Cisplatin−DNA Adducts Inhibit Ribosomal RNA Synthesis by Hijacking the Transcription Factor Human Upstream Binding Factor†

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ABSTRACT: Several eukaryotic cellular proteins recognize DNA modified by the anticancer drug cisplatin (*cis*-diamminedichloroplatinum(II) or *cis*-DDP); among these proteins is a class of DNA-binding molecules containing the HMG (high-mobility group) box DNA recognition motif. We have previously reported the extraordinarily high binding activity to cisplatin adducts by human upstream binding factor (hUBF), an HMG box containing transcription factor that stimulates ribosomal RNA synthesis (Treiber et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5672−5676). In the present study, we discovered that (1) hUBF interacted selectively with DNA lesions formed by therapeutically effective platinum compounds [Pt(en)Cl_2] and [Pt(dach)Cl_2], in addition to the lesions formed by *cis*-DDP, suggesting a possible association with their anticancer effect; (2) multiple HMG boxes contributed additively to the hUBF-adduct interaction, providing a possible explanation for the unusually high affinity of hUBF for *cis*-DDP adducts as compared to the lower affinities of other HMG box proteins; and (3) ribosomal RNA transcription in a reconstituted system is specifically inhibited in the presence of *cis*-DDP adducts. In this third experiment, a ratio of adducts/promoter of ∼4:1 completely abolished the transcription activated by hUBF. Taken together, these data lend support to the view that transcription factors involved in cellular growth regulation, such as ribosomal RNA transcription, may be hijacked by *cis*-DDP adducts resulting in functional inhibition.

The anticancer drug cisplatin (*cis*-diamminedichloroplatinum(II) or *cis*-DDP;[1] Figure 1) has shown outstanding success in clinical regimens that produce nearly complete remission of testicular cancer (*1*). *cis*-DDP also is frequently used in combination with other drugs to treat a variety of other cancers (*2*, *3*). 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 (*4*). The major DNA adducts formed by *cis*-DDP have been characterized as the 1,2-intrastrand d(GpG) (65%), 1,2-intrastrand d(ApG) (25%), and 1,3-intrastrand d(GpNpG) (6%) cross-links (*5*); numbers in parentheses indicate relative distributions of adducts. By contrast, the clinically ineffective trans isomer of *cis*-DDP forms 1,3- and 1,4-intrastrand cross-links, monofunctional adduct and, at a low frequency, interstrand cross-links (*6*). *trans*-DDP is unable, however, to form cross-links at adjacent DNA bases. On that basis it was proposed that the 1,2-intrastrand cross-links represent the DNA lesions most responsible for the anticancer activity of *cis*-DDP (*7*). While there is ample evidence that DNA damage is a necessary prelude to cell death caused by *cis*-DDP, the biological events

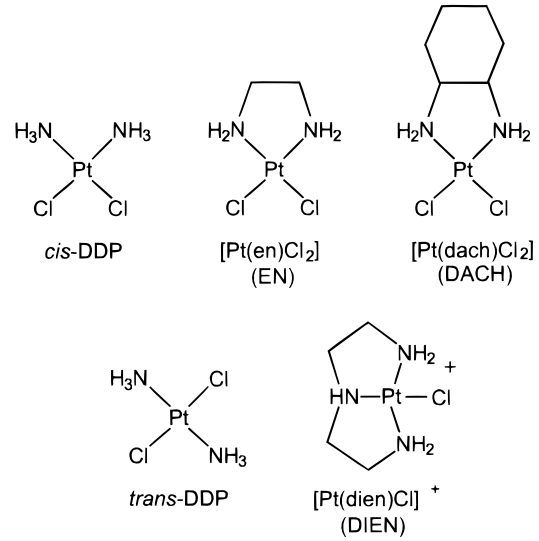

FIGURE 1: Structures of platinum compounds. On the top row are the antitumor compounds *cis*-DDP, [Pt(en)Cl_2], and [Pt(dach)Cl_2]; to the bottom are therapeutically inactive compounds *trans*-DDP and [Pt(dien)Cl]$^+$.

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); DACH, 1,2-diamminocyclohexane; EN, ethylenediamine; DIEN, diethylenetriamine; SDS, sodium dodecyl sulfate; hUBF, human upstream binding factor; PAGE, polyacrylamide gel electrophoresis; r_b, drug-to-nucleotide ratio; TBP, TATA-binding protein; TAF_I, TBP associated factors for RNA polymerase I; xUBF, *Xenopus* upstream binding factor.

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after 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 (*8*−*10*).

Recently a class of cellular proteins that contains a DNA-binding motif homologous to nuclear HMG proteins has been shown to recognize specifically DNA lesions formed by *cis*-DDP (*11*−*13*). 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 (*14*). One characteristic of HMG box proteins shared by both subfamilies is their ability to cause bending and looping of the promoter DNA once they bind to their cognate binding site in the promoter (*15*). Moreover, these proteins also bind nonspecifically to DNA with bent structures such as four-way junction DNA (*16*, *17*). The ability to approach and modulate specific DNA structures and to interact with other nuclear, structural, or transcriptional components thus suggests an architectural role for HMG box proteins (*15*, *18*, *19*).

More than 120 HMG box proteins are now known and there are at least 14 different HMG box genes in humans (*20*). Among the known HMG proteins is the ribosomal RNA transcription factor, hUBF. Interestingly, almost all of the HMG box proteins investigated so far, including hUBF (*13*), 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 (*11*), calf and human HMG1/2 (*12*, *21*), human structure-specific recognition protein SSRP1 (*22*), yeast mitochondrial cytochrome *c* oxidase transcription repressor Ixr1 (*23*, *24*), human mitochondrial transcription factor mtTFA (*25*), mouse testis-specific HMG-domain protein (tsHMG) (*26*), and human sex determination factor hSRY (*27*). 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 (*25*). Several models have been introduced to describe possible roles of hUBF and other HMG box proteins as mediators of anticancer activity of *cis*-DDP (*13*, *26*, *28*). 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 cross-link, *cis*-$[Pt(NH_3)_2]^{2+}$-d(GpG), the major adduct formed by *cis*-DDP, with a striking affinity ($K_{d(app)} \sim 60$ pM) that is comparable to that measured for its cognate rDNA promoter ($K_{d(app)} \sim 18$ pM) (*13*). On the basis of 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.

Here we present further evidence that hijacking may contribute to the mechanism by which *cis*-DDP exerts its anticancer activity. First, we demonstrate that hUBF recognizes platinum compounds with stereochemistries similar to that of *cis*-DDP and that the high affinity of hUBF for *cis*-DDP adducts is likely the result of additive contributions from multiple HMG boxes participating in the binding. Furthermore, titration of hUBF away from its cognate binding site within the rDNA promoter by *cis*-DDP-modified DNA (but not *trans*-DDP-modified DNA) abolishes transcription from the rDNA promoter in a reconstituted RNA polymerase I transcription system.

EXPERIMENTAL PROCEDURES

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 (Figure 1) were carried out as described (*29*). Platinum compounds were the gift of Professor S. J. Lippard. The drug-to-nucleotide ratio (r_b) of the platinated DNA fragment was determined by atomic absorption and UV absorption spectroscopies (*29*). The platinated DNA decoys were generated from plasmid pBR322 DNA modified by *cis*-DDP and *trans*-DDP at a wide range of r_b's following the same reaction procedures.

In Vitro Synthesis of hUBF Wild-Type and Deletion Mutants. The N-terminal deletion mutants, 84N, 182N, 284N, and 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 that have been previously described (*30*). Wild-type hUBF protein (WT) and other C-terminal deletion mutants, 489C, 408C, 282C, and 204C, were synthesized from one vector, pTβGUBF1 (*31*), which was linearized with *Eco*RI, *Bgl*II, *Bgl*I, *Sty*I, and *Pvu*II, 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 of RNA was used in a 50 μL reaction containing 4 μL of ^{35}S-methionine (1200 Ci/mmol, Amersham) and 33 μL of nuclease-treated rabbit reticulocyte lysate (Promega). Following the in vitro translation reaction, 2−4 μL of the mixture was separated on a 5%−15% mini-gradient SDS/polyacrylamide gel and proteins were transferred to nitrocellulose membrane (BIO-RAD minigel electrophoresis and transfer system). Quantification of in vitro generated hUBF was determined by PhosphorImager analysis (Molecular Dynamics) of hUBF bands compared with ^{35}S-methionine standards. Numbers representing mutant hUBF proteins denoted sites of truncation at the N or C terminus. Mutants db1, db2, db3, db4, and dbx were internal deletions of individual HMG boxes corresponding to amino acids 101−108 (box 1), 205−284 (box 2), 284−371 (box 3), 371−491-(box 4), and 492−670 (box 5 and 6), respectively (*30*).

Southwestern Analysis. Southwestern analysis was carried out as previously described (*29*) with minor modifications. Proteins were resolved on gradient SDS/polyacrylamide gels (5%−15% or 5%−20%) and transferred to nitrocellulose membranes. Air dried 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 422 bp DNA at (1−5) × 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 mutant protein 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 ^{32}P-labeled DNA. Computation of binding strength was further corrected by determining the actual molar amount of mutants present on the membrane by ^{35}S-methionine quantification. During ^{32}P autoradiography, a 0.254 mm thick copper sheet was used to block ^{35}S emissions selectively from in vitro translated proteins.

Ribosomal RNA Transcription in Vitro in a Reconstituted System and Nuclease S1 Mapping. In vitro 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 (*32*) with minor modifications on account of the presence of platinum-modified DNA. For the *cis*-DDP series, 3 ng of pBR322 modified to r_b values of 0, 0.0017, 0.0086, 0.019, 0.039, and 0.078 was used in each reaction; for the *trans*-DDP series, 10 ng of pBR322 with r_b values of 0, 0.0018, 0.0090, 0.019, 0.042, and 0.071 was present. In vitro transcription reactions contained recombinant hUBF, the linearized transcription vector template (100 ng of pHu3, a derivative of pBR322 containing the *Eco*RI/*Bam*HI fragment of human rDNA that includes sequences from −500 to +1500 (*33*)) 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, and the reaction was incubated for 30 min at 30 °C in a final reaction volume of 25 μL.

RESULTS

hUBF Binds Selectively to DNA Modified by cis-DDP and Two Other Therapeutically Active Platinum Drugs. Figure 1 shows structures of the five different platinum compounds used in this study. The DNA adduct spectra formed by platinum compounds in which the chemical reaction leaving groups are in 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) cross-links (*34*, *35*). Monoclonal antibodies raised against *cis*-DDP damaged DNA exhibit cross-reactivity toward adducts formed by these molecules, suggesting that these adducts adopt similar configurations (*36*). The high therapeutic indices of this category of compounds are well-documented (*4*). In contrast, *trans*-DDP and DIEN, both of which are therapeutically inactive, generate adduct spectra that notably lack 1,2-intrastrand cross-links. DIEN can only form monofunctional adducts owing to its single chloride leaving group.

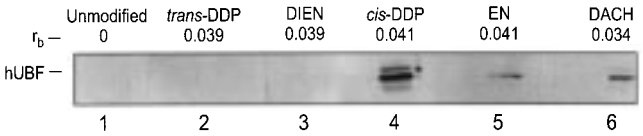

FIGURE 2: hUBF selectively binds to DNA modified by *cis*-DDP and other antitumor platinum compounds that can form 1,2-intrastrand cross-links. Identical blots of in vitro translated hUBF proteins were probed in a Southwestern analysis with a ^{32}P-labeled 422 bp *Ava*I fragment from M13mp18 (*28*) that was unmodified or modified by different platinum compounds. The drug-to-nucleotide ratios (r_b) for the unmodified fragment and 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.

We explored the specificity of hUBF for binding to platinum adducts of different structures by probing identical blots of in vitro translated hUBF with DNA fragments modified by the five compounds indicated above. Our Southwestern results (Figure 2) revealed that hUBF selectively recognized DNA modified by *cis*-DDP, EN, and DACH, but not by unmodified control DNA or DNA modified by *trans*-DDP or DIEN, confirming the distinctive ability of the compounds with cis geometry to form lesions that attract HMG proteins. 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). At similar levels of platination (r_b), hUBF binds to DNA modified by EN and DACH with reduced avidity compared to DNA modified by *cis*-DDP, reflecting a less favorable adduct-DNA structure for binding. Thus the efficacy of these compounds is consistent 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 an $r_b \sim 0.038$ used in this study contained an average of 32 adducts/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) (*13*, *37*). 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 an 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 noncooperative nature of binding between hUBF and a single platinum adduct (*13*) and the presumed 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.

Multiple HMG Boxes Participate in Binding of hUBF to Platinated DNA. The 97 kDa hUBF is involved in initial promoter recognition during ribosomal RNA transcription (*30*). 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 multicomponent complex that is responsible for promoter selectivity (*30*, *31*). The functional domains for SL1 interaction, transcription activation, and nonspecific DNA

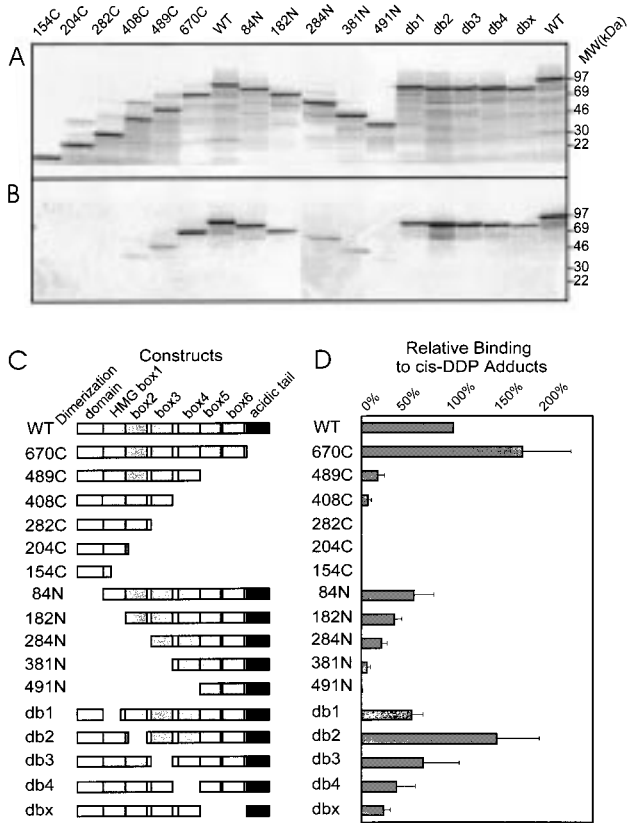

FIGURE 3: Multiple HMG boxes participate in binding of hUBF to platinated DNA. (A) In vitro translated hUBF and hUBF mutants used for Southwestern analysis. Approximately equal molar amounts of ^{35}S-labeled hUBFs proteins were present on the blots. (B) Southwestern analysis of hUBF proteins using ^{32}P-labeled 422 bp DNA modified by *cis*-DDP ($r_b \sim 0.038$) as the probe. The intensity of the band reflects the avidity of binding. (C) Schematic representation of full-length hUBF and various deletion mutants. The amino terminal dimerization domain, the six HMG box motifs, and the acidic domain are illustrated. (D) Binding of hUBF mutants to ^{32}P-labeled DNA modified by *cis*-DDP was quantitied and compared to that of wild type (normalized to 100% binding). Three independent experiments were performed.

binding overlap at the C-terminal half of the protein. To investigate which domains in hUBF are important for its binding to *cis*-DDP adducts, we produced a panel of hUBF deletion mutants (Figure 3C), including successive deletions from the N-terminus and C-terminus as well as internal deletions of individual HMG boxes by in vitro transcription and translation. The ability of these proteins to bind to *cis*-DDP-modified DNA was analyzed by Southwestern analysis.

Membranes blotted with approximately equimolar amounts of hUBF wild type and mutant proteins (Figure 3A) were probed with ^{32}P-labeled 422 bp DNA globally modified with *cis*-DDP ($r_b \sim 0.038$), and the results are shown in Figure 3B. 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 3D. In the analysis, we assumed no differential degree of protein renaturation on the basis of the reasoning that most of the hUBF truncation mutants are generated between HMG box repeats. None of the mutants exhibited any affinity for the control DNA (data not shown).

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, A and B. 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.

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 (db1), but not other HMG boxes, abolished the footprinting pattern (*30*). This mutant hUBF still interacts with platinated DNA with avidity similar to most other deletions of individual HMG boxes (Figure 3B, db1).

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 (*38*).

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 (*13*). 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.

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 (*13*). This result suggested that binding of a *cis*-DDP adduct by hUBF may negatively affect rRNA transcription by titrating hUBF away from its normal site of action; this titration effect could work in concert with the "DNA repair blocking" mechanism previously suggested and demonstrated in vitro for other HMG box proteins (*39*). A demonstration of functional inhibition of ribosomal RNA synthesis, however, is necessary to establish biological feasibility of the "titration" mechanism.

Transcription from rDNA requires, besides hUBF, the selectivity factor SL1 and RNA polymerase I. Although SL1

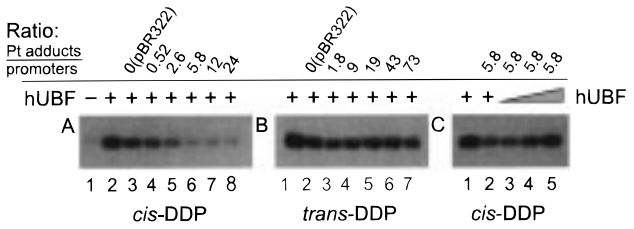

FIGURE 4: *cis*-DDP DNA inhibits the stimulative effect of hUBF on ribosomal RNA synthesis in an in vitro reconstituted system. Purified recombinant hUBF was incubated with template rDNA gene in the presence of pBR322 that was modified by *cis*-DDP (A) or *trans*-DDP (B) prior to the addition of both SL1 and RNA polymerase I in order to initiate rRNA transcription. The resulting transcription was detected by nuclease S1 analysis. Constant amounts of pBR322 with different r_b levels were used in the reactions as indicated by the molar ratio of platinum adducts over promoters (3 and 10 ng total pBR322 for each reaction in 4A and 4B, respectively). (C) Elevated levels of hUBF restore transcription inhibited by *cis*-DDP adducts. Equal amounts of *cis*-DDP-modified DNA at an adduct/promoter ratio of 5.8 were used in each reaction, and transcription inhibition can be relieved by increasing the level of hUBF at 7.5, 15, 30, and 45 ng, respectively.

does not bind to DNA by itself, it cooperatively interacts with UBF, which binds to the rDNA promoter and tethers SL1 to the DNA template (*40*). Recent studies show that SL1, composed of the TATA-box-binding protein TBP and its three associated factors (TAFs), has to interact through its smallest TAF subunit with UBF in order to form a functional transcription initiation complex (*32*). To test our hypothesis directly, we therefore investigated the effect of platinated DNA on ribosomal RNA transcription in a reconstituted in vitro transcription system. 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 to 0.078. Transcripts were detected by nuclease S1 mapping (Figure 4).

Under standard conditions, hUBF was able to stimulate transcription of an rDNA minigene significantly above background (Figure 4A, lanes 1 and 2). An increase in the *cis*-DDP adduct/promoter ratio greatly decreased hUBF-activated transcription in a dose-dependent manner (lanes 3−8). The in vitro rRNA transcription returned to the basal level similar to that seen in the absence of recombinant hUBF when *cis*-DDP adducts were present at 2.6−5.8-fold above that of rDNA promoters (Figure 4A, lanes 5 and 6). By contrast, the rRNA transcription level remained more or less constant upon successive increases in the amount of *trans*-DDP adducts, from a *trans*-DDP adduct/promoter ratio of 1.8 to one as high as 73 (Figure 4B, lanes 2−7). Since we increased the concentration of platinum adducts by increasing the degree of modification while maintaining a constant level of DNA, the dose-dependent inhibition of rRNA transcription by *cis*-DDP-modified DNA but not by *trans*-DDP adducts reflected the specific interaction between hUBF and *cis*-DDP adducts. The observation that an average of about four *cis*-DDP adducts was needed to inhibit transcription from one rDNA template correlated very well with our previous data indicating that hUBF binds with one-third of the avidity to a 1,2-d(GpG) *cis*-DDP adduct ($K_{d(app)} \sim 60$ pM) as compared to an rDNA promoter ($K_{d(app)} \sim 18$ pM).

If simply out-titrating hUBF by *cis*-DDP causes a decrease in transcription from the rDNA promoter, then elevated levels of hUBF should restore transcription initiation. Starting at a state where rRNA transcription was reduced in the presence of *cis*-DDP and hUBF (Figure 4C, lane 2), we increased the concentration of hUBF in the transcription reaction while maintaining the *cis*-DDP concentration constant (lane 3−5). The level of transcription was nearly restored to the level seen in the absence of *cis*-DDP adducts (lane 5). These results suggested that hUBF alone is indeed able to reverse the inhibitory effect of *cis*-DDP adducts on rDNA transcription.

In summary, our 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. It is worthy of note that the K_d's determined in our earlier work were obtained from hUBF footprinting experiments in the absence of SL1. From the correlation of data between the binding assay and our present functional assay, *cis*-DDP DNA adducts are able to titrate UBF away from a multifactor transcription initiation complex just as well as from a simpler UBF−promoter complex, thus further lending in vivo feasibility to the titration mechanism.

DISCUSSION

We have demonstrated that human upstream binding factor recognizes the DNA adducts formed by a set of therapeutically effective platinum compounds that share certain 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 other HMG box proteins, including SSRP1, HMG1, and SRY (*11*, *27*, *29*). In a separate experiment, we observed that hUBF did not bind to DNA modified by the platinum compound *cis*-[Pt(NH_3)$_2$(*N*3-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, recognition of DNA modified by platinum compounds that can engage in cis bifunctional binding to purines is likely to be the common characteristic among HMG box proteins. Interestingly, HMG1 has recently been shown to bind a *cis*-DDP interstrand GC/CG adduct equally as well as to an intrastrand 1,2-d(GpG) *cis*-DDP adduct, while no binding is observed for the interstrand cross-link adduct formed by *trans*-DDP (*41*). 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 different platinum compounds 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 (*42*). While EN and DACH 1,2-d(GpG) intrastrand adducts have also been shown to bend DNA (*43*), monoadduct-forming compounds DIEN and *cis*-[Pt(NH_3)$_2$(*N*3-cytosine)]$^+$ do not (*44*, *45*). The crystal structure of a 1,2-d(GpG) *cis*-DDP adduct in a dodecamer DNA provides the most informative structural analysis to date (*46*). In addition to DNA bending and unwinding, the X-ray structure of the major cisplatin 1,2-d(GpG) cross-link 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 cross-link 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 (*46*).

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 (*47*−*49*). 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 UBF 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) major groove modification by methylation does not interfere with binding (*50*). Therefore, the major adducts formed by *cis*-DDP, EN, and DACH, which cross-link adjacent guanines in the major groove, may be situated on the opposite side of the 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 to 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 Ixr1, 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 (*13*, *37*, *51*). Thus, the actual contact with a single adduct by the multiple HMG box proteins hUBF and Ixr1 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 (6×10^{-11} M), indicating that other factors must contribute to the interaction (*13*, *25*). 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* system 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 is extended from the enhancer element (*52*). 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 (*53*). Similarly, a single HMG box induces a sharp bend after binding to a *cis*-DDP adduct (*25*). 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 (there are a total of 12 HMG boxes in an hUBF dimer) 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.

Even though our study shows that deletion of a single individual HMG box at different positions does not prevent the residual protein from binding to platinated DNA, 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 a deletion causes a shift in the relative position of the remaining HMG boxes, resulting in a new HMG box to assume the preferential domain for platinum binding. The reduced binding in successive deletion mutants may reflect a loss of nonspecific binding that accompanied the loss of an additional HMG box.

Finally, our data with the reconstituted rRNA transcription system demonstrated that the model for transcription factor titration is viable in vitro (Figure 5). 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) cross-link by the human DNA repair excision nuclease is specifically inhibited by HMG1, mtTF1, tsHMG, and SRY (*27*, *39*, *54*). A yeast strain deficient in HMG box protein Ixr1 exhibits a *cis*-DDP resistant phenotype (*23*), and such differential desensitization is nearly abolished by additional mutations in several nucleotide excision repair genes (*55*). Although it is possible that hUBF contributes to cytotoxicity by this same mechanism, the present work and our previous biochemical studies suggest that *cis*-DDP adducts may serve as highly effective decoys for hUBF, thereby disrupting in vitro rRNA transcription. It is possible, of course, that both mechanisms are operative. 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 as compared to the affinity of the protein for *cis*-DDP adducts (Figure 5). In the case of hUBF, this difference in affinities is a mere 3-fold, rendering hUBF a good candidate for playing a role in the "titration" model (*13*). In the present study, we found that the ratio of adduct/promoter at which transcription was abolished correlated well with the respective difference in binding constants. This ratio is also consistent with our previous prediction, based on the

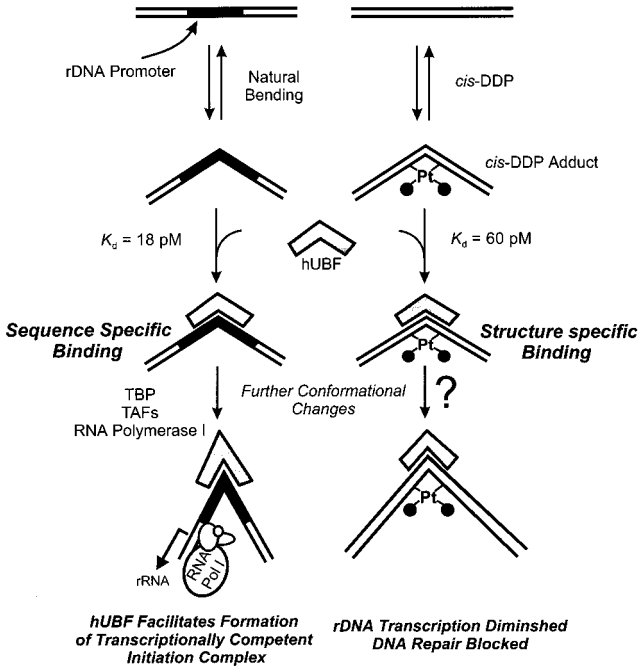

FIGURE 5: Transcription factor hijacking: schematic diagram illustrating possible mechanisms for the involvement of hUBF in *cis*-DDP toxicity. The comparable avidity with which hUBF binds the rDNA promoter and a *cis*-DDP adduct renders it a unique candidate for playing roles in both titrating rRNA synthesis and blocking DNA repair (see Discussion).

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. Thus we provide evidence for the model that an HMG box protein could mediate *cis*-DDP toxicity via a transcription factor hijacking mechanism. In contrast, yeast studies show that 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 (*24*). One possible explanation for this observation is that the modest affinity of Ixr1 for *cis*-DDP adducts ($K_d \sim 2.5 \times 10^{-7}$ 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 was not determined. It is known, however, that the HMG box domain from LEF-1 binds a T-cell antigen receptor (TCR) enhancer motif with an affinity ($K_d \sim 10^{-9}$ M) that is 2 orders of magnitude higher than the value that was determined for a single *cis*-DDP 1,2-d(GpG) adduct ($K_d \sim 10^{-7}$ M) (*25*, *56*). Another SRY-like HMG box protein, Sox-4, binds this same DNA element with even higher affinity ($K_d \sim 3 \times 10^{-11}$ M) (*57*). 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 be unlikely to be disrupted by *cis*-DDP adducts in these specific cases.

Enhanced ribosomal RNA synthesis is an important requirement for cell proliferation and tumor growth (*58*−*62*). The correlation between altered rRNA synthesis and changes in the level of mRNA or degree of phosphorylation in mammalian UBF has been observed by in vitro induction of cell growth and differentiation (*63*). hUBF has also been shown to bind the protein product of the tumor supressor gene Rb, and such binding causes reduced rRNA synthesis and a severely impaired ability for hUBF to bind cruciform DNA as well as the rDNA promoter (*64*, *65*). 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 50000 (data not shown), a number that is similar to the 10000−100000 adducts per cell found in cancer patients during treatment with *cis*-DDP (*66*). Considering that ∼560 copies of rDNA upstream promoters are present in HeLa cells (*67*), 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. The two principal models recently 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 proteins that possess an affinity for *cis*-DDP adducts that is comparable to that for the cognate promoter. Experiments by Chao and co-workers in human A549 lung tumor cells showed a specific change of distribution pattern of HMG1/2 upon treatment of *cis*-DDP, from nucleoli and cytosol to nuclei, suggesting a possible titration by *cis*-DDP adducts (*68*). The study seems to support the hijacking model for HMG1/2 proteins, which also fit the criteria of comparable affinity for promoter and *cis*-DDP adducts. However, interpretation of the change in distribution of UBF reported in the study by Chao should be taken with caution because of the extremely high doses of *cis*-DDP used (which would be expected to be lethal) in the experiment.

Recently, Vichi et al. showed that the TATA box binding protein TBP/TFIID binds selectively to *cis*-DDP or UV-damaged DNA (*69*). They found that *cis*-DDP- or UV-damaged DNA can be used as a highly efficient competitive binding site to inhibit in vitro transcription from an RNA polymerase II promoter. TBP has been shown to participate in initiation of basal transcription of all three eukaryotic RNA polymerases (*70*−*72*), and it is therefore a good target for becoming hijacked by adducts formed by DNA damaging agents that cause significant helix bending. It would be of interest to explore whether the spectrum of platinum adducts to which TBP can bind correlates with the anticancer potential of these compounds as opposed to general cytotoxicity. It is noteworthy that SL1 contains TBP, and thus, if the binding affinity and concentration of this protein complex were high enough, cisplatin adducts could affect transcription by hijacking SL1. Note, however, that our work shows that the addition of an excess of hUBF can offset the hijacking phenomenon. If TBP were a limiting factor (i.e., easily hijacked by cisplatin adducts), one would have expected that it would have been more difficult to restore transcriptional activity with hUBF alone. Interestingly, very recent work demonstrates that cisplatin treatment of HeLa cells causes a redistribution of hUBF within the nucleolus, which would be consistent with a hijacking event in vivo (*73*); this study complements that shown in Figure 4 where

we show hijacking in a reconstituted transcription system in vitro.

The model of transcription factor hijacking, either by homologous DNA-binding sequences or by DNA modified by exogenous agents, has recently been the subject of heightened interest. In *Saccharomyces cerevisiae*, the discovery that extrachromosomal rDNA circles (ERCs) accumulate in aging mother cells has provided new insights into the molecular mechanism of aging. It is proposed that a high number of ERCs may serve to titrate or hijack important factors necessary for replication or transcription, which ultimately limits lifespan (*74*). A similar model has been proposed to describe some of the biological effects of DNA adducts produced by an electrophilic derivative of benzo[a]pyrene; these adducts hijack the RNA polymerase II transcription factor Sp1 (*75*, *76*). These other examples of hijacking, taken together with our studies, underscore the fact that DNA damage or the aging process can cause possible disruptions in patterns of gene regulation. Exploitation of the ability of chemicals to cause such disruptions may be of practical application in the design of novel pharmaceutical agents.

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