or modified with cis-DDP, trans-DDP was added. The mixture was incubated at either 0 °C or room temperature for 5 min before transferring into a 30 °C water bath for optimal transcription. After 30 min, the reaction mixture was digested with RNase-free DNase I at high salt concentration at 30 °C and subsequently with proteinase K at 42 °C. ³H-RNA marker was added at 2-3 x10⁵ cpm per sample prior to phenol/chloroform extraction and TCA precipitation on GF/C filters using a Millipore manifold. After a repeated digestion by DNase I and Proteinase K, the RNA was eluted from the filters, alkaline denatured and precipitated by isopropanol. One ml of TE instead of 2-[tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid (TES) solution was used to resuspend the RNA. Three to four μl of RNA was counted in liquid scintillation fluid using a dual label cpm program to separate ³H and ³²P counts. The counts of ³H marker was used to adjust difference in recovery.
De novo rRNA synthesis was determined by slot blot analysis following a published protocol with minor modifications (Aubudul, 1993). pHr10SK, a plasmid containing the human 18S rRNA gene and pHFbA-1, which contained the human β–actin gene was linearized by Kpn I and Bam H1, respectively, alkaline denatured, and neutralized by 6x SSC. 10-12.5 μg of rDNA and 2.5-5 μg of actin gene DNA was immobilized to nitrocellulose using a Schleicher and Schuell slot blot apparatus. The membrane was air dried and baked at 80 °C oven for 2 h and subsequently probed with $^{32}$P labeled total RNA using the Stratagene Quickhyb solution and protocol. The membranes was air dried and analyzed using the Phosphorlmager system.

17. Quantitative PCR

Quantitative PCR was conducted according to a protocol developed by H. Zarbl’s laboratory at MIT. HeLa cells transiently transformed with pCMV-βgal vector were harvested after 24 or 48 h and lysed by incubating on ice for 10 min with 2 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$ and 0.5% NP-40). Nuclei were pelleted by centrifugation for 5 min at 2200 rpm and 0 °C. Cells were resuspended with 50 μl TE per 5 x $10^6$ cells, and incubated at 37 °C for 1 h with 500 μl extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, pH 8.0 and 0.5% SDS) and 2 μl 10 mg/ml DNase-free pancreatic RNase. Proteinase K was subsequently added to a final concentration of 100 μg/ml and the mixture was incubated at 50 °C for 3 h before cooling down to room
temperature. An 18 gauge needle was used to sheer the DNA by passing the mixture through the needle 12 times. The resulting solution was incubated with 32 µl 5 M NaCl and 720 µl isopropanol at room temperature for 15 min and centrifuged for 10 min at 12,000 x g, 4 °C. The pellet was re-dissolved in 180 µl TE, and re-precipitated with ethanol before finally being resuspended in 100 µl TE.

Quantitative PCR was performed to amplify 197 bp fragment in the 3’ end of the β–gal gene using two primers n3 and n4 kindly provided by J. Mello. External standards contained template pCMV-βgal at 5 concentrations ranging from 0.4-25 ng. Reactions were carried out in a volume of 100 µl, including 10 µl of previously isolated genomic DNA as template, 100 pmol of each primer, 200 µM final concentration of each dNTP, 1 μCi of [α-32P]dCTP (800 - 6000 Ci/ mmol) and 1 U of Stratagene pfu polymerase along with its reaction buffer. The PCR cycle consisted of 1 min at 94 °C, 2 min at 53 °C, and 2 min 70 °C. The 197 bp fragment synthesized after 25-30 cycles was resolved on a 7% polyacrylamide gel and analyzed by PhosphorImager.
RESULTS

1. Controlling hUBF overexpression.

A mammalian expression vector pcDNAUBF was constructed by subcloning the hUBF gene from the baculovirus expression vector pFLAGUBF into the Invitrogen vector pcDNA1Neo, using a 16/14-mer duplex adapter to connect two incompatible restriction ends. The schematic diagram of pcDNA1Neo and the cloning procedure were shown in Figure 4-1. The hUBF gene was placed under the control of the human cytomegalovirus (CMV) promoter to achieve a high level of transcription. The final ligation product was transformed into the *E. coli* strain MC1061/P3, which harbored the P3 episome (kan<sup>R</sup>, amber tet<sup>R</sup>, amber amp<sup>R</sup>). The cloning vector provided the ColE1 origin of replication and supF suppressor tRNA, thereby allowing transformed bacteria to survive in selection media containing tetracycline and ampicillin.

pcDNAUBF was transformed into HeLa cells by lipofection and the neo selection marker on the vector allowed direct selection of G418 resistant clones. Extracts prepared from individually expanded positive clones were examined by Western analysis using a rabbit antibody raised against recombinant hUBF. A total of 33 clones was examined for cellular hUBF protein levels. No overexpression was found in any of the clones compared to the HeLa parental cells. In a parallel transformation experiment, the empty pcDNA1Neo was
transformed into HeLa cells and subsequent clonal selection resulted in approximately the same number of G418 resistant clones as that of pcDNAUBF. This suggested that the expression of pcDNAUBF vector was not toxic to the cells. An RNase protection assay was used to measure mRNA levels. pcDNAUBF was linearized by EcoN I and a 438 bp hUBF antisense RNA was synthesized from 3’ of the gene using Sp6 RNA polymerase. Total RNA isolated from three of the pcDNAUBF clones and two pcDNA1Neo control clones was examined for hUBF mRNA levels. No significant elevation of hUBF transcription was found in pcDNAUBF clones compared to that of pcDNA1Neo clones or parental HeLa cells.

2. Immunofluorescent staining of HeLa nucleoli upon treatment of cells with cis-DDP.

Since hUBF is localized in the nucleolus, while cis-DDP can form DNA-adducts in the entire genome, treatment of cells with the drug may cause dispersion of hUBF from the nucleolus into the nuclei and, additionally, prevent newly synthesized hUBF from being transported into the nucleolus. Therefore, the possibility to observe nucleolar delocalization of hUBF was explored by immunofluorescence (Fig. 4-2).

The LD_{50} for cis-DDP and trans-DDP in HeLa cells were 3 μM and 75 μM, respectively under current experimental conditions. The hUBF in HeLa nucleoli was visualized by incubation of HeLa cells with a human autoimmune serum containing anti-NOR90, followed by a secondary antibody coupled with fluorescein. Cells were treated for
one hour at various Pt doses ranging from 0.01-50 μM for cis-DDP, and 0.1-100 μM for trans-DDP. The treated cells were stained at various time points from 4-144 h after treatment. At all doses and time points, the hUBF was localized in the nucleoli. Heavy doses of both cis-DDP and trans-DDP seemed to cause nucleolar segregation and a slight diffusion of the fluorescence in the nuclei, indicating a general toxic effect by both platinum compounds. This conclusion was reaffirmed in a personal communication with Professor Lan Bo Chen, a biochemist at Dana Farber Institute with extensive experience in cellular microscopic structures.

3. Inhibition of rRNA synthesis in vivo by cis-DDP adducts

Recently, McStay and coworkers reported construction of a vector containing an internal ribosomal entry site (IRES) (Jang et al., 1989) from encephalomyocarditis virus (EMCV) at the 5' leader of an RNA polymerase I transcript with an additional 3' SV40 polyA signal (Palmer et al., 1993). The EMCV IRES is a region of secondary structure in the leader sequence of the transcript that stimulates initiation of translation by increasing ribosomal binding (Jung et al., 1989). Such a vector, pHENA, when transformed into human cells, is able to drive efficient expression of neomycin phosphotransferase (neo resistance) at levels comparable to that produced from an RNA polymerase II driven expression vector utilizing a retroviral enhancer/promoter. Thus, if cis-DDP adducts could titrate hUBF away from an rDNA promoter, the neo resistance gene under the control of hUBF and RNA polymerase I
will be preferentially inhibited resulting in the sensitization of cells to cis-DDP treatment (Fig. 4-3).

HeLa cells were transformed separately with pHENA and a RNA polymerase II controlled neo selection vector, pSV2neo by lipofection. Stable clones were selected and expanded to perform a cis-DDP toxicity test. However, the polymerase I driven neo resistant clones were highly unstable, losing the selection marker within a few weeks and dying subsequently.

In a transient lipofection assay, pHENA or pSVneo were co-transformed with pBR322, unmodified or modified with cis-DDP or trans-DDP, along with pCMV-βgal. Cell extracts were prepared after 24-48 h and examined for neomycin phosphotransferase activity. Cellular β-galactosidase activity was determined to calibrate the transformation efficiency. The results showed no clear pattern of preferential inhibition of RNA polymerase I driven promoter, or any dose dependent pattern of inhibition.

4. Measurement of total RNA synthesis in the presence of platinated DNA by dual labeling

An experiment comparing the efficiencies of different DNA transformation methods was conducted. Under optimized conditions, the overall expression efficiency of electroporation can be two-fold that of lipofection (data not shown), although the percentage
of cells that received the expression vector may be higher in lipofection according to the manufacturer. The limit of DNA input that may have resulted in the failure in pHENA/pBR322/pCMV-βgal co-transformation experiment can therefore be partially overcome by electroporation, although an increased transformation efficiency generated by extreme electroporation conditions caused a general reduction in cell survival (data not shown).

The rate of rRNA synthesis is about 45% of the total RNA synthesis in cultured cells (Marzluff and Huang, 1985). The following preliminary experiment was designed to test the titration model in cells using a dual label experiment that measures total RNA synthesis in the presence of platinated DNA.

In an initial experiment, genomic DNA from 5x10^6 AD293 cells transformed with 50 μg CMV-βgal vector was recovered 24 hr after electroporation and quantitative PCR was performed with external CMV-βgal standards, using two primers that generate a 192 bp DNA after 30 cycles of amplification (Fig. 4-4). The amount of CMV-βgal DNA in the cells (~14% survival) can then be extrapolated from the standard curve and the amount of platinum adducts that can be introduced per cell can be assessed. An estimated upper limit of 6.9 fg plasmid was present per nucleus. If the DNA were platinated at an r_v of 0.04, this would translate into 5 x10^5 adducts /cell, well within the range of clinical relevance.
Human AD293 cells were pre-labeled with \(^3\)H-thymidine to identify the nuclei DNA. Up to 50 μg of pBR322, unmodified or modified by \textit{cis}-DDP or \textit{trans}-DDP was electroporated into cells. At different time points, aliquots of the samples were pulse labeled with \(^{14}\)C-uridine to mark the \textit{de novo} total RNA synthesis. The cells were subsequently spotted onto GF/C filters, washed free of unincorporated label and analyzed with the dual label dpm program in a liquid scintillation counter. The total RNA synthesis was quantified normalizing \(^{14}\)C dpm with \(^3\)H dpm, which accounts for the variance in cell number between samples. A decrease of 41 to 57% in total RNA synthesis was observed for cells transformed with platinated DNA compared with unmodified pBR322 within 36 h after electroporation (Fig. 4-5). In another experiment, 18 h and 24 h after electroporation, the total RNA synthesis in the presence of 50 μg of \textit{cis}-DDP modified pBR322 (\(r_b=0.112\)) per 5 x10^6 cells was inhibited by 37% and 47% respectively, compared to unmodified pBR322, whereas \textit{trans}-DDP modified pBR322 (\(r_b=0.141\)) caused approximately 20% and 28% inhibition, respectively. After 24 to 36 h, the cells transformed with unmodified DNA reached confluence, and at which point RNA synthesis began to decrease due to contact inhibition (Fig. 4-5). Interestingly, the \textit{trans}-DDP adduct containing DNA transformed cells showed an average 19% higher survival rate than \textit{cis}-DDP transformed cells. In a third experiment, pBR322 modified at approximately half of the \(r_b\) for by both \textit{cis}-DDP and \textit{trans}-DDP were compared with previously used platinum decoys. At adduct levels half of previous input (equivalent to 48 μg at \(r_b=0.055\)), total RNA synthesis was inhibited by \textit{cis}-DDP and \textit{trans}-DDP to a similar degree (32% for \textit{cis}-DDP adducts, and 35% for \textit{trans}-DDP adducts). When
the adduct level was doubled to a level of 48 μg at r_b=0.112 was repeated, the preferential inhibition became apparent, with 46% inhibition by cis-DDP and 21% inhibition by trans-DDP adducts. This confirmed the results of the previous two experiments. A possible explanation is that the titration model specific for cis-DDP adducts is only operative above a certain adduct level. In a further investigation of total RNA transcription inhibition, no correlation was observed when r_b was increased above 0.15; it is possible that the extraordinarily high levels of adduction have severely altered the DNA structure. An estimation of cell survival by counting cells that remained attached to the tissue culture plate at the time of the harvest of the second experiment suggested that cis-DDP adducts were more toxic than trans-DDP adducts.

5. Nuclear run-off experiment and slot blot analysis

In order to overcome the problem of insufficient adduct input, a nuclear run-off experiment was carried out to examine de novo rRNA synthesis by mammalian nuclei. In this case, nuclei can be directly incubated with increasing amounts of platinated DNA without the barrier of a cell membrane. Highly radioactive 32P can be used without the additional concern for its toxicity to cultured cells.

Isolated AD293 cell nuclei were incubated in the presence of [α-32P]-UTP, the four NTPs and pBR322 modified by platinum compounds. Total RNA was isolated and the
recovery of total RNA was monitored by addition of a $^3$H-RNA marker. Radioactive rRNA was detected by slot blot analysis, in which total RNA was hybridized with the denatured rDNA gene blotted to a nitrocellulose membrane. As a control, $\beta$–actin transcription was also analyzed. No significant decrease in total RNA synthesis was detected, even when the $cis$-DDP was added to a level of $10^8$ adducts per nuclei, a level at which the nuclei tended to aggregate after a short time. There also was no preferential inhibition of rRNA synthesis compared to $\beta$–actin mRNA synthesis. However, this result was understandable because a positive control of using promoter sequence to titrate away hUBF showed no differential RNA synthesis. The rDNA promoter region was amplified by PCR and incubated during nuclear run-off synthesis. In this case, up to $10^6$ copy/nucleus promoter could be added before nuclei began to aggregate. No significant change in total RNA synthesis or specific rRNA synthesis was detected.
DISCUSSION

1. hUBF overexpression experiment

Overexpression of platinum-DNA recognition proteins in cis-DDP resistant cell lines or in tissues in which the therapeutic effects of cis-DDP are manifested has been reported (Chao et al., 1991; Bissett et al., 1993; Mello et al., 1996). The titration model predicts that overexpression of hUBF would help to compensate for its diversion from the promoter by cis-DDP adducts, allowing cells to be more resistant to the drug treatment. However, the "repair blocking" model predicts that overexpression of hUBF would cause the adducts to persist longer on the DNA thus sensitizing cells to cis-DDP. Therefore, by comparing cis-DDP toxicity in isogenic cell lines with different hUBF levels, it may be possible to estimate the relative contribution of these two mechanisms.

Clones overexpressing hUBF were not detected. One possibility is that the exogenous hUBF gene in the pcDNAUBF was lost in cells while the neo resistance gene remained under G418 selection pressure. Another explanation is that the cells might have a feedback mechanism to regulate the level of cellular rRNA by controlling hUBF expression. Indeed, in muscle cell differentiation, there is a coordinated decrease in the hUBF mRNA level and subsequent rRNA synthesis (Larson et al., 1993). Multiple cAMP response elements and serum responsive elements are present upstream of mouse hUBF promoter.
region (-1183 to -343). In an assay of pUBF-CAT reporter gene expression, higher levels of CAT expression upon serum stimulation are observed compared to a pUBF-CAT construct without the additional upstream sequences (Nishimura et al., 1994). Future experiments in hUBF overexpression studies may explore the use of an inducible expression system to manipulate better hUBF expression.

2. Immunofluorescent staining of HeLa nucleoli upon treatment of cells with cis-DDP

Nucleolar segregation, an indicator of disrupted rRNA synthesis, has been observed after treatment with many drugs (Routeille et al., 1982). Although this phenomena did occur at high doses of cis-DDP and trans-DDP treatment, a clear pattern of hUBF delocalization from the nucleoli to the nuclei was not observed in correlation with the specific anticancer effect of cis-DDP. It is possible that the level of hUBF delocalization necessary to inhibit rRNA synthesis is below the limit of immunofluorescence detection. Another explanation may lie in the fact that cis-DDP adducts are enriched in the nucleolar DNA due to the GC-rich content in rDNA genes (Khan et al., 1978). Therefore, even if the hUBF molecules were diverted away from the rDNA promoter region, they may simply be attracted to the abundant cis-DDP adducts in the nucleoli.

3. Test the disruption of rRNA synthesis in vivo by cis-DDP adducts
The failure to maintain permanent neo resistant clones may be directly related to the intrinsic instability of RNA polymerase I controlled protein expression. However, transient expression of pHENA along with platinated DNA and pCMV-βgal also failed to yield meaningful results. One possibility is that lipofection permitted only limited amount of DNA to enter a cell during transformation and the chances of all three vectors getting into the same cell in sufficient quantity may have been too low. Another possibility is that the high background of the neomycin phosphotransferase assay may have limited sensitivity.

4. Dual label experiment to test total RNA synthesis in the presence of platinated DNA

In order to separate the effect of titration and repair blocking, it was important to test each model independently. Rather than directly damaging chromosomal DNA, DNA adducts were introduced in trans to minimize potential complications caused by adducts formed on genomic DNA that might obscure the results. The mouse (Pikaard et al., 1990) and Xenopus oocyte (Labhart and Reeder, 1984) enhancers for RNA polymerase I compete promoter sequences in trans and inhibit rRNA synthesis in vivo. It may be possible to demonstrate titration by transforming platinated DNA into cells and then determining the level of rRNA synthesis inhibition.

The data suggest that cis-DDP adducts can indeed inhibit total RNA transcription in vivo by 41-57%; moreover, the inhibition was approximately twice as efficient than for trans-
DDP adducts. Given the fact 45% of total RNA synthesis can be attributed to rRNA synthesis, it is highly likely that ribosomal RNA synthesis was severely inhibited. Moreover, the cell survival data suggest that cis-DDP adducts were 19% more toxic than trans-DDP adducts, which was in turn more toxic than unmodified pBR322. This inhibitory effect on growth supports but certainly does not definitively prove the titration model. This type of differential toxicity of cis-DDP adducts versus trans-DDP adducts is distinguished from previous experiments reported in literature in that in this experiment, the adducts were situated in a bacterial plasmid that was not expressed in mammalian cells, and since the cells were not treated with the drugs, the genomic DNA was adduct-free. The complications caused by a combined effect of blockage of DNA synthesis, RNA synthesis, and DNA repair were avoided; instead, the differential toxicity may be directly attributed to reduced total RNA synthesis by introduction of exogenous adducts, which lends support to the transcription factor hijacking hypothesis.

It should be noted that there may be multiple HMG box proteins present in the cells studied and they also bind cis-DDP adducts. These observations may represent a collective effect of hijacking of all the HMG box containing transcription factors. It is possible that the real cytotoxic effect was determined by the titration of another HMG box transcription factor that regulates the expression of a second critical gene.

5. Nuclear run-off experiment and slot blot analysis
A nuclear run-off experiment has been used to demonstrate the inhibition of rRNA synthesis by the retinoblastoma protein (Rb) (Cavanaugh et al., 1995). However, in a similar experiment in which cis-DDP adducts were incubated with the run-off reaction, no rRNA transcription inhibition was observed. Since a high excess of rDNA promoters did not cause inhibition, the nuclear run-off does not appear to be sensitive enough to measure a decrease in rRNA synthesis in the present experimental system.

One explanation for the above result is that during nuclear run-off, new transcription is not initiated; instead, transcription that has already been initiated is faithfully elongated to give a measure of the level of transcription at the time of cell lysis (Greenberg and Ziff, 1984). The exogenous platinated DNA or rDNA promoter sequences have no effect on the transcription complexes that had already been formed (and hence no longer require hUBF). High levels of elongation may mask any effects of the platinum decoys. The observed rRNA synthesis inhibition by Rb protein, however, may be attributable to the ability of Rb to directly bind to the transcription initiation complex.
CONCLUSIONS

The experiments described in this chapter primarily investigated the titration model of human upstream binding factor hUBF in mediating the cytotoxicity of cis-DDP \textit{in vivo}. In particular, experiments were designed to examine the effect of platinum decoys in titrating hUBF away from rDNA promoters in mammalian cells. The preliminary results from the dual label experiment suggest that, relative to unmodified DNA, the \textit{cis}-DDP DNA adducts inhibited total RNA synthesis significantly, and that the inhibition was more severe than that caused by \textit{trans}-DDP adducts. In addition, a differential survival of cells transformed with DNA adducts formed by the two platinum compounds was observed. This effect was directly related to the introduction of exogenous platinum adducts without disrupting the genomic integrity, and thus the data support a transcription factor hijacking model. These results do not directly suggest the titration of hUBF, nor do they reveal that titration of hUBF is responsible for the observed toxicity. However, based on the fact that 45\% of ongoing total RNA synthesis is attributable to ribosomal RNA synthesis, an observed 40\% inhibition of total RNA synthesis would with good likelihood imply inhibition of ribosomal RNA synthesis. Considering the unusually high affinity of hUBF for \textit{cis}-DDP adducts compared to that of other HMG boxes, these results are consistent with the notion that titration of hUBF may be involved in causing the observed rRNA synthesis inhibition and the toxic effect of \textit{cis}-DDP adducts.
Several other experiments to explore preferential rRNA synthesis in vivo yielded negative data owing to experimental limits and the results are therefore inconclusive.
FUTURE EXPERIMENTS

Future experiments to investigate the role of hUBF in mediating the cytotoxicity of cis-DDP should be aimed at several areas:

First, to examine the titration model, sensitive methods to measure specific de novo rRNA synthesis need be developed in order to differentiate rRNA synthesis from total RNA synthesis. The pulse-chase experiment designed to label de novo rRNA synthesis described in this work could then be extended to detect rDNA transcripts (as in slot blot analysis), instead of counting total RNA. Alternatively, rRNA and nuclei could be independently isolated and counted following dual labeling.

Second, overexpression of hUBF in an inducible system may be used in order to fine-tune transiently the level of hUBF at appropriate times in order to avoid a constitutive overexpression vector that may establish a feedback mechanism to suppress hUBF synthesis. The results of a toxicity test in these cells transiently overexpressing hUBF may be used to assess the contribution of the titration and repair blocking models, since the titration model predicts a resistant phenotype whereas the repair blocking model suggests a sensitized phenotype.
Third, a sequence in the rRNA precursor may be used to probe total RNA following the transformation of cis-DDP adducts. Since the rRNA precursor is processed within hours of transcription (Sirlin, 1970), the presence of this specific sequence may reflect de novo RNA synthesis in vivo.

Fourth, although the titration model may play an important role in the operation of hUBF, a repair blocking effect may also contribute to the toxicity of cis-DDP. The high affinity of hUBF for cis-DDP adducts makes it a good candidate as a repair-shielding agent. Reconstituted repair-competent UvrABC system and mammalian cell free extracts can be used to study the cis-DDP adduct repair in the presence of hUBF and assess the magnitude of the repair blocking effect relative to that observed in other HMG box proteins.
REFERENCES


Chu G. and Chang, E. (1988) Xeroderma pigmentosum group E cells lack a nuclear factor that binds to damaged DNA. Science 242, 564-567


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**Figure 4-1.** Schematic diagram illustrating the cloning of the hUBF overexpression vector, pcDNAUBF. The hUBF gene was excised from the baculovirus expression vector pFLAGUBF and subcloned into the Invitrogen vector pcDNA1Neo, using a 16/14-mer duplex adapter to connect two incompatible restriction ends.
Construction of Mammalian Expression Vector pcDNAUBF
Figure 4-2. Schematic diagram illustrating the experiment examining a possible immunodelocalization of nucleolar hUBF of cells upon treatment of cells with *cis*-DDP. Treatment of cells with the drug may cause dispersion of hUBF from nucleolus and, additionally, prevent newly synthesized hUBF from being transported into the nucleolus. The effect may be visualized through immunofluorescence.
Possible Immunodelocalization of Nucleolar hUBF upon Treatment of Cells with cis-DDP

Detection of hUBF: antiserum against hUBF followed by a secondary antibody coupled with fluorescein
Figure 4-3. Schematic diagram illustrating the experiment designed to investigate a possible preferential inhibition of rRNA synthesis by *cis*-DDP DNA adducts. The encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) can drive efficient expression of neomycin phosphotransferase (neo resistance) at levels comparable to that produced from an RNA polymerase II driven expression vector. Thus, if *cis*-DDP adducts could titrate hUBF away from a rDNA promoter, the neo resistance gene under the control of hUBF and RNA polymerase I will be preferentially inhibited, resulting in the sensitization of cells to *cis*-DDP treatment.
Is RNA Pol I Promoter Driven Reporter Gene Expression Inhibited by \textit{cis-DDP}?

Hypothesis: RNA pol I promoter driven reporter will be preferentially inhibited by treatment with \textit{cis-DDP}.
Figure 4-4. Schematic diagram showing the experimental design testing the titration model in vivo. Exogenous platinum adducts were electroporated into mammalian AD293 cells and the de novo total RNA synthesis was examined by a dual label scintillation counting method.
Inhibition of Total RNA Synthesis by cis-DDP DNA Adducts

Human AD290 Cells

\[ ^3 \text{H-thymidine} \]
\[ \text{Label DNA} \]

Electroporation of Pt-DNA adducts

\[ \text{pBR322} \quad \text{cis-DDP-pBR322} \quad \text{trans-DDP-pBR322} \]

\[ ^{14}\text{C-Uridine} \]
\[ \text{Label new RNA synthesis} \]

Harvest cells
Bind to filters
Remove free label
Count \(^3\text{H}\) and \(^{14}\text{C}\) separately

\[ ^{14}\text{C} / ^3\text{H} \] represent normalized total RNA synthesis
Figure 4-5. Estimation of platinum adduct input by electroporation. Genomic DNA from 5×10^6 AD293 cells transformed with no DNA, 20 μg and 50 μg CMV-βgal vector (Ctl, B1 and B4, respectively) was recovered 24 hr after electroporation and quantitative PCR was performed with external CMV-βgal standards, using two primers that generate a 192 bp DNA after 30 cycles of amplification. The amount of CMV-βgal DNA in the cells can then be extrapolated from the standard curve and the amount of platinum adducts that can be introduced per cell can be estimated.
Estimation of Adduct Input after Electroporation by Quantitative PCR

<table>
<thead>
<tr>
<th>CMV-βgal Template (ng)</th>
<th>Standards</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 5 1 0.2 0.04</td>
<td>Ctl B1 B4</td>
</tr>
</tbody>
</table>

192 bp:

Estimation: $\sim 5 \times 10^5$ Pt/cell at rb=0.04
Figure 4-6. Inhibition of total RNA synthesis by cis-DDP adducts. AD293 cells were pre-labeled with 3H-thymidine followed by electroporation of plasmid pBR322, unmodified or modified with cis-DDP or trans-DDP. At various time points after electroporation, a set of samples were pulse labeled with 14C-uridine in order to de novo label total RNA synthesis. Dual label scintillation counting was performed as described in Figure 4-4. Total RNA synthesis was preferentially inhibited by cis-DDP DNA adducts. Furthermore, the cis-DDP adducts were directly associated with a reduced survival of the cells after electroporation.
Inhibition of Total RNA Synthesis by cis-DDP Adducts

![Graph showing inhibition of RNA synthesis](image)

Survival of Cells after Transformation with Platinum Adduct

<table>
<thead>
<tr>
<th>Hours</th>
<th>cis-DDP</th>
<th>trans-DDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>*62%</td>
<td>100%</td>
</tr>
<tr>
<td>24</td>
<td>40%</td>
<td>63%</td>
</tr>
</tbody>
</table>

* normalized against survival of cells transformed with unmodified pBR322
BIOGRAPHY

The author was born in 1964 and raised by her grandparents in Shanghai, China. She entered the University of Science and Technology of China (USTC) in 1983, where she majored in molecular biology and participated in research work in a biophysics laboratory. In 1989, the author came to United States to pursue an advanced degree in biology in Marquette University, Wisconsin. A year later, her interest in applied biological sciences led her to join the Division of Toxicology as a Ph.D. student. Her graduate work was conducted under the guidance of Prof. John Essigmann, and involved a protein that recognizes drug-bound DNA. After completing her Ph.D. at MIT, she will attend medical school at Oregon Health Sciences University.