Binding to Cisplatin-Modified DNA by the *Saccharomyces cerevisiae* HMGB Protein Nhp6A†

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ABSTRACT: Nhp6A is an abundant non-histone chromatin-associated protein in *Saccharomyces cerevisiae* that contains a minor groove DNA binding motif called the HMG box. In this report, we show that Nhp6Ap binds to cisplatin intrastrand cross-links on duplex DNA with a 40-fold greater affinity than to unmodified DNA with the same sequence. Nevertheless, Nhp6Ap bound to cisplatinated DNA readily exchanges onto unmodified DNA. Phenanthroline−copper footprinting and two-dimensional NMR on complexes of wild-type and mutant Nhp6Ap with DNA were employed to probe the mode of binding to the cisplatin lesion. Recognition of the cisplatin adduct requires a surface-exposed phenylalanine on Nhp6Ap that promotes bending of DNA by inserting into the helix from the minor groove. We propose that Nhp6Ap targets the cisplatin adduct by means of intercalation by the phenylalanine and that it can bind in either orientation with respect to the DNA lesion. A methionine, which also inserts between base pairs and functions in target selection on unmodified DNA, plays no apparent role in recognition of the cisplatin lesion. Basic amino acids within the N-terminal arm of Nhp6Ap are required for high-affinity binding to the cisplatin adduct as well as to unmodified DNA. Cisplatin mediates its cytotoxicity by forming covalent adducts on DNA, and we find that Δ*nhp6a/b* mutants are hypersensitive to cisplatin in comparison with the wild-type strain. In contrast, Δ*nhp6a/b* mutants are slightly more resistant to hydrogen peroxide and ultraviolet irradiation. Therefore, Nhp6A/Bp appears to directly or indirectly function in yeast to enhance cellular resistance to cisplatin.

The high-mobility group (HMG) proteins are a class of non-histone chromosomal proteins that were originally characterized by their highly charged nature and fast migration on denaturing protein gels. They are classified into three major families (HMGA, HMGB, and HMGN) with each containing a signature motif (*1*−*3*). HMGB proteins contain one or more copies of the 80-amino acid HMG box, which associates with DNA in a moderately sequence-specific or in a sequence-neutral manner (*1*, *4*, *5*). The HMG DNA binding domain consists of an extended peptide segment with three α-helices folded into an L-shaped structure (*5*, *6*). The concave surface of the folded domain binds to the minor groove of DNA, resulting in considerable bending and untwisting of the DNA helix (*7*−*9*). One or two surface-exposed hydrophobic residues within the minor groove binding interface are often inserted into the base pair stack to generate localized kinks. The Nhp6A protein from *Saccharomyces cerevisiae* contains one HMG box with hydrophobic residues Met 29 and Phe 48 that have been shown to modulate Nhp6Ap-induced bending of DNA (Figure 1) (*9*; J. E. Masse et al., manuscript in preparation). Nhp6Ap also contains an additional 16-amino acid segment at its N-terminus that is rich in basic residues and is essential for binding to linear DNA (*10*).

HMGB proteins are involved in a number of biological processes. The abundant non-sequence-specific HMG box protein, HMGB1, has been shown to stimulate RAG1/2 cleavage in V(D)J recombination (*11*−*13*) and to promote binding of sequence-specific transcription factors (*6*). For example, HMGB1 promotes the cooperative assembly of an "enhanceosome" complex involving viral-specific transactivators at the Epstein Bar virus lytic gene promoter (*14*). The sequence-specific class member, LEF1, functions both as a direct transcription activator and as an architectural factor at the T cell receptor α enhancer (*15*). Yeast Nhp6Ap has been shown to be required for proper regulated expression of a number of genes that are transcribed by RNA polymerase II or III (*16*−*20*). A hallmark of HMGB proteins is their preferential association with altered nucleic acid structures such as Holliday junctions (*21*, *22*), DNA bulges (*23*, *24*), DNA entry−exit points on nucleosomes (*25*), DNA microcircles (*10*, *26*), and cisplatin-modified DNA (*27*, *28*).

Cisplatin [*cis*-diamminedichloroplatinum(II)] is one of the most successful and widely used anticancer drugs (*29*).

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FIGURE 1: (A) Sequence alignment of *S. cerevisiae* Nhp6Ap, rat HMGB1 domain A, and domain B. The amino acids whose side chains have been shown (Nhp6Ap and HMGB1 domain A) or are implicated (HMGB1 domain B) in insertion into DNA upon binding are highlighted in bold (*52*; J. E. Masse et al., manuscript in preparation). The locations of the basic tail (residues 1−16 with the two basic patches underlined in the Nhp6A sequence) and extended N-terminal segment and three α-helices of the HMGB domain are denoted for Nhp6Ap (*9*). (B) NMR solution structure of Nhp6Ap bound to the SRY DNA recognition sequence (J. E. Masse et al., manuscript in preparation). The three α-helices and extended N-terminal segment, which form the core of the protein, are shown in orange. The side chains of methionine 29 and phenylalanine 48, which insert into the DNA helix from the minor groove, are shown in red. The N-terminal tail is shown in cyan with amino acids 8−10 and 13−16, which comprise the two basic patches, highlighted in blue.

Cisplatin exerts its cytotoxic activity by forming a covalent adduct between the N7 positions of two successive guanines or a guanine plus adenine within the major groove of DNA (*30*, *31*). This covalent modification results in a large bend toward the DNA major groove with an accompanying widening of the minor groove (*32*, *33*). A number of DNA binding proteins have been found to recognize this altered DNA structure in vitro (*34*, *35*). Some function in DNA repair, such as XPA, Msh2, DNA photolyase, and RPA. Others, such as the HMG box proteins, HMGB1, mtTFA, and tsHMG, have been shown to inhibit repair of the cisplatin adduct by the nucleotide excision repair (NER) machinery in vitro (*36*, *37*). By stably binding to the cisplatin-modified DNA, the HMGB proteins have been proposed to shield the lesion from the repair proteins (*38*, *39*).

The in vivo relationship between HMG box protein levels and cisplatin toxicity is equivocal. There are several reports which indicate that the presence of HMGB proteins sensitizes cells to cisplatin. For example, overexpression of HMGB1 induced by addition of steroid hormone in breast cancer cells was recently shown to correlate with higher cisplatin sensitivity, and clinical trials to evaluate the efficacy of the combined treatment are ongoing (*40*). On the other hand, many cancer cell lines that have developed resistance to cisplatin often have elevated levels of HMGB1 (*41*). Yeast mutants deficient in components of the nucleotide excision repair (NER) pathway are extremely sensitive to cisplatin, confirming that reactivity of DNA to cisplatin is the major mechanism of its cytotoxicity and that the dominant cisplatin lesion is primarily corrected by the NER pathway (*39*). Deletion of yeast *ixr1*, encoding a transcriptional repressor that contains two HMG boxes, results in 2−6-fold increased resistance to cisplatin depending on the strain background, supporting the idea that HMGB proteins sensitize cells to cisplatin (*38*, *39*). In contrast, an *Schizosaccharomyces pombe* mutant deficient in the HMGB protein Cmb1 is more sensitive to cisplatin treatment (*42*).

Several sequence-neutral HMGB proteins are present in the yeast nucleus, of which Nhp6Ap and its close paralog Nhp6Bp are probably the most abundant (*16*). The amino acid sequences of the HMG boxes of Nhp6Ap and Nhp6Bp are also the most similar to the two domains of mammalian HMGB1/2 (*43*). Therefore, we decided to explore the possible interactions between Nhp6Ap and cisplatin-modified DNA in vitro, and the relationship of these proteins to cisplatin cytotoxicity in vivo. We find that Nhp6Ap selectively binds to cisplatinated DNA and determine the structural features of the protein that are responsible for mediating this specificity using a combination of mutagenesis, gel mobility shift assays, footprinting, and NMR. In addition, we show that yeast mutants lacking Nhp6A/Bp are moderately hypersensitive to cisplatin treatment but display slightly enhanced resistance to other genotoxic agents.

MATERIALS AND METHODS

Yeast Strains and Plasmids. All *S. cerevisiae* strains used in this study were derived from SEY6210 (MATα *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ201 lys2-801 suc2-Δ9 gal3 mel*). RJY6009 (SEY6210 *nhp6A::URA3 nhp6B::LEU2*) (*16*) was transformed with the YCp plasmid pRS314 (*TRP1*) (*44*) and plasmids derived from pRS314 expressing Nhp6A wild-type, M29A, and F48A proteins from the *NHP6A* promoter to make strains RJY6569, RJY6567, RJY6565, and RJY6566, respectively (ref *10* and this work). Levels of Nhp6A proteins expressed by these strains were indistinguishable from that of the endogenous chromosomal copy in SEY6210 as determined by immunoblotting (ref *10* and this work).

Proteins. Wild-type and mutant Nhp6A proteins were purified as described previously (*10*). Nhp6A mutants M29A, F48A, Y28A, Δ(2−12), and Δ(2−16) were described in refs *9* and *10*, and Ala(13−16) was described in ref *45*. M29A, F48A, M29F, and F48M were constructed as described previously (*10*). HMGB domain A contains amino acids Met 1−Lys 88 (*46*).

Preparation of Cisplatinated DNA. DNA was modified with cisplatin essentially as described previously (*33*). Cisplatin (Sigma) was dissolved in dimethylformamide and activated by reacting with $AgNO_3$ at molar ratio of 1:2

overnight at room temperature. The 19-base oligonucleotide (200 μM) (Figure 2A) containing a single GG dinucleotide was reacted with 600 μM activated cisplatin for 6 h at 37 °C in the dark. The cisplatinated DNA was purified from unmodified DNA on 15% polyacrylamide−7 M urea gels. The modified strand was then 5′ end-labeled with ^{32}P by T4 polynucleotide kinase (New England Biolabs) with [γ-^{32}P]-ATP, chromatographed through a Sephadex G25 spin column, and annealed with an equal amount of complementary strand. The modified DNA duplex was then isolated using a native 20% polyacrylamide gel.

Gel Mobility Shift Assays. Gel mobility shift assays were performed essentially as described in ref *47* with some minor modifications. Reaction mixtures (20 μL) containing 10 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM spermidine, 0.2 mg/mL acetylated BSA (Promega), 0.05% NP40, and 10 pM unmodified or cisplatinated 19 bp ^{32}P end-labeled probe were incubated with increasing amounts of protein for 30 min at 30 °C. Free and bound species were resolved on a 15% polyacrylamide (59:1 acrylamide:bisacrylamide ratio) gel in 45 mM Tris-borate buffer (pH 8.3) at 15 mA for 2 h at 23 °C. Binding was quantitated using a phosphorimager and ImageQuant software (Molecular Dynamics, Inc.). K_d was calculated by plotting $\log[b/(1-b)]$ against log[P], where b is the fraction of labeled probe bound and [P] is the protein concentration, and the x-intercept was determined. K_d was then calculated according to the equation $K_d = 10^{x-\text{intercept}}$.

Binding of wild-type and the mutant Nhp6A proteins listed in Table 1 was also evaluated using our previous binding conditions [20 mM Hepes (pH 7.5), 40 mM NaCl, 1 mM EDTA, 0.1 mg/mL acetylated BSA, and 5% glycerol] (*9*, *10*, *48*). Under these conditions, Nhp6Ap bound to the cisplatinated and unmodified 19 bp probe with affinities of 20 and 174 nM, respectively. The mutants behaved in the same relative manner as reported in Table 1.

Phenanthroline−Copper Footprinting. Binding reactions (20 μL) were performed in 10 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 100 mM NaCl, 50 mM LiCl, 1 mM spermidine, 0.2 mg/mL acetylated BSA (Promega), and 0.05% NP40 (*47*) with 1 nM 5′ ^{32}P-labeled DNA probe and increasing concentrations of Nhp6Ap. The reaction mixtures were incubated on ice for 30 min followed by addition of 3 μL each of 1.25 mM 5-phenyl-1,10-phenanthroline (GFS Chemicals, Inc.) copper (4:1), 0.003% H_2O_2, and 100 mM ascorbic acid (Sigma). After 5 min at room temperature, the reaction was quenched by adding 4 μL of 28 mM neocuprine (Sigma), 3.5 μL of 3 M sodium acetate, 20 μg of tRNA, and 96 μL of H_2O. The samples were then extracted with phenol and chloroform (1:1), precipitated with ethanol, and resuspended in formamide loading buffer (*49*). Electrophoresis was through an 18% polyacrylamide−6 M urea gel at 50 W. The A + G formic acid reaction (*49*) was performed on the same DNA probes and electrophoresed in parallel to align the sequence.

NMR Sample Preparation and Spectroscopy. The DNA oligonucleotides dCTGAATGGATACCGC (top strand) and dGCGGTATCCATTCAG (bottom strand) were synthesized using standard phosphoramidite chemistry. Unmodified DNA oligonucleotides were purified either by column chromatography on a Sephadex G50 column eluted with water as described previously (*9*) (trityl off) or by HPLC using a

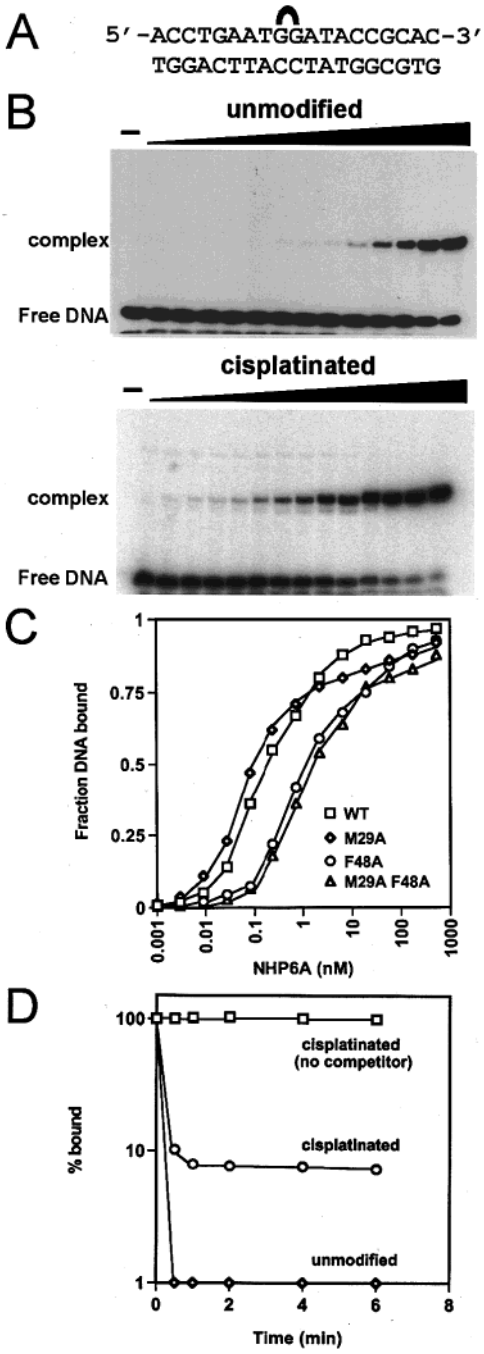

FIGURE 2: (A) DNA sequence of the 19 bp oligonucleotide used as a probe for the DNA binding studies. The top strand contains a single pair of guanines, which were cross-linked at their N7 positions by cisplatin. (B) Preferential binding of Nhp6Ap to cisplatin-modified DNA over unmodified DNA. The 19 bp DNA probe was 5′ end-labeled with ^{32}P on the modified strand (top) and incubated with increasing amounts of Nhp6Ap in 2-fold increments from 0.001 to 5 nM. Nhp6Ap−DNA complexes were separated from free DNA by native gel electrophoresis. (C) DNA binding isotherms for binding of wild-type and mutant Nhp6A proteins to the cisplatin-modified 19mer. (D) Stability of Nhp6Ap complexes with cisplatin-modified and unmodified DNA. Nhp6Ap was incubated with the cisplatinated or unmodified ^{32}P-labeled 19mer (30 pM) for 30 min at 23 °C. Sonicated salmon sperm DNA (2.5 μg/mL) was then added, and aliquots of the binding mix were applied to a polyacrylamide gel immediately before and various times after addition of competitor DNA. The percentage of labeled probe bound by Nhp6Ap immediately before addition of competitor DNA was set at 100%, and the percentage of complexes remaining at different times after competitor addition was calculated accordingly.

Table 1: Binding of Wild-Type and Mutant Nhp6Ap to Cisplatin-Modified DNA

protein	inserting residue(s)[a]	K_d(unmod)[b]	K_d(cispl)[b]	K_d(unmod)/ K_d(cispl)[b]
Nhp6Ap (WT)	M F	3.9 ± 1.2	0.10 ± 0.04	39.0
M29A	A F	3.0 ± 1.3	0.085 ± 0.02	35.3
F48A	M A	5.2 ± 0.8	1.5 ± 0.08	3.5
M29A/F48A	A A	4.7 ± 3.3	0.91 ± 0.2	5.2
M29F	F F	2.9 ± 1.3	0.048 ± 0.008	60.4
F48M	M M	3.9 ± 0.5	0.43 ± 0.09	9.1
Y28A	M F	27.6 ± 4.0	1.8 ± 1.1	14.8
Δ(2−12)	M F	15.1 ± 4.1	0.16 ± 0.06	93.4
Δ(2−16)	M F	400000	15000	26.7
Ala(13−16)	M F	10000	1.3 ± 0.6	8000
HMGB1 domain A	A F	2000	1.7 ± 0.06	1000
Hmo1p	V L F[c]	499 ± 84	169 ± 67	3

[a] Amino acids present at positions 29 and 48 of Nhp6Ap, respectively, or at the equivalent positions in the case of HMGB1 domain A and Hmo1p. [b] K_d values (nanomolar) were determined by gel mobility shift assays using the unmodified (unmod) or cisplatinated (cispl) 19mer in Figure 2A. [c] The second HMG box of Hmo1p contains only one potential inserting residue, which is located at the position equivalent to Met 29 of Nhp6Ap.

Hamilton PRP1 column eluted with an acetonitrile gradient (from 0 to 25%) (trityl on). Activated cisplatin [$Pt(HN_3)_2(H_2O)_2^{2+}$] was prepared as described above and reacted at a 4:1 molar ratio with the purified top strand (1 mM) overnight at room temperature in the dark. The singly cross-linked product was separated by electrophoresis on preparative 19% denaturing polyacrylamide gels and purified by anion exchange (50% DEAE-Sepharose, 50% DEAE-Sephacel) followed by size-exclusion chromatography on a Sephadex G25 column. The DNA duplexes were prepared by titration of the bottom strand into the top strand until a 1:1 ratio was reached as assayed by one-dimensional (1D) and TOCSY NMR spectroscopy of the samples in D_2O. NMR spectra were acquired at 500 or 600 MHz on Bruker DRX spectrometers. Uniformly ^{15}N-labeled Nhp6A and mutant proteins were prepared as described previously (*9*). Complexes of wild-type Nhp6A and mutant F48A and M29A proteins with DNA were prepared by titrating ^{15}N-labeled proteins into the DNA duplexes and monitoring complex formation by NMR as previously described (*9*). After titration, samples were lyophilized, resuspended, and dialyzed into 10 mM NaCl and 2 mM $NaPO_4$ (pH 5.8) so that the buffer conditions of all samples were identical. Final sample concentrations were ~0.15 mM in complex. $^1H-^{15}N$ HSQC spectra along with 1D spectra were used to analyze the differences in the wild-type and mutant Nhp6A protein binding to the cisplatin DNA and to unmodified DNA. Assignments of cross-peaks in the $^1H-^{15}N$ HSQC spectrum of Nhp6Ap bound to unmodified DNA were obtained by comparison with the assignments of the Nhp6Ap complex with SRY DNA (*9*) since the protein exhibited only small chemical shift changes in the complexes formed with these two different DNA sequences.

Sensitivity of Yeast Cells to Cisplatin, H_2O_2, UV, and Methylmethanesulfonate. The cisplatin sensitivity assay was based on the method used by Lippard and co-workers (*39*). RJY6009 (Δ*nhp6a/b*) containing *TRP1* plasmids expressing wild-type and mutant Nhp6A proteins were grown in SD without tryptophan (*50*), subcultured into YPD, and grown until saturated. The cells were then collected and resuspended in 1 mL of 0.67% yeast nitrogen base (YNB) at an OD_{600} of 0.4. Cisplatin (Sigma) was added to a final concentration of 0, 0.2, 0.4, and 0.8 mM, and incubation was continued for 2 h at 30 °C. The cells were then serially diluted and plated onto SD plates without tryptophan, and colony-forming units were determined after 2−3 days at 30 °C. Sensitivity to cisplatin treatment was also measured in SEY6210 (*NHP6A NHP6B*) and RJY6009 (SEY6210 *nhp6A::URA3 nhp6B::LEU2*). The increased sensitivity of RJY6009 compared with SEY6210 paralleled the difference between RJY6569 (Δ*nhp6a/b* pRS314) and RJY6567 (Δ*nhp6a/b* pRS314-*NHP6A*) reported in Figure 5.

To measure sensitivity to treatment with H_2O_2, SEY6210 and RJY6009 were grown in YPD to an OD_{600} of 0.6−1.0. The cells were collected, resuspended in 1 mL of YNB at an OD_{600} of 0.4, and incubated with 0, 5, 10, or 20 mM H_2O_2 for 1 h at 30 °C. Dilutions were plated onto YPD plates and colony-forming units measured after growth for 2−3 days at 30 °C. Sensitivity to treatment with methylmethanesulfonate (MMS) was measured similarly except that cells in YNB media were incubated with 0, 0.02, 0.04, or 0.08% (v/v) MMS for 30 min at 30 °C and then plated. To measure UV sensitivity, exponentially growing cells were diluted and plated on YPD. The plates were irradiated with short-wave UV light at 6 J s^{-1} m^{-2} for 0, 30, 60, and 90 s. Survival was evaluated after incubation at 30 °C for 2−3 days.

RESULTS

Preferential Binding of Nhp6Ap to Cisplatin-Cross-Linked DNA. The properties of Nhp6Ap binding to DNA cross-linked with cisplatin were initially evaluated using gel mobility shift assays. A 19mer duplex that contained two centrally located guanines in the top strand was used as a probe (Figure 2A). To generate the cisplatinated probe, the top strand was reacted with cisplatin and the single cross-linked oligonucleotide was purified by gel electrophoresis prior to annealing with the bottom strand. The affinity of Nhp6Ap for DNA varies with solution conditions, but under the conditions used in Figure 2 and Table 1 (see Materials and Methods), Nhp6Ap formed a single complex on the unmodified duplex with an apparent K_d of 3.9 nM. When the 19mer duplex contained the cisplatin-modified strand, its affinity for Nhp6Ap increased approximately 40-fold (Figure 2B and Table 1). The constant for binding of Nhp6Ap to the cisplatinated 19 bp fragment is 0.1 nM, which is 17 times greater than that measured for HMGB1 domain A under the same conditions (see also ref *47* and Table 1).

Nhp6Ap forms dynamic complexes on B-DNA that readily exchange with other DNA segments. In the following experiment, we show that complexes formed on cisplatinated DNA are similarly dynamic. Nhp6Ap was prebound to 30 pM ^{32}P-labeled cisplatinated or unmodified 19 bp duplexes, and salmon sperm DNA was then added as a competitor. Complexes remaining on the labeled probe at various times after addition of competitor DNA were assessed by gel electrophoresis. As shown in Figure 2D, essentially all of the complexes which were initially formed between Nhp6Ap and unmodified DNA dissociated within 30 s after addition of 2.5 μg/mL salmon sperm DNA. Surprisingly, complexes formed between Nhp6Ap and cisplatinated DNA also exchanged rapidly with competitor DNA. At equilibrium,

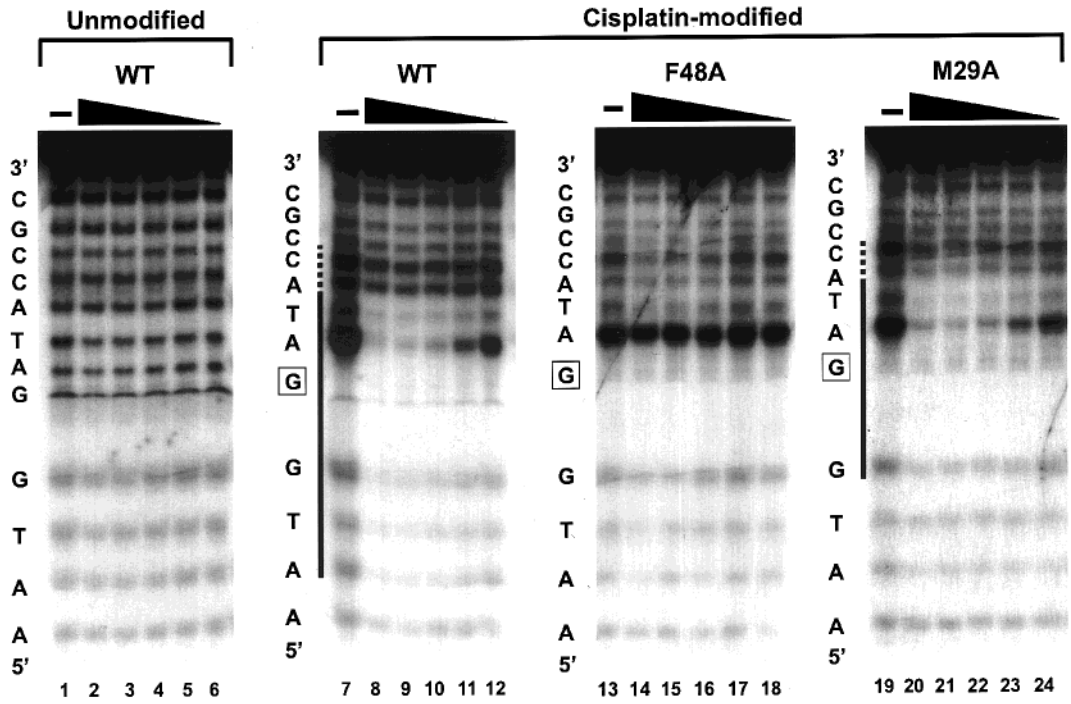

FIGURE 3: Footprinting using 5-phenyl-1,10-phenanthroline−copper on the Nhp6Ap−cisplatin-modified DNA complexes. The unmodified (lanes 1−6) or cisplatin-modified (lanes 7−24) ^{32}P-labeled 19 bp DNA in Figure 2A (top strand) was used as the probe in a binding reaction with wild-type Nhp6Ap, F48A, and M29A, as designated. Nhp6Ap was added at 4-fold increasing concentrations from 8 nM to 2 μM (right to left). No Nhp6Ap was added to the lane marked −. Strong and weak protections are indicated on the DNA sequence at the left of each panel by solid and dashed lines, respectively. The cisplatin-cross-linked guanine that displays a low level of scission by the phenanthroline−copper is boxed.

approximately 8% of the starting level of complexes was bound to the cisplatinated DNA. As expected, the relative proportion of complexes bound to cisplatinated versus unmodified competitor DNA at equilibrium depended on the concentration of competitor DNA added (data not shown).

Localization of Nhp6Ap Binding to Cisplatinated DNA by Phenanthroline−Copper Footprinting. Phenanthroline−copper footprinting was used to examine Nhp6Ap binding to cisplatin-modified DNA because this reagent specifically probes the minor groove where Nhp6Ap predominantly associates. Furthermore, the efficiency of DNA scission by phenanthroline−copper often has been found to be sensitive to perturbations in DNA structure (*51*). As shown in lane 7 of Figure 3, 5-phenyl-1,10-phenanthroline−copper cleavage of the cisplatin-modified strand of the 19 bp oligonucleotide resulted in a strong hypersensitive site at the adenine nucleotide 3′ to the cross-linked guanines when compared with the unmodified strand (lane 1). In addition, cleavage products corresponding to the 3′ guanine nucleotide are strongly reduced. Addition of Nhp6Ap resulted in strong protection of a six-base region centered over the cross-linked guanines with weaker protection extending over an additional one to two nucleotides in the 3′ direction (lanes 8−12). No evidence of specific binding was observed over this range of added Nhp6Ap with the unmodified DNA (lanes 2−6). Phenanthroline−copper cleavage of the unmodified strand of the cisplatinated duplex did not display altered cleavage sensitivities, and the protection by Nhp6Ap was much less pronounced (data not shown). As discussed further below, the protection pattern extending on both sides of the cisplatin adduct on the top strand may reflect binding of Nhp6Ap in either orientation about the cisplatin adduct.

NMR Analysis of the Binding of Nhp6Ap on Unmodified versus Cisplatinated DNA. To gain further insight into the mode of binding of Nhp6Ap on cisplatinated versus unmodified DNA, the complexes were also studied by NMR spectroscopy. The 15 bp DNA sequence was identical to the 19 bp duplex used for the footprinting except that the terminal two base pairs on each end were not included. Formation of Nhp6Ap−DNA complexes was initially assayed by monitoring changes in the DNA imino proton resonances and the exchangeable Trp 59 Hϵ resonance (downfield shift from 10.04 ppm) in spectra acquired on the sample in H_2O (data not shown). Titration of Nhp6Ap with unmodified DNA resulted in the appearance of a single Trp resonance at 10.53 ppm. In contrast, two Trp resonances at 10.73 and 10.54 ppm appeared for the complex formed with cisplatinated DNA (see Figure 4A). The chemical shifts of the protein resonances in the ^{1}H−^{15}N HSQC spectrum of ^{15}N-labeled Nhp6Ap bound to the unmodified DNA are very similar to those observed for the complex of Nhp6Ap with SRY DNA (*9*), consistent with binding of the protein at a single site on the DNA. Comparison of this spectrum with that obtained for ^{15}N-labeled Nhp6Ap in complex with cisplatin DNA shows that while overall the spectra are similar, there are significant differences (Figure 4A). Several of the resonances, in addition to Trp 59, are split into two different peaks, indicating that Nhp6Ap is forming two different complexes on the cisplatinated DNA. Furthermore, many of the cross-peaks, including the backbone amides of Thr 12, Ala 24, Ala 27, Trp 59, Phe 30, Arg 40, Ile 46, Val 51, and Lys 53, and the side chain amide of Asn 43, have chemical shifts significantly different from those found with the unmodified DNA. This indicates that both of the binding

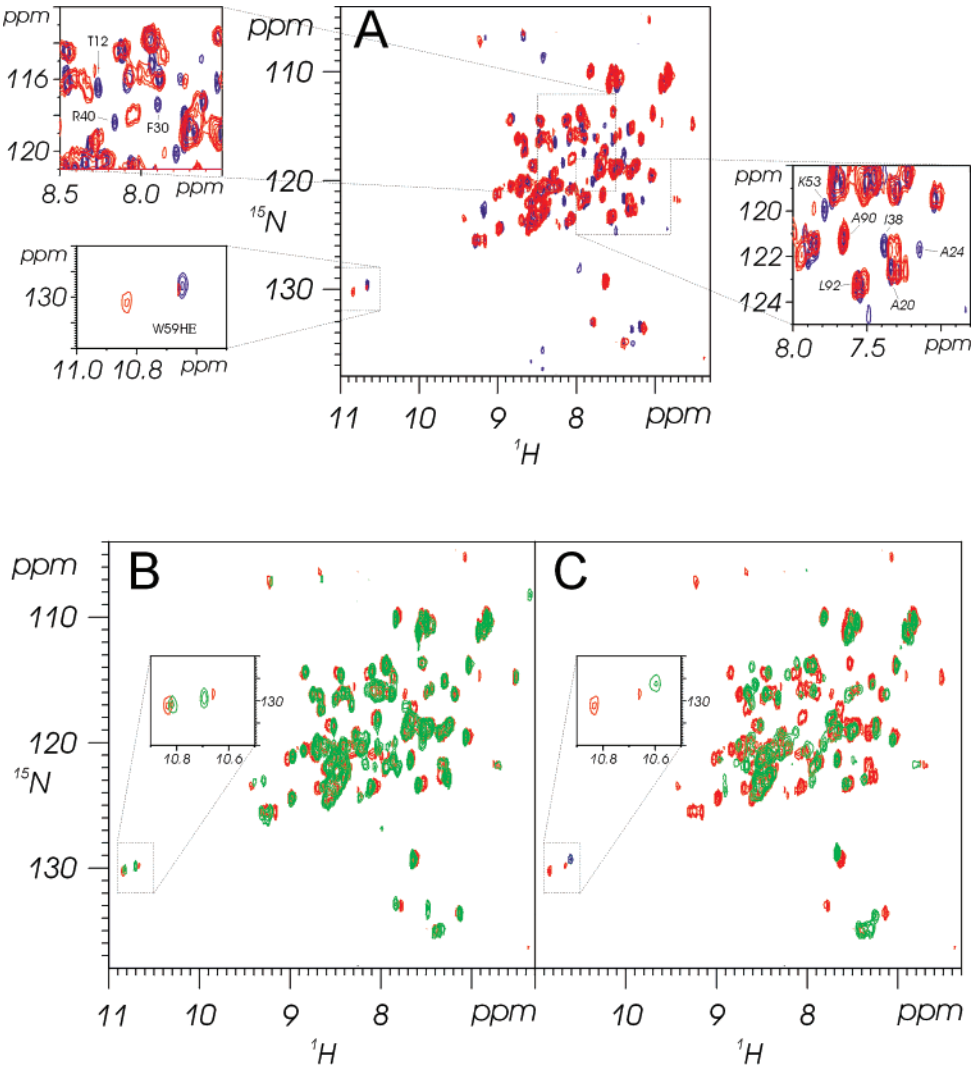

FIGURE 4: ^{1}H−^{15}N HSQC spectra of 1:1 complexes of the ^{15}N-labeled wild-type or mutant Nhp6Ap bound to unmodified or cisplatinated duplex DNA (dCTGAATGGATACCGC) at 37 °C. The cisplatination site is the two central guanines in the DNA sequence. (A) Overlay of Nhp6Ap with unmodified (blue) and cisplatinated (red) DNA. Boxed regions are expanded to show differences in the spectra of the two complexes. Note that many of the resonances, including W59 Hϵ, are doubled in the complex with cisplatinated DNA. (B) Overlay of spectra of Nhp6Ap mutant M29A (green) and the wild-type (red) bound to cisplatinated DNA. (C) Overlay of spectra of Nhp6Ap mutant F48A (green) and the wild-type (red) bound to cisplatinated DNA.

sites on the cisplatin DNA are different from the single binding site on the unmodified DNA. The NMR data indicating two cisplatin-specific complexes, together with the protection from phenanthroline−copper cleavage extending on both sides of the cross-linked guanines, suggest that Nhp6Ap can bind to the cisplatin adduct in either orientation.

Effects of Phe 48 and Met 29 Mutations on Binding of Nhp6Ap to Cisplatin DNA. The solution structure of Nhp6Ap bound to DNA revealed that Met 29 and Phe 48 are inserted into base pair steps within the minor groove interface (*9*; J. E. Masse et al., manuscript in preparation; Figure 1). Both of these residues strongly influence Nhp6Ap-induced bending of DNA and co-activation of transcription (*9*, *10*; B. Wong and R. C. Johnson, unpublished results). In HMGB1 domain A, Phe 37, which is analogous to Phe 48 in Nhp6Ap (see Figure 1A), is inserted between the cisplatin-cross-linked guanines and has been shown to be critical for specific binding to cisplatinated DNA (*52*). To evaluate the relative roles of Phe 48 and Met 29 in targeting of Nhp6Ap to cisplatinated DNA, we examined the effects of amino acid substitutions at these positions by gel mobility shift assays, phenanthroline−copper footprinting, and NMR. As described below, we conclude that Phe 48 performs a critical role in the specific targeting of Nhp6Ap to the cisplatin adduct. Met 29 has no detectable role in selective binding to cisplatinated DNA, although it appears to influence the orientation of the bound Nhp6Ap under some conditions.

(a) Gel Mobility Shift Assay and Phenanthroline−Copper Footprinting. An Nhp6A mutant protein in which Phe 48 was substituted with alanine (F48A) lost nearly all specificity for binding to cisplatinated versus unmodified DNA, even though its affinity for the unmodified 19mer was essentially unchanged from that of the wild type (Figure 2C and Table 1). Moreover, addition of F48A to cisplatinated DNA yielded no significant protection from phenanthroline−copper cleavage (Figure 3, lanes 13−18), confirming the lack of specific binding over the cisplatin adduct. On the other hand, Nhp6Ap M29A bound to the cisplatinated DNA with an affinity that was very similar to that of the wild type (Figure 2C and Table 1). M29A generated strong protection from phenanthroline−copper cleavage extending from the cross-linked guanines to three to four bases on the 3′ side (Figure 3, lanes

19−24). However, unlike the wild-type pattern, nucleotides to the 5′ side of the cross-linked guanines (A7 and T8) showed little protection, suggesting that M29A is predominantly binding asymmetrically over the 3′ side of the cisplatin adduct.

The binding properties of additional mutants containing changes at residues 29 and 48 were also investigated (Table 1). A double mutant containing alanine substitutions at both residues 29 and 48 behaved like F48A; it exhibited no enhanced affinity for cisplatinated DNA versus unmodified DNA. A phenylalanine in place of methionine at position 29 bound slightly better to both the unmodified and cisplatinated duplexes, but the discrimination for cisplatinated DNA was similar to that of the wild type. These results provide evidence that position 29 does not play a significant role in selective binding to the cisplatin adduct. A mutant containing a methionine in place of phenylalanine at position 48 retained some enhanced binding to the cisplatinated DNA, but its binding affinity was 5-fold lower than that of the wild type.

(b) Effects of M29A and F48A Mutations on NMR Spectra of Complexes Formed with Cisplatin DNA. The binding of Nhp6Ap M29A and F48A to cisplatinated and unmodified DNA was also investigated by NMR. We find that Met 29 is involved in binding site selection on the unmodified DNA sequence used here and on the SRY DNA sequence (J. E. Masse et al., manuscript in preparation) since comparison of the $^{1}H-^{15}N$ HSQC spectra of M29A and wild-type Nhp6Ap on unmodified DNA shows significant differences. In contrast, the $^{1}H-^{15}N$ HSQC spectra of M29A and wild-type Nhp6Ap on cisplatinated DNA are very similar (Figure 4B). There appear to be two binding sites for both proteins on the DNA. Most of the resonances that are doubled in the spectrum of the complex with wild-type Nhp6Ap are also doubled in the spectrum with the M29A mutant, although a few doubled cross-peaks are converged to a single peak in the complex with M29A. This may be due to a small difference in the exchange rate between the two binding sites on the DNA for M29A versus wild-type Nhp6Ap. It also appears that one of the two binding sites is somewhat more populated in the complexes with M29A than with wild-type Nhp6Ap (e.g., compare the relative intensities of the Trp 59 cross-peaks). Taken together, these observations are consistent with the hypothesis that Met 29 is not important for recognition and binding at a cisplatin lesion, even though it does influence binding site selection on unmodified DNA.

NMR spectra indicate that substitution of Phe 48 with Ala has a large effect on the binding of Nhp6Ap to cisplatinated DNA (Figure 4C). In contrast to wild-type Nhp6Ap, the F48A mutant protein appears to bind at only one site on the modified DNA. For example, only a single resonance is observed for Trp 59 Hϵ. This site does not appear to be the same as either of the wild-type sites. Mutation of Phe 48 to Ala also appears to affect the rate of exchange of the protein with unmodified DNA, moving it into the intermediate exchange regime and resulting in a significant line broadening of the imino proton resonances of the DNA. This effect is also seen in the complexes of F48A with cisplatinated DNA, although to a lesser extent (data not shown). These results provide support for the hypothesis that Phe 48 is an important determinant for DNA binding, and is directly involved in the recognition of the cisplatin lesion.

Role of the N-Terminal Segment of Nhp6Ap. Previous studies have pointed to the importance of the basic N-terminus of Nhp6Ap for high-affinity binding and biological activity (*9*, *10*, *45*). This 16-amino acid region, which is disordered in solution, associates with the major groove of DNA within an Nhp6Ap−DNA complex (*9*; J. E. Masse et al., manuscript in preparation). As described below, the N-terminal segment has little, if any, role in selective binding to cisplatinated DNA, although it does strongly enhance overall binding affinity.

Removal of the entire 16-amino acid region [$\Delta(2-16)$] results in a minimal HMG box that displays extremely poor binding, but retains specificity for the cisplatinated 19mer (Table 1). The N-terminal segment contains two patches of basic residues ($K_8K_9R_{10}$ and $R_{13}K_{14}K_{15}K_{16}$), of which the latter has been shown to be the most important for Nhp6Ap binding to linear DNA (*10*, *45*). When all four residues in the second basic patch are changed to alanines, the mutant Nhp6Ap Ala(13−16) exhibited very weak binding to the unmodified 19mer but much stronger binding to cisplatinated DNA (Table 1). Because the binding to unmodified DNA is so poor, the 8000-fold discrimination of cisplatinated DNA by Ala(13−16) is much greater than that of the wild type. A mutant deleted for the first basic patch, Nhp6Ap $\Delta(2-12)$, displayed a moderately reduced affinity for the unmodified 19mer but an affinity for the cisplatinated DNA similar to that of the wild type.

Sensitivity of nhp6a/b Mutants to Cisplatin and Other DNA-Damaging Agents. Previous work has suggested that high-affinity binding of HMGB proteins to cisplatin-modified DNA results in "shielding" of the damaged DNA from the cellular repair machinery. For example, overproduction of HMGB1 in breast cancer cells resulted in enhanced sensitivity to cisplatin, and a deletion of the *S. cerevisiae* HMGB protein Ixr1p resulted in increased resistance of yeast cells to cisplatin (*38*, *40*). Therefore, we were interested in determining how yeast mutants deleted for *nhp6a* and *nhp6b*, encoding the major HMGB proteins in the nucleus, would respond to treatment with cisplatin. RJY6569 (Δ*nhp6a/b*) and RJY6567 (*NHP6A*) were incubated for 2 h at 30 °C with different concentrations of cisplatin, and the survival efficiency was determined. As shown in Figure 5A, the Δ*nhp6a/b* mutant was more sensitive to the cisplatin treatment than the otherwise isogenic *NHP6A* strain. Yeast cells expressing Nhp6Ap F48A displayed a sensitivity that was intermediate between those of the Δ*nhp6a/b* and *NHP6A* cells. On the other hand, the sensitivity of cells expressing the Nhp6Ap M29A mutant was indistinguishable from that of *NHP6A* cells (Figure 5B). As a control, *IXR1* was inactivated in the same yeast background, and the resulting strain was tested for cisplatin sensitivity. Consistent with previous reports (*38*), the *ixr1* mutant yeast exhibited approximately 2-fold enhanced resistance to cisplatin treatment (data not shown).

Δ*nhp6a/b* mutants grow slower than *NHP6A* yeast and may be generally more sensitive to stress conditions. To address whether the increased sensitivity to cisplatin-mediated DNA damage reflected a general property of the mutants, sensitivities to other DNA-damaging agents were measured. As shown in panels C and D of Figure 5, Δ*nhp6a/b* mutants were slightly more resistant to incubation with H_2O_2 and UV radiation than the *NHP6A/B* control.

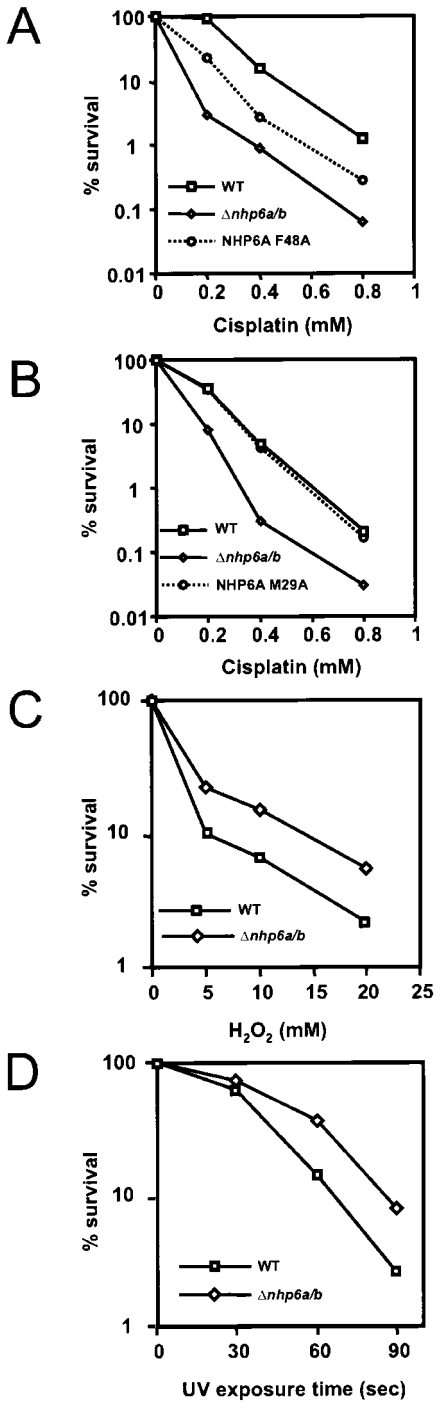

FIGURE 5: In vivo sensitivity of Nhp6A wild-type and mutant yeast strains to cisplatin and other genotoxic agents. (A and B) Cisplatin sensitivity. Δ*nhp6a/b* yeast strains containing plasmids expressing Nhp6Ap-WT (RJY6567), Nhp6Ap-F48A (RJY6566), Nhp6Ap-M29A (RJY6565), or no Nhp6Ap (RJY6569) were incubated with increasing amounts of cisplatin for 2 h in YNB at 30 °C. Cells were then plated onto SD media, and the number of colonies was scored after 2−3 days at 30 °C. Survival was calculated relative to the number of colony-forming units obtained upon incubation without cisplatin, which was set at 100%. (C) Hydrogen peroxide sensitivity. SEY6210 (WT) and RJY6009 (Δ*nhp6a/b*) were incubated in YNB for 1 h at 30 °C with increasing amounts of H_2O_2. The treated cells were plated onto YPD media, and survival was calculated as described above. (D) UV sensitivity. Cultures of SEY6210 (WT) and RJY6009 (Δ*nhp6a/b*) were diluted and plated onto YPD media. Plates were exposed to short-wave UV light (6 J s^{-1} m^{-2}) for different amounts of time, and the number of colony-forming units was determined after incubation for 2−3 days at 30 °C.

Similarly enhanced resistance to methylmethanesulfonate by the Δ*nhp6a/b* mutants was also observed (data not shown). The reason(s) for the slightly enhanced resistance to these different genotoxic agents is not known, but it is possible that the repair machinery is slightly induced in Δ*nhp6a/b* mutants. Nevertheless, these results suggest that the mutants are not generally compromised in the repair of DNA damage.

DISCUSSION

We find that Nhp6Ap binds with high affinity to DNA containing a cisplatin intrastrand cross-link between two guanines over unmodified DNA of the same sequence. Under the binding conditions described by Lippard and co-workers, Nhp6Ap binds to a cisplatinated 19 bp fragment with an affinity of 0.1 nM, which is 17 times greater than the affinity of HMGB1 domain A, the more selective of the two HMG DNA binding domains present in HMGB1 (*47*, *53*). The 40-fold discrimination for modified over unmodified 19 bp DNA displayed by Nhp6Ap is less than the value of 1200-fold measured for HMGB1 domain A (Table 1 and refs *47* and *53*). However, this difference largely reflects the 500-fold stronger binding of Nhp6Ap to unmodified DNA. Whereas preferential binding to cisplatinated DNA is a characteristic of many HMGB proteins (*31*, *35*), not all family members share this property. For example, the yeast HMGB proteins Hmo1p (Table 1) and Rox1p (*54*) do not preferentially bind to cisplatinated DNA.

Even though Nhp6Ap binds to cisplatinated DNA with a low apparent K_d, the complexes are dynamic, and Nhp6Ap can rapidly exchange onto excess unmodified DNA (Figure 2D). Parallel experiments with HMGB1 domain A gave similar results with domain A being rapidly released from prebound cisplatinated DNA complexes (data not shown). The unstable nature of the cisplatinated DNA complexes may have important ramifications regarding potential mechanisms by which HMGB proteins may modulate sensitivity of cells to cisplatin treatment.

Binding to Cisplatinated DNA Is Targeted by Phe 48 and Stabilized by the N-Terminus. Nhp6Ap has two hydrophobic residues, Met 29 and Phe 48, that insert into the minor groove of DNA upon binding (*9*; J. E. Masse et al., manuscript in preparation; Figure 1). Both of these residues introduce positive roll at the base pair step where they insert, and loss of either residue reduces the extent of DNA bending as measured by microcircle formation (*9*, *10*). It seemed possible that either or both of these residues could function to target Nhp6Ap to the cisplatin adduct, which creates a similar distortion in DNA (*32*). However, on the basis of the results presented here, Phe 48 appears to be exclusively responsible. A similar conclusion has recently been reached by Lippard and co-workers in studies of HMGB1 domain A (*47*). In domain A, which does not have a long chain aliphatic residue at the position equivalent to Met 29 (residue 16 is an alanine), the residue equivalent to Phe 48 (Phe 37) is inserted between the cross-linked guanines in a crystal structure with cisplatinated DNA, and a F37A substitution abolishes binding in solution (*52*). In the F37A background, introduction of a phenylalanine at position 16 enabled very weak binding to cisplatinated DNA. We did not observe an effect of Met 29 on Nhp6Ap targeting to cisplatinated DNA in the presence or absence of Phe 48. In addition, Nhp6Ap

Y28A selectively bound to cisplatinated DNA, although its affinity for both unmodified and modified DNA is reduced over that of the wild type (Table 1). Tyr 28 functions together with Met 29 to form a "hydrophobic wedge" that modulates Nhp6Ap-induced bending (*9*). Interestingly, a mutant containing a phenylalanine at position 29 together with the native Phe 48 resulted in an ~2-fold enhanced affinity for both cisplatinated and unmodified DNA, whereas the analogous mutant of domain A displayed a reduced level of binding. HMGB1 domain B normally has a phenylalanine at position 16 and an isoleucine at position 37. Loss of Phe 16 in an otherwise wild-type domain B reduces the affinity of binding 3-fold but in an I37A background abolishes binding to cisplatinated DNA (*47*). Thus, unlike in Nhp6Ap, a significant role of both hydrophobic residues is implicated in binding to cisplatinated DNA by domain B.

The prominent role of Phe 48 in anchoring Nhp6Ap to the cisplatin cross-link suggests a mode of binding that is analogous to that of the HMGB1 domain A−cisplatinated DNA crystal structure (*52*). However, domain A is positioned asymmetrically over the cisplatin adduct such that the protein-associated DNA extends to the 3′ direction with respect to the modified strand. This asymmetric binding mode was confirmed by solution footprinting studies (*52*). In contrast, NMR results indicate that Nhp6Ap apparently forms two complexes unique to the cisplatinated DNA, and the phenanthroline−copper protection studies show that Nhp6Ap generates a nearly symmetric footprint over the cross-linked guanines. To account for these results, we propose that Nhp6Ap specifically targets the cisplatinated DNA by insertion of Phe 48 between the cross-linked guanines and that it can bind in either orientation. Models of the two proposed modes of binding of Nhp6Ap to cisplatinated DNA are shown in Figure 6.

Interestingly, when the Met 29 side chain is absent on Nhp6Ap, the solution footprinting experiments indicated a preference for binding on the 3′ side of the adduct, as observed for HMGB1 domain A (*47*, *52*). Under the high protein−DNA concentrations used for NMR, both complexes appeared to be present, although one form may be over-represented relative to the wild type (see Figure 4B). The reason the presence of Met 29 appears to augment binding in one orientation within the DNA duplex is unknown. However, we note that NMR data indicate that this residue plays a role in target selection in the absence of a static bend (*9*; J. E. Masse et al., manuscript in preparation).

The N-terminal basic arm is essential for the formation of a complex on cisplatinated DNA since mutant Δ(2−16) missing this region displays nearly undetectable binding. Within this region, the second basic patch between residues 13 and 16 is clearly sufficient since Δ(2−12) binds with high affinity. The importance of residues 13−16 is highlighted by the Ala(13−16) mutant where each of the four basic residues was converted to alanine. This mutant binds with a 13-fold decrease in affinity relative to that of the wild type, but its 1 nM binding constant is at least 10 000 times lower than that measured with Δ(2−16). These results suggest that the first basic patch that includes Lys 8, Lys 9, and Arg 10 can also function to stabilize the complex. HMGB1 domain A contains several lysines at its N- and C-termini, but these do not interact with DNA in the X-ray structure of the cisplatin complex (*52*). On the other hand,

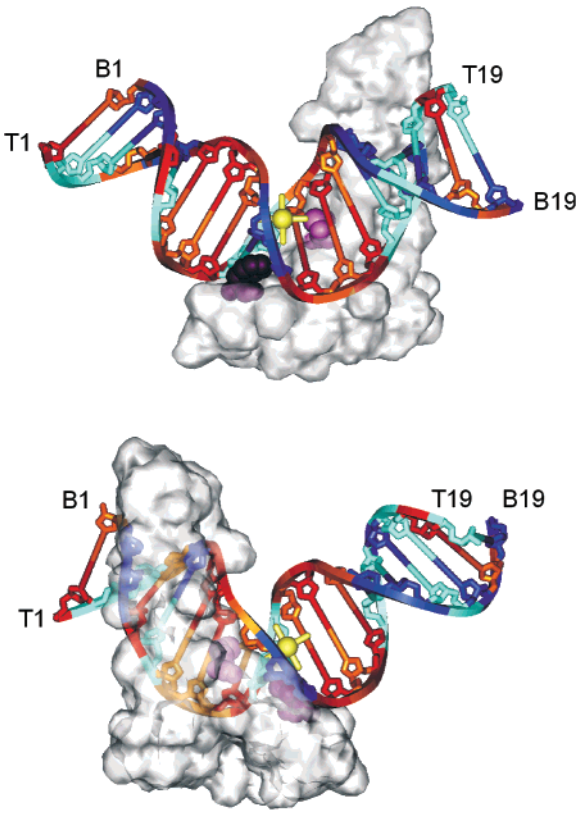

FIGURE 6: Schematic representation of two modes of binding by Nhp6Ap to cisplatin-modified DNA. A surface representation of Nhp6Ap is shown bound in either orientation to the cisplatin-modified 19 bp DNA sequence in Figure 2A, with Phe 48 inserted between the cross-linked guanines. The protruding side chains of Phe 48 and Met 29 are shown in purple and pink, respectively, and the cisplatin moiety is in yellow. The DNA bases (top strand, T1−T19; bottom strand, B1−B19) are color-coded: adenine in red, thymine in gold, guanine in blue, and cytosine in cyan. The basic N-terminal tail is not displayed for clarity. These models were generated using Insight II software (Accelrys, Inc.) and are based on the Nhp6Ap−15 bp DNA^{sry} structure (J. E. Masse et al., manuscript in preparation) except that B-DNA extensions were added to the ends, and the bases are substituted with those of the 19 bp oligonucleotide used in this study.

mutational analysis has shown that the basic region within the linker connecting domains A and B enhances binding of domain B to cisplatinated DNA (*55*).

Our findings with Nhp6Ap, together with those of HMG-D and HMGB1 domains A and B, indicate that a bulky hydrophobic residue at the beginning of helix 2 is a critical determinant for recognition of distorted DNA (*6*, *56*). The precise mechanism by which HMGB proteins target binding to the distortion, however, may depend on the particular lesion. Cerdan et al. have recently proposed an NMR-based model for HMG-D binding to a distorted DNA segment containing a two-base bulge (*24*). A set of intermolecular NOEs localized Thr 33, which is equivalent to Nhp6A Phe 48, to a base pair step to the 5′ side of the AA bulge, not the lesion itself.

Loss of Nhp6A/Bp Sensitizes Yeast to Cisplatin Treatment. Several proteins, including histone H1, TBP, Msh2p, Ku70/80, RPA, and other HMGB proteins such as Ixr1p, selectively bind to cisplatinated DNA (*34*, *35*). Given the abundance of Nhp6A/Bp in the nucleus and its affinity for cisplatin-cross-linked DNA in vitro, it seems likely that it may be the predominant nuclear protein recognizing cisplatin-damaged

DNA in yeast cells. In mammalian cells, the dominant HMGB protein, HMGB1, has been proposed to bind sufficiently tightly to cisplatin-modified DNA to block repair by the nucleotide excision repair pathway, the primary mechanism by which cisplatin adducts are removed (*39*, *40*). However, our in vitro results indicate that HMGB1 domain A and Nhp6Ap form relatively unstable complexes with cisplatin lesions. In support of the shielding model, addition of large amounts of HMGB1 or several other HMGB proteins to an in vitro nucleotide excision repair assay inhibited the reaction (*36*, *37*). Moreover, increasing HMGB2 levels in cultured adenocarcinoma cells by transfection (*57*) or HMGB1 levels in breast cancer cells by steroid hormone treatment resulted in enhanced sensitivity to cisplatin (*40*). Therefore, we were surprised to observe that the absence of Nhp6A/Bp from yeast cells resulted in hypersensitivity to cisplatin. Loss of phenylalanine 48 impaired the ability of Nhp6Ap to mediate resistance, consistent with the lack of cisplatin-directed binding by the mutant. However, the F48A mutant still mediated some level of resistance. Consistent with the in vitro binding studies, cells expressing Nhp6Ap M29A displayed a resistance to cisplatin treatment similar to that of the wild type. The differential effects of F48A and M29A on cisplatin resistance contrast with the similar properties of these mutants regarding co-activation of RNA Pol II transcription and growth rate at 30 °C. Our findings with Nhp6A/Bp in *S. cerevisiae* are consistent with the recent report that a deletion of the *S. pombe* gene encoding HMGB protein Cmb1 also resulted in hypersensitivity to cisplatin (*42*).

There are a number of possible mechanisms that can account for the modest hypersensitivity of *nhp6a/b* mutants to DNA damage by cisplatin. Nhp6A/Bp could directly or indirectly facilitate repair of the cisplatin lesion. For example, Nhp6A/Bp could direct one or more components of the NER system to the cisplatin lesion. An indirect role could be to facilitate remodeling of chromatin that may be required for efficient repair in vivo, as chromatin structure is known to be a barrier to the DNA repair machinery (*58*), and ATP-dependent chromatin remodeling activities have been shown to enhance NER in vitro (*59*). Nhp6Ap has recently been found to promote binding of the SPN or CP complex, the yeast equivalent to the mammalian chromatin remodeling FACT complex, to nucleosomes (*60*, *61*). Alternatively, *nhp6a/b* mutants may have altered chromatin structure that could enhance exposure of DNA to cisplatin reactivity. However, the DNA would have to be uniquely sensitive to cisplatin since the mutants are not hypersensitive to hydrogen peroxide and methylmethanesulfonate. Nhp6A/Bp could also enhance expression of a gene encoding a component of the NER machinery that functions in repair of cisplatin lesions. This seems unlikely, however, since Met 29 and Phe 48 appear to have equivalent roles in promoting gene expression in the cases investigated thus far.

Finally, we note that Ixr1p, not Nhp6Ap, was identified in a screen for cisplatin binding proteins in yeast extracts (*38*). Paradoxically, Ixr1p is reported to bind to 92 bp cisplatinated fragments with a K_d of 250 nM (*54*), as compared with a K_d of 0.1 nM for Nhp6Ap binding to 19 bp cisplatinated fragments. Perhaps Ixr1p associates with cisplatinated DNA in a more stable manner than Nhp6Ap, and this property may be responsible for the opposite phenotypes of *ixr1* and *nhp6a/b* mutants with respect to cisplatin sensitivity. In vitro, Nhp6Ap and mammalian HMGB1 domain A behave similarly with respect to affinity constants, off rates, and modes of targeting to the cisplatin lesion, implying that Nhp6Ap is a relevant model for mammalian HMGB1. Continued studies on the interactions of different HMGBs as well as other cisplatinated DNA binding proteins and their mutants will be required to fully understand the cellular responses to cisplatin treatment, a necessary prerequisite for improved chemotherapy by this drug.

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