Binding of Ixr1, a Yeast HMG-Domain Protein, to Cisplatin−DNA Adducts in Vitro and in Vivo†

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ABSTRACT: Ixr1 is a yeast HMG-domain protein that binds specifically to DNA adducts formed by the antitumor drug cisplatin. Interruption of the *IXR1* gene in yeast desensitizes cells to cisplatin. This effect is unrelated to a natural function of Ixr1, which is to repress the transcription of *COX5b*. Ixr1 interacts specifically and preferentially with DNA modified by cisplatin. In the present work, Ixr1 was purified from a clone expressed in *Escherichia coli*. The dissociation constant for Ixr1 binding site-specifically to a 92-bp probe containing a single *cis*-[Pt(NH_3)$_2${d(GpG)-N7(1) -N7(2)}] intrastrand cross-link was measured to be 2.5 ($\pm$0.1) $\times$ 10^{-7} M, similar to that found for HMG1. Ixr1 binds at least an order of magnitude more tightly to cisplatin−DNA adducts than to unmodified DNA. Hydroxyl radical footprinting revealed that Ixr1 protects an area of platinated DNA that is approximately 15 bp in size and centered at the platinum adduct. The binding of HMG-domain proteins to cisplatin−DNA adducts has been proposed to divert these proteins from their natural DNA-binding sites, disrupting transcription. This hypothesis was tested for Ixr1 in yeast. The protein was not titrated away from the Cox5b promoter sufficiently well to disrupt transcription either of Cox5b mRNA from genomic DNA or of the β-galactosidase gene under control of the promoter in a plasmid DNA transformed into yeast.

The antitumor drug *cis*-diamminedichloroplatinum(II), or cisplatin,[1] is used to treat many different types of tumors (Olin, 1994). Cisplatin forms bifunctional adducts on DNA (reviewed in Bruhn et al., 1990), the predominant one being an intrastrand d(GpG-N7(1)-N7(2)) cross-link. This adduct is specifically recognized by proteins containing the HMG-domain DNA binding motif (Whitehead & Lippard, 1995). HMG-domain proteins do not bind to DNA adducts of the inactive trans isomer of cisplatin, *trans*-DDP, or to UV-induced adducts (Pil & Lippard, 1992). When a yeast HMG-domain protein, Ixr1, was interrupted to form *ixr1* strains, the yeast cells were desensitized to cisplatin (Brown et al., 1993; McA'Nulty & Lippard, 1996). Thus, Ixr1 can mediate cisplatin cytotoxicity in vivo.

There are several ways in which HMG-domain proteins might effect cisplatin cytotoxicity (reviewed in McA'Nulty & Lippard, 1995; Zamble & Lippard, 1995). One possibility is that protein binding may shield excision repair of cisplatin−DNA adducts. Addition of excess HMG1, a small HMG-domain protein, to mammalian cell extracts blocked repair of cisplatin intrastrand d(GpG), and d(ApG) cross-links (Huang et al., 1994). Only adducts to which HMG1 bound were not repaired. This mechanism was also supported by work with the *IXR1* and *ixr1* strains of *Saccharomyces cerevisiae*. The absence of Ixr1 in the *ixr1* strain desensitized the cells to cisplatin. This decreased sensitivity in the *ixr1* strain is dependent upon intact excision repair, specifically, upon the presence of the DNA-damage recognition complex (McA'Nulty & Lippard, 1996). Interruption of *IXR1* did not desensitize cells to *trans*-DDP or UV light. Thus Ixr1 does not affect excision repair in general, just the excision repair of cisplatin−DNA adducts, probably by shielding them from recognition by the repair complex.

HMG-domain proteins could affect cisplatin cytotoxicity in a manner other than by shielding repair of DNA adducts. Many HMG-domain proteins are transcription factors, and cisplatin treatment might disturb the transcription of the genes they regulate (Donahue et al., 1990). When HMG-domain proteins bind to cisplatin−DNA adducts, they are less available to bind to their natural target sites. The affinity of HMG-domain proteins for cisplatin−DNA adducts is similar to their affinity for the natural binding sites. In cells undergoing cisplatin chemotherapy, there are more platinum−DNA adduct binding sites than natural binding sites. Titration of HMG-domain proteins away from the latter might disrupt expression of the gene(s) they regulate.

This theory has received some support by work with hUBF, a transcription factor for ribosomal RNA which is critical for cell proliferation and survival (Jantzen et al., 1990). hUBF, which binds to a site-specific cisplatin−d(GpG) adduct (Treiber et al., 1994), was removed from its natural binding site by physiological levels of cisplatin−

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); [Pt(en)Cl_2], ethylenediaminedichloroplatinum(II); [Pt(dien)Cl]$^+$, diethylenetriaminechloroplatinum(II); HMG, high mobility group; Ixr1, intrastrand cross-link recognition protein; HMG1, high mobility group protein 1; Ord1, oxygen/oxidase regulating defective, an alternative name for Ixr1; SSRP1, structure specific recognition protein; SRY, sex determining factor; hUBF, human upstream binding factor; *COX5b*, gene for cytochrome *c* oxidase Vb; UV, ultraviolet light; 92Ct, unmodified 92-bp oligonucleotide; 92Pt, platinated 92-bp oligonucleotide; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; NP40, nonidet P40; BSA, bovine serum albumin; TE, Tris−EDTA buffer.

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Table 1: *S. cerevisiae* Strains

strain	genotype
IXR1	*MAT*α, *ura3-52, ade2, trp1-1, lys2-201, his3-200, leu2-3,112*
ixr1	IXR1 *ixr1::LEU2*
JM43	*MAT*α, *his4-580, trp1-289, leu2-3,112, ura3-52*
JM43Δixr1	JM43 *ixr1::LEU2*
JM43Δrox1	JM43 *rox::LEU2*
Ixr1-1	JM43 *cox5a::URA3, ixr1-1*; also known as Ord1-1[a]

[a] Okamoto & Cumsky, 1995.

DNA adducts. These studies were performed in vitro, however, and thus were unable to reveal any response that cells might make following removal of hUBF from its sites on the genome. For instance, cells treated with the drug might raise the expression levels of hUBF in response to cisplatin−DNA adduct formation.

Recently, a natural function for Ixr1 was identified, allowing this titration hypothesis to be evaluated in vivo. Although it could play other roles, Ixr1, alternatively called Ord1 for oxygen/oxidase regulating defective protein (Lambert et al., 1994), regulates the transcription of cytochrome *c* oxidase subunit V (CoxV) by binding to the Cox5b promoter (Zitomer & Lowry, 1992; Lambert et al., 1994). There are two isoforms of CoxV: CoxVa, which is used under aerobic conditions, and CoxVb, which is used under hypoxic conditions (Hodge et al., 1989). The genes encoding the CoxV proteins are called *COX5a* and *COX5b*. Expression of the CoxV isoforms is regulated by several different proteins. Ixr1 represses the expression of the *COX5b* gene but does not affect the expression of *COX5a* (Lambert et al., 1994). The conditions that relieve Ixr1 repression are unknown. Rox1, another HMG-domain protein, represses the expression of *COX5b* in an oxygen-dependent manner (Zitomer & Lowry, 1992). Both *ixr1* and *rox1* mutants express *COX5b*, and the two *COX5* genes are repressed by glucose. Rox1 represses transcription from the sequence YYYATTGTTCTC on several proteins used for respiration under hypoxic conditions (Lowry & Zitomer, 1988; Lowry et al., 1990). None of the other hypoxic or aerobic respiration genes investigated were regulated by Ixr1 (Lambert et al., 1994).

In the present study, we report the purification of Ixr1 from a clone expressed in *Escherichia coli* and the details of its binding to cisplatin−DNA adducts. We also tested the titration hypothesis by examining the effect of cisplatin−DNA adducts on transcription from the Cox5b promoter both in a plasmid system and in genomic DNA. Since the increased expression of Cox5b in *ixr1* cells might affect cisplatin cytotoxicity, we further examined the effect of cisplatin treatment on other cell lines that express Cox5b. This information facilitates comparison of the effects of Ixr1 with those of mammalian HMG-domain proteins which bind to cisplatin DNA adducts. The results of these experiments significantly enhance our understanding of how Ixr1 modulates cisplatin cytotoxicity and contribute to our general knowledge of how HMG-domain proteins may potentiate the chemotherapeutic properties of cisplatin and its analogs.

MATERIALS AND METHODS

Yeast Strains and Plasmids. The yeast strains in Table 1 were obtained or constructed. The *E. coli* strain BL21(DE3) harbors the T7 RNA polymerase gene and pSB4 is a plasmid containing the *Ixr1* gene (Brown et al., 1993). Plasmid pMet is an *E. coli* plasmid that includes the Met promoter in the polylinker and was obtained from the laboratory of G. Fink, at MIT. pCD43, obtained from the laboratory of L. Guarente, at MIT, and pYes2 (Invitrogen) are yeast expression plasmids. Plasmid pSL301 (Invitrogen) is a plasmid with a superlinker containing 50 restriction enzyme sites. pCox5a is a yeast expression plasmid containing the *COX5a* gene linked to its genomic promoter and was obtained from the laboratory of M. Cumsky, UC Irvine. pET3a is an *E. coli* expression plasmid (New England Biolabs). Plasmid pYep contains the COX5b promoter inserted before the β-galactosidase gene on a yeast plasmid (obtained from the Cumsky laboratory). All media types are described elsewhere (Ausubel et al., 1994).

Expression of Ixr1 in E. coli. pET3a-Ixr1 is an *E. coli* plasmid that expresses the *IXR1* gene from a T7 RNA polymerase promoter. The *IXR1* gene was obtained from the plasmid pSB4. Primers were designed (5′-primer: 5′-GTACGTCTATCATATGAACACCGGTATCTCG-3′; 3′-primer: 5′-GTACGTCTATGGATCCTTATTCATTTTT-TATGAT-3′) for the polymerase chain reaction (PCR) that included *Nde*I and *Bam*HI restriction enzyme sites to enable insertion of the gene in the correct orientation into pET3a. The gene was amplified by PCR and purified with a Qiagen PCR purification kit. The PCR product and the pET3a vector were digested with *Nde*I and *Bam*HI restriction enzymes to yield *Nde*I and *Bam*HI overhanging ends. The digested PCR product and pET3a plasmid were purified on a 0.8% agarose gel. The *Ixr1* insert was ligated into the plasmid. A restriction enzyme digest analysis was performed by using *Nde*I and *Bam*HI to ascertain that the desired gene product was retained in the plasmid.

Purification of Ixr1. *E. coli* BL21(DE3) pLysS cell lines containing pET3a-Ixr1 were inoculated into LB media + 2.5% glucose + ampicillin. Cells were grown at 37 °C to an OD_{600} of 0.7−0.8 and induced with 1 mM IPTG for an additional 3 h. The cells were harvested by centrifugation (GSH rotor, Sorvall) and resuspended in 30 mL of lysis buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 0.1% Tween, 1 mM PMSF, and 1 mg/mL Pefabloc (Boehringer Mannheim). The cells were lysed by running them through a French pressure cell twice at 1100 psi. The lysed cells were spun at 40 000 rpm in a TI45 ultracentrifuge rotor (Beckman) to remove cell debris. The supernatant was flash frozen in liquid nitrogen and stored at −80 °C.

Aliquots of the frozen cell extracts were thawed, diluted with buffer A to reduce the salt concentration to below 60 mM, and loaded onto a heparin cartridge (Bio-Rad) at a flow rate of 0.5 mL/min (buffers: A = 50 mM Tris, pH 7.0, 10% glycerol, 10 mM β-mercaptoethanol, 1 mM EDTA; and buffer B = buffer A + 1 M NaCl). After the cell extracts were loaded, the column was washed with buffer A to remove any nonbinding proteins. Bound proteins were eluted by using a nonlinear salt gradient from 100 mM to 1 M NaCl, and 10 mL fractions were collected. The eluted fractions were analyzed by silver stained SDS−PAGE (Sambrook et al., 1989) and Southwestern blotting (Toney et al., 1989).

The low salt fractions containing Ixr1 were pooled and diluted by adding an equal volume of buffer A. The resulting solution was incubated for 10 min at 4 °C with 440 μg of chicken erythrocyte DNA as a competitor and loaded onto

a prepared dsDNA affinity column (see below). Upon completion of loading the protein solution, the column was washed with buffer A to remove any nonbinding proteins. The Ixr1 protein was eluted from the column by using a nonlinear salt gradient from 0 to 1 M NaCl. Ten milliliter fractions were collected and analyzed for the presence of Ixr1 by silver stained SDS−PAGE and Southwestern blotting. The band corresponding to Ixr1 by SDS−PAGE and Southwestern analysis was analyzed by N-terminal amino acid sequencing at the MIT Biopolymers laboratory.

Preparation of a dsDNA Affinity Column. Because Ixr1 failed to bind to various types of column material for protein isolation (S-Sepharose, CM Sepharose, DEAE cellulose, Q-Sepharose, phenyl Sepharose, hydroxylapatite), a dsDNA affinity column containing the consensus binding site of several HMG-domain proteins (AACAAAG; van de Wetering et al., 1993; Harley et al., 1994) and CL-2B sepharose was prepared according to the method of Kanodaga and Tjian (1986). Two complementary oligonucleotides (5′-GATCAACAAAG-3′ and 5′-GATCCTTTGTT-3′) were synthesized (PS250 DNA synthesizer from Cruachem) and purified by HPLC (Waters 486, C18 reverse phase column). The oligonucleotides were annealed, phosphorylated, and ligated to form longer pieces of DNA. The DNA was cross-linked to CL-2B Sepharose (Pharmacia) (Kadonaga & Tjian, 1986). The resulting column material was used to make a 15 mL (2.5 cm) column and equilibrated with buffer A (50 mM Tris-HCl, pH = 7.0, 10% glycerol, 10 mM β-mercaptoethanol). The column was run as described.

DNA Probes. A 123-bp probe, used for Southwestern and gel mobility shift assays, was isolated, platinated, and labeled as previously described (Andrews & Faller, 1991). The 92-bp unplatinated (92Ct) and site-specifically platinated (92Pt) probes (Pil & Lippard, 1992; Chow et al., 1995) were obtained from Dr. C. S. Chow and used for gel mobility shift assays and for hydroxyl radical footprinting. The 92-bp probes were labeled by incubation with polynucleotide kinase in the presence of [γ-^{32}P]ATP (NEN). The DNA was extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), and the unincorporated label was removed by passing the sample over a Sephadex G25 spin column (Boehringer Mannheim). The 92-bp probes were prepared for footprinting by digesting with *Avа*I for 1 h at 37 °C, extracting the DNA with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), and removing the small nucleotides with a Sephadex G25 spin column. The cut DNA was then 3′ radiolabeled on the Pt damaged strand. The enzyme and unincorporated label were removed.

Gel Mobility Shift Assays. Gel mobility shift assays were accomplished as described previously, with minor modifications (Carthew et al., 1985; Donahue et al., 1990). Protein was obtained from Ixr1-containing fractions eluted from each column; unless stated otherwise, all binding assays employed the most highly purified material eluted from the DNA affinity column. The protein solutions were concentrated and resuspended in 20 mM Tris, pH 7.0. Various amounts of Ixr1 and radiolabeled DNA probe (0.5 ng) were incubated in a 20 μL volume containing 2 μg of competitor DNA, 10 mM HEPES, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 4% glycerol, 1 mM spermidine, 0.05% NP40, and 200 μg/mL BSA on ice for 20 min in order to assure that the complex reached equilibrium. For the competition studies, unlabeled 92-bp probe, either 92Ct or 92Pt, was added to each reaction increasing from a 0- to 500-fold excess over labeled DNA probe. To each sample was added 2 μL of loading buffer (10 mM Tris-HCl, pH 7.0, 30% glycerol, 0.025% (w/v) each of xylene cyanol and bromophenol blue), and the protein−DNA complexes were resolved on pre-run, nondenaturing 5% or 10% TBE polyacrylamide (29:1 monomer:bis) gels. Electrophoresis was carried out in 1× TBE for 3−4 h at 300 V, while the temperature was maintained at 5 °C using a recirculating bath. On completion of the electrophoresis, the gels were dried, exposed to X-ray film, and analyzed by using a Molecular Dynamics phosphorimager.

Hydroxyl Radical Footprinting. Hydroxyl radical footprinting was performed according to the method of Tullius et al. with minor revisions (Tullius & Dombrowski, 1986; Tullius et al., 1987). Binding buffers were modified to resemble those used for standard gel mobility shift assays and contained 10 mM HEPES (pH 7.0, 7.5, 7.9), 1 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$ 1 mM spermidine, and 1% NP40. Binding reactions were performed at all pH levels using 60 ng of DNA probe, and either 0, 1.7×10^{-7}, or 7.8×10^{-7} M of Ixr1. Binding reactions were carried out on ice for 20 min. Equal amounts of a freshly prepared 0.2 mM Fe(EDTA) solution were mixed with 20 mM sodium ascorbate and 2.4% H_2O_2. A 30 μL aliquot of this solution was added to each binding reaction, and the cleaving reaction was allowed to proceed for 8 min on ice. The digestion was stopped by the addition of 20 μL of 100 mM thiourea, and the DNA was ethanol precipitated. The DNA pellets were dissolved in TE buffer, pH 8.0, extracted with phenol/$CHCl_3$/isoamyl alcohol (25:24:1), ether extracted, and ethanol precipitated. The DNA pellets were washed with cold 70% ethanol and dried in a Speedvac (Savant SC110). The samples were resuspended in formamide loading dye (Sambrook et al., 1989) and heated to 90 °C for 2 min prior to loading onto the denaturing gel. Maxam−Gilbert sequencing of the 92-bp DNA probes was performed as described, with minor modification (Sambrook et al., 1989).

Dried DNA samples from Maxam−Gilbert sequencing and footprinting reactions were counted in a Beckman scintillation counter (Model #LS 6500). The pellets were dissolved in an appropriate amount of formamide loading dye solution (Sambrook et al., 1989) to yield equal numbers of counts when 2 μL of the solution was loaded onto an 8% denaturing (7 M urea) polyacrylamide gel (0.4 mm thick). Electrophoresis was carried out for 2−3 h at 1500 V (45−50 °C). Gels were dried onto filter paper (Hoefer Scientific Instruments) and exposed to film (Kodak X-OMAT AR) at room temperature. Gels were analyzed by using a Molecular Dynamics phosphorimager.

Construction of Ixr1 Expression Plasmids. pMM1 contains *IXR1* expressed by the Met promoter. The Met promoter was excised from pMet with *Xba*I and *Eco*RV and isolated on an agarose gel. The *IXR1* gene was excised from pSB4 with *Acc*I and *Sna*BI and isolated by agarose gel electrophoresis. pSL301 was digested with *Eco*RV and *Sac*I, and the vector fragment was isolated. The two fragments were ligated to form pSL301-Ixr1, with the *IXR1* gene in the polylinker. pSL301-IXR1 and pYes2 were digested with *Bam*HI and *Xho*I, and the appropriate fragments were isolated and ligated to form pYY (pYes2-Ixr1). pYY was cut with *Bam*HI which was filled in with Klenow. The linear plasmid was then cut with *Spe*I. This procedure removed the Gal promoter from pYY. The Met promoter was then ligated to this vector. The resultant plasmids were analyzed for the correct size and restriction enzyme cleavage patterns, con-

firming that *IXR1* and the Met promoter had been inserted into the correct locations.

Cytotoxicity Assays. Colony-counting assays were performed on yeast cells grown to saturation that were treated with cisplatin for 2 h in SD media (McA'Nulty & Lippard, 1996). Each strain was assayed in three to five separate experiments, and results from a representative experiment are shown in the figures. The percent error indicated in the figures shows a 2σ level derived from colony counts on the three identical plates and does not represent multiple experiments. A line was fit to the log of the percent survival data. The slope of the line is k in the expression percent survival $= \exp(-k[\text{cisplatin}])$. The reported values for k are an average of three to five separate experiments. The same medium was always used for growing and plating the cells.

Anaerobic Cytotoxicity Assays. All solutions used were degassed by bubbling argon through them for 0.5 h for 5 mL volumes or 1 h for larger volumes. Cells were grown by inoculating the media aerobically into 16 mm glass screw cap tubes, and then degassing the cells plus media with a sterile cannula through a sterile septum. The tubes were transferred into an anaerobic chamber (wet box) so that the screw cap could be put on the tube; parafilm was then wrapped around the cap. The cells were grown in a shaking incubator at 30 °C for 3 days. The ability of this system to keep the media oxygen-free was tested by using oxygen sensitive probes (BBL Gas Pak disposable anaerobic indicator). Cisplatin treatment was performed as described in the previous paragraph except manipulations were performed at ≈25 °C on a rocker in the wet box. The cells were spun down in a centrifuge and plated, also as detailed above, except that all procedures were carried out in the wet box. Prior to being brought into the box, the plates were treated overnight in the BBL Gas Pak oxygen removal system; the removal of oxygen was confirmed with oxygen sensitive probes (BBL Gas Pak disposable anaerobic indicator). The plates were pumped into the box while in the BBL Gas Pak apparatus (Becton, Dickinson, Inc.), which is basically a large jar that can hold the appropriate atmosphere. After the cells were put on the plates, they were transferred back into the BBL Gas Pak apparatus, removed from the box, and grown for 3 days at 30 °C. Subsequently, they were removed and grown aerobically so that the colonies could be readily counted. The oxygen indicators present while the plates were growing did not change color until the plates were removed from the apparatus.

Testing the Titration Hypothesis. Plasmid Based Test. A plasmid, pYep, with the Cox5b promoter linked directly to the β-galactosidase gene, was transformed into yeast. Freshly transformed cells were used in the assays since the β-galactosidase activity drops off after about a week. JM43 and JM43Δixr1 cells containing the plasmid were inoculated into SCΔUra (2% galactose) media, and the cells were grown to log phase. The cells were then pelleted and resuspended in 5 mL of fresh media containing varying levels of cisplatin. The cells were incubated for 6 h at 30 °C in culture tubes and then frozen at −80 °C. The assay was adapted from that in *Current Protocols in Molecular Biology* (Ausubel et al., 1994). β-Galactosidase activity was calculated according to the standard equation $U = 1000 \times (\text{OD}_{420})/[(t)(V)(\text{OD}_{600})]$, where t = time the reaction ran (min) and V = volume of cell culture used (mL). Each experiment was performed 3−5 separate times; the error bars in the figures represent the variation among these experiments at 2σ.

Cox5b mRNA Analysis. Yeast cells were treated with cisplatin in YPGE media for 6 h at 30 °C. The 10 mL aliquots were then pelleted and resuspended in 1 mL of cold water. The cells were transferred to Eppendorf tubes and pelleted. The cell pellets were quickly frozen by dropping the tubes in liquid nitrogen and stored in the −80 °C freezer. The next day, total RNA was prepared by the acidic phenol method (Ausubel et al., 1994). A_{260} (absorbance) and A_{280} readings were taken to confirm the purity and yield of the sample, and the RNA was stored at −20 °C. All solutions were treated with diethyl pyrocarbonate (DEPC) and autoclaved to inactivate any contaminating RNAse. Approximately 30 μg of each sample was run on a 1.8% formaldehyde−agarose gel (Sambrook et al., 1989). The gel was run for 3−5 h at 50−70 V. The RNA was transferred to nitrocellulose using the Schleicher & Schuell Turboblotter rapid downward transfer system. The membrane was dried in a vacuum oven. The blot was prehybridized and hybridized in Stratagene's Quickhyb. The blot was probed with $(7-10) \times 10^6$ cpm Cox5b probe for 1 h at 50 °C. The blot was rinsed twice in 3× SSC, 0.1% SDS, for 15 min, and exposed to film overnight. The bands were quantitated with a phosphorimager. The blot was reprobed as above using the probe for actin. The probes used were 5′-GCACGAGGAGCGTCGTCACCGGCA-3′ for actin and a 200 bp fragment of *COX5b* for Cox5b mRNA (Hodge et al., 1989). The probes were labeled with [γ-^{32}P]ATP and kinase, or with random priming with [α-^{32}P]dCTP and Klenow, respectively. The graphs presented in the figures represent individual experiments. The numbers associated with the Cox5b mRNA levels were arbitrary units, and there was no numerical correspondence between different graphs since the numbers are based on the phosphorimager output.

RESULTS

Purification of Ixr1 to Near Homogeneity. A T7 RNA polymerase expression plasmid was constructed with the *Ixr1* gene and expressed in *E. coli*. Southwestern blotting revealed the presence of Ixr1 in a minipreparation of cell extracts. From the translated sequence of *IXR1*, the p*I* of Ixr1 was calculated to be 8.45 (Brown et al., 1993), so various cation and anion exchange resins were used at different pH values in attempts to purify this protein, but Ixr1 did not bind to any of them. Heparin was the first affinity resin tried that bound Ixr1 strongly enough to be used for initial purification. Nonbinding proteins were eluted in the wash. The chromatogram and buffer profile for this affinity resin are shown in Figure 1a. The diagonal lines indicate fractions containing Ixr1 as determined by Southwestern analysis. The protein elutes in two distinct peaks, one low affinity form at 100 mM NaCl and the other, high affinity, fraction at 250−800 mM NaCl.

Additional purification of Ixr1 was accomplished by using a dsDNA affinity column containing the sequence, AACAAAG. The chromatogram and buffer profile for this column are presented in Figure 1b. Nonbinding proteins were eluted in the wash. Ixr1 eluted at 190−320 mM NaCl and could be separated from some impurities by this salt gradient, as indicated. Ixr1 isolated from the more slowly eluting band (Figure 1b) was analyzed by N-terminal amino acid sequencing. The sequence was identical to that expected from the open reading frame of *IXR1* (Brown et al., 1993).

Interactions of Ixr1 with DNA Modified by Cisplatin. The low and high salt protein fractions containing Ixr1 from the

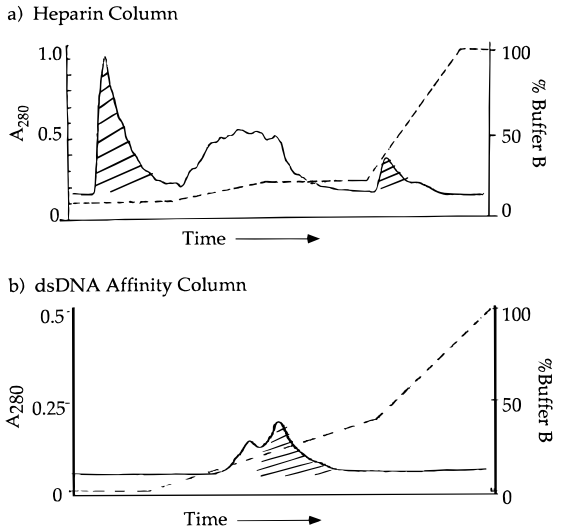

FIGURE 1: Chromatograms from the purification of Ixr1, which was detected by Southwestern analysis in the peaks identified by diagonal lines. (a) Elution profile from the heparin cartridge. The NaCl concentration was increased from 100 mM to 1 M in a nonlinear fashion. (b) Elution profile from the dsDNA affinity column. Ixr1 was found in the second peak seen to elute from this column. The solid lines show the A_{280} values of the protein elution profiles and the dashed lines the buffer profiles.

heparin column were evaluated by gel mobility shift assays with globally platinated DNA. The low salt fraction bound specifically to a 123-bp probe modified with cisplatin, reducing its mobility, whereas the high salt fraction did not (data not shown). Neither protein fraction bound to unmodified DNA or DNA that was modified with *trans*-DDP. The low salt fraction did not bind to a 123-bp probe modified with chlorodiethylenetriamineplatinum(II) or treated with UV light. Lanes containing the low salt fraction showed a ladder of bands, indicating the presence of several different species of Ixr1−cisplatin−DNA adducts in the binding solution. As more protein from the low salt fraction was added to the binding reaction, the Ixr1−DNA complexes decreased in mobility, and eventually failed to enter the gel.

The observation that the high salt fraction bound platinated 123-bp DNA in a Southwestern analysis but not in a gel mobility shift assay was explored further. The Southwestern assay includes denaturation/renaturation steps to remove any SDS bound to the protein. The protein fraction eluting at high salt was therefore treated with 7 M guanidine-HCl, as in the Southwestern assay, and dialyzed against Tris-HCl buffer, pH 7.0. The resulting protein solution was active in a gel mobility shift assay with 123-bp probe treated with cisplatin (data not shown). The same ladder of bands was present for this high salt treated fraction as had appeared for the low salt fraction.

Figure 2 presents the results of 92Ct and 92Pt gel mobility shifts in the presence of increasing Ixr1 concentrations ranging from (0−6.5) × 10^{-6} M. The most striking difference between the two gels is the presence of a fast migrating band in the right panel of Figure 2 attributed to specific binding of Ixr1 to the *cis*-[Pt(NH_3)$_2${d(GpG)-N7-(1)-N7(2)}] cross-link on DNA. Bands of lower mobility present in both gels are assigned to non-platinum-dependent binding of Ixr1 to the DNA probes. The pattern of one specific band, identified with Ixr1 binding to 92Pt, and lower mobility bands visible for the binding of Ixr1 to both probes was observed both with and without the presence of competitor DNA.

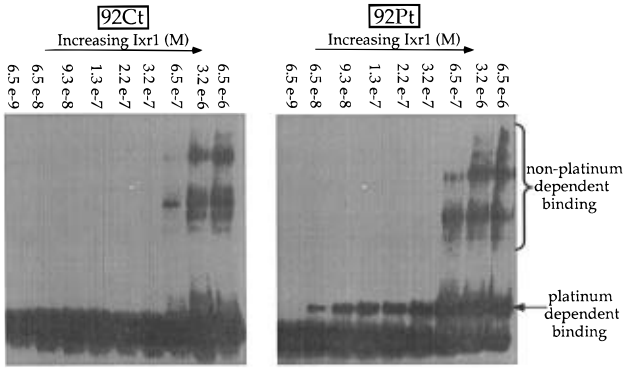

FIGURE 2: Gel mobility shift assays used to determine the dissociation constant of Ixr1 for the 92-bp probes. The Ixr1 concentration ranged from (0−6.5) × 10^{-7} M, as indicated. All binding reactions contained 0.5 ng of DNA probe and no competitor DNA.

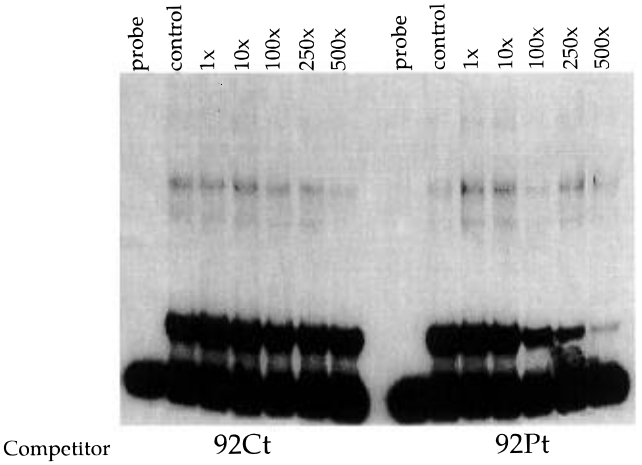

FIGURE 3: Competition studies of Ixr1 binding to 92Pt. The bandshift is competed with unlabeled 92Ct (left) and 92Pt (right). Lanes 1 and 8 contain the DNA probe, lanes 2−7 and 9−14 contain 0.06 ng of *92Pt, 6.5 × 10^{-7} M Ixr1 and increasing amounts of cold competitor 92Ct or 92Pt. The 1:1 platinum specific band is the only band to be competed away by 92Pt.

Dissociation constants (K_d values) were determined for both the specific Ixr1−Pt−DNA adduct as well as for the lower mobility, platinum-independent, bands. The latter values were 2.1 and 1.9 (±0.1) × 10^{-6} M for 92Ct and 92Pt, respectively. The specific binding of Ixr1 for the Pt−DNA adduct was 2.5 (±0.1) × 10^{-7} M, an order of magnitude stronger than the nonspecific binding. K_d values were calculated assuming 1:1 protein:DNA stoichiometry, as previously described (Pil & Lippard, 1992).

Competition studies were performed to investigate further the nature of the multiple bands seen in gel mobility shift assays with Ixr1 and the 92-bp DNA probes (Figure 3). Addition of up to 500-fold excess of 92Ct to solutions containing Ixr1 and radiolabeled 92Ct (not shown) and 92Pt (Figure 3, left) did not affect the band shifts seen by autoradiography, indicating the formation of a strong complex for these platinum-independent bands. Addition of 500-fold excess 92Pt to solutions containing Ixr1 and radiolabeled 92Ct did not affect the complexes detected by autoradiography (not shown), but addition of unlabeled 92Pt to solutions of Ixr1 and radiolabeled 92Pt (Figure 3, right) inhibited only the band of highest mobility. We therefore assign this band to the specific complex of Ixr1 with the cisplatin−DNA lesion.

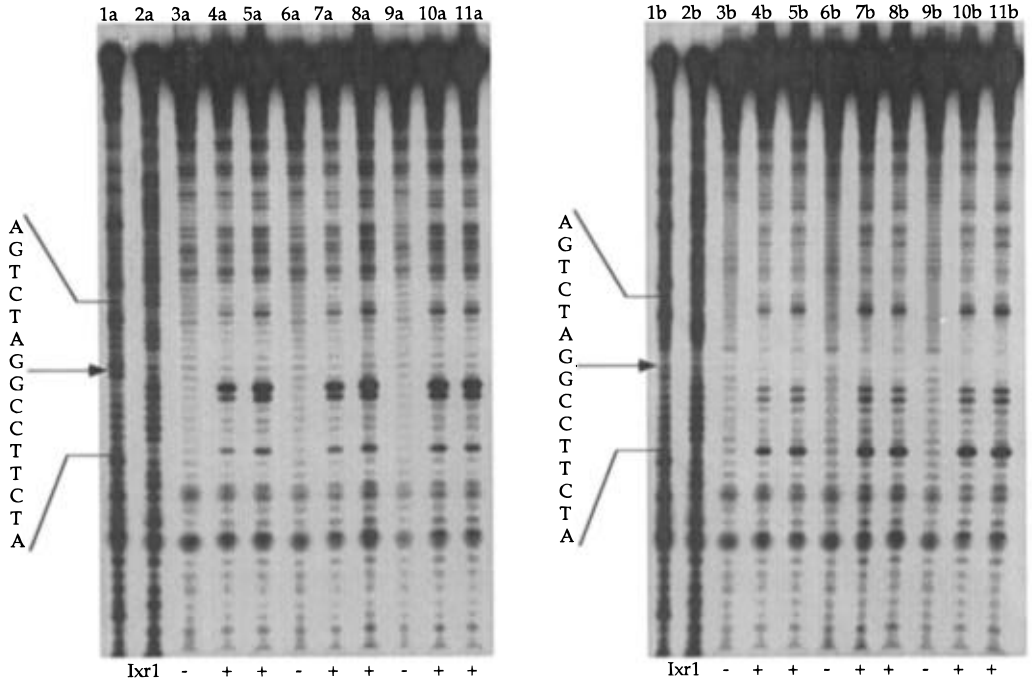

FIGURE 4: Hydroxyl radical footprint analysis of Ixr1 with 92Ct (a) and 92Pt (b). Lanes 1a and 1b contain the Maxam−Gilbert G reaction and lanes 2a and 2b the C+T reactions for 92Ct (left) and 92Pt (right), respectively. Lanes 3−5 on each gel show the results of footprinting reactions run at pH 7.0, lanes 6−8 at pH 7.5, and lanes 9−11 at pH 7.9. Ixr1 concentration in lanes 3, 6, and 9 is 0 M; in lanes 4, 7, and 10, 1.7×10^{-7} M; and in lanes 5, 8, and 11, 7.8×10^{-7} M.

Hydroxyl radical footprints of Ixr1 bound to 92Ct and 92Pt probes are shown in Figure 4, and a phosphorimager analysis of the data is depicted in Figure 5. A change in pH ranging from 7.0 to 7.9 did not affect the footprints. Two Ixr1 concentrations, 1.7 and 7.8×10^{-7} M, were examined. At the lower concentration there was only specific Ixr1 binding to the Pt−DNA adduct, whereas at the higher concentration both specific and nonspecific binding occurred. As noted on the gel, in the area of the cisplatin d(GpG) cross-link there was a distinct "toeprint", or absence of cutting, indicating that the platinum adduct protects the two guanosine residues from digestion. A similar toeprint was seen in the Maxam−Gilbert G reaction lane of the platinated 92-bp probe.

The autoradiograph and its phosphorimager analysis (Figures 4 and 5) (Dixon et al., 1991) clearly reveal a change in the hydroxyl radical cleavage pattern upon addition of Ixr1 to the 92Ct and 92Pt DNA probes. A 15 bp area of protection by Ixr1 is evident for both 92-bp probes, encompassing the GG site. Although the Ixr1 footprints on the two probes are similar, there are distinct differences in the particular bases that are best cleaved by the hydroxyl radical. The most sensitive sites of hydroxyl radical cleavage for the 92Ct probe are at the two cytosine bases adjacent to the two guanosine residues. Although these two bases are also cleaved with high frequency in the reaction of Ixr1 complexed 92Pt, there are two adenosine residues, one seven nucleotides 3′ to the Pt−GG adduct and the other 6 nucleotides 5′ to the Pt−GG adduct, that are even more sensitive to cleavage by hydroxyl radical. Differences in the relative intensities of these bands can be quantitatively evaluated from Figure 5.

Ixr1 Overexpression Studies. Ixr1 was expressed in yeast cells to demonstrate further that it is responsible for the differential sensitivity of the *IXR1* and *ixr1* strains to cisplatin. Previously, cisplatin cytotoxicity assays were performed when the cells had grown to saturation or reached the post-diauxic phase (Brown et al., 1993; McA'Nulty & Lippard, 1996). Galactose-induced Ixr1 expression did not occur in cells that were at saturation, so other expression

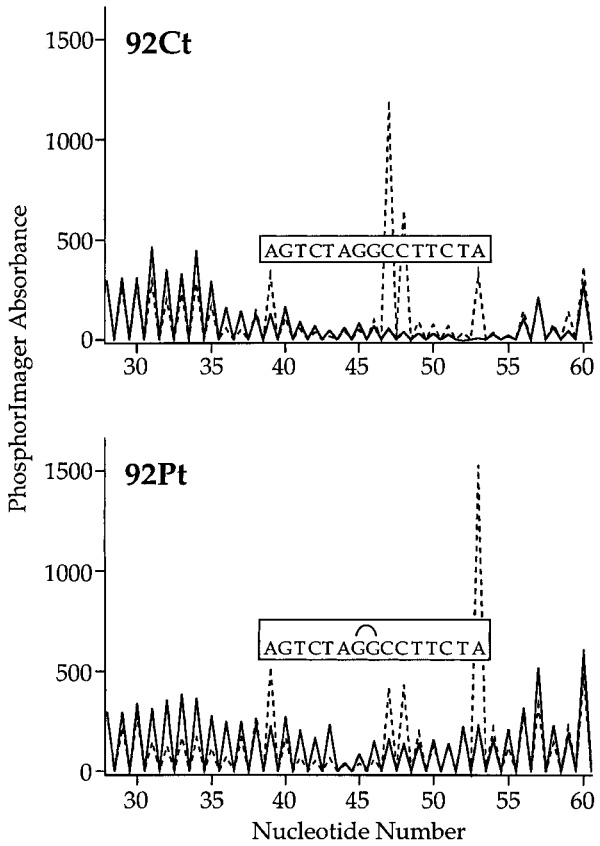

FIGURE 5: Analyses of the hydroxyl radical footprint of Ixr1 with 92Ct (top) and 92Pt (bottom) at pH 7.9. The data from this figure were background corrected and normalized for comparison. The GG site of platination spans nucleotides 45−46. Analysis of hydroxyl radical digestion of the 92-bp probes with 0 M Ixr1 (—) and 1.7×10^{-7} M Ixr1 (- - -).

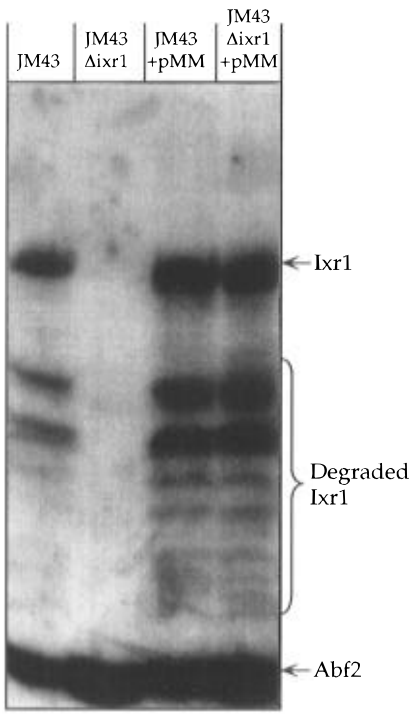

FIGURE 6: Ixr1 overexpression from the Met promoter. Southwestern showing the levels of overexpression obtained from the pMM Ixr1 overexpression plasmid. The same amount of cells were used in each lane.

systems were investigated. Attempts to express the protein from a plasmid under control of its genomic promoter did not give significant levels of Ixr1 in cells at saturation. We next investigated the Met promoter, which can be induced by growing the cells in media lacking methionine (Mountain & Korch, 1991). This promoter was chosen because cells grown to saturation have exhausted dextrose in the media and may be depleting other components as well. The vector was constructed by placing the Met promoter upstream from the *Ixr1* gene. Cells were grown on SCΔUraΔMet (dextrose) media to preserve the plasmid and to express Ixr1 off the promoter. The cells do not need exogenous methionine to grow. Southwestern blots demonstrated that Ixr1 expression was present in cells grown to saturation (Figure 6). JM43 and JM43Δixr1 were grown with either the control plasmid pYes2 or the expression plasmid pMM and treated with cisplatin. There was a clear difference in survival between the JM43+pYes2 and JM43Δixr1+pYes2 strains (slope ratio of 2.7, Figure 7 and Table 2). The JM43Δixr1+pMM strain was still less sensitive than JM43+pYes2, but substantially more sensitive than the JM43Δixr1+pYes2 strain. This result indicates that Ixr1 can sensitize cells to cisplatin. There was no difference between the two JM43 strains, probably because the level of Ixr1 expression was not sufficiently high. In the Southwestern blot (Figure 6), the difference between the JM43 strains is not nearly as great as the difference between the two JM43Δixr1 strains. Unfortunately, there is no method for raising the levels of expression from this promoter, which has already proved to be better than the more commonly employed GAL promoter. The relative sensitivities of strains to cisplatin were quantitated as detailed in the Materials and Methods section, and the exponential slope of the equation, % survival = $\exp(-k[\text{cisplatin}])$, was determined (Table 2). The slopes were compared because it is inaccurate to compare the relative sensitivities at a specific cisplatin concentration. The slope can be easily

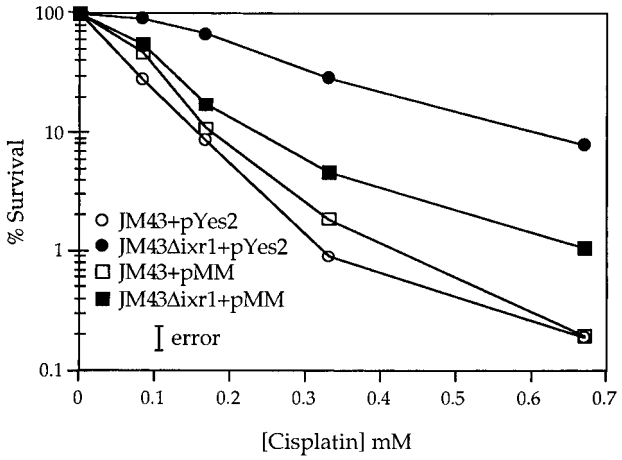

FIGURE 7: Cytotoxicity assays performed on JM43 and JM43Δixr1 with the control plasmid pYes2 and the Ixr1 overexpression plasmid pMM. The cells were treated with cisplatin after being grown to saturation in SCΔUraΔMet media.

Table 2: Ixr1 Overexpression Cytotoxicity Assay

strain	slope (k)	k(J+pYes2)/k(other)	range of ratios
JM43+pYes2	11.4 ± 1.5		
JM43Δixr1+pYes2	4.2 ± 0.4	2.7 ± 0.3	2.4−2.9
JM43+pMM	11.5 ± 2.0	1.0 ± 0.09	0.9−1.1
JM43Δixr1+pMM	8.0 ± 1.2	1.4 ± 0.05	1.4−1.5

Table 3: Cisplatin Cytotoxicity Assay Results

WT strain	slope (k)	mutant	k(WT)/k(mutant)	range of ratios
JM43	4.3 ± 1.0	Δixr1	3.9 ± 2	2.0−6.5
		Δrox1	1.1 ± 0.2	0.78−1.2
		Ixr1-1	0.76 ± 0.1	0.66−0.84
anaerobic	9.1 ± 2.4	Δixr1	2.4 ± 0.8	1.7−3.2
IXR1	7.9 ± 1.6	Δixr1	1.5 ± 0.2	1.3−2.1

converted into LD50 values (LD50 = ln(0.5)/k), or any other similar measure.

Rox1 and Cisplatin Sensitivity. Rox1 also represses *COX5b* expression, so if the decrease in cisplatin cytotoxicity of the *ixr1* strains is due to increased levels of CoxVb, *rox1* cells should have the same phenotype. Alternatively, *rox1* strains could be less sensitive to cisplatin because the HMG-domain protein Rox1 is missing, which may cause cisplatin−DNA adducts to be less shielded from excision repair. Rox1 has an unusual HMG-domain with five amino acids in the middle dividing it into two separate pieces that are homologous to other HMG-domains. A JM43Δrox1 strain was obtained and treated with cisplatin. There was no significant difference between JM43 and JM43Δrox1 in cisplatin sensitivity (Table 3). The unusual sequence and unexpected behavior of this HMG-domain protein led us to obtain a Rox1 expression vector and determine whether it bound to cisplatin−DNA adducts. Up to this time, all HMG-domain proteins that had been analyzed bound to cisplatin-modified DNA. Results from two laboratories (N. L. Raju, J. P. Whitehead, and S. J. Lippard, unpublished data, and Lambert et al., 1994) indicate that Rox1 does not bind to cisplatin−DNA adducts. Furthermore, no band of the correct molecular weight for Rox1 is visible in Southwestern blots performed on yeast cell extracts of *ixr1* strains (Figure 6, for example). These results indicate that Rox1 does not actually bind to cisplatin−DNA adducts, and thus no difference in cisplatin sensitivity could be expected following the removal of its HMG-domain. Rox1 is currently the only HMG-domain

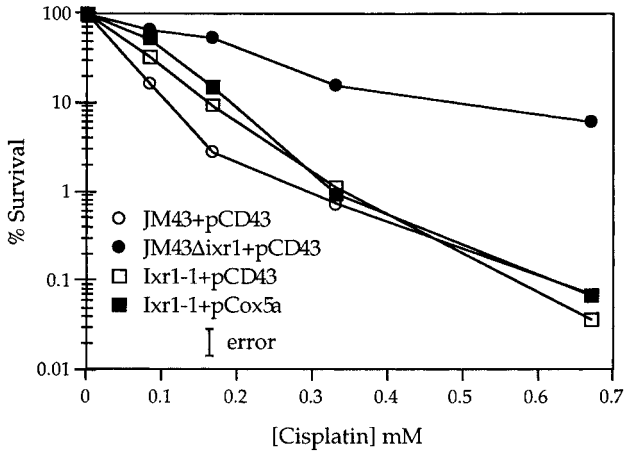

FIGURE 8: Cytotoxicity assays performed on *Ixr1-1* strains. Assay was performed on JM43, JM43Δixr1, and *Ixr1-1* with the control plasmid pCD43 and on *Ixr1-1* with the CoxVa expression plasmid pCox5a. Cells were grown to saturation on SCΔUra media.

Table 4: Ixr1-1 Cytotoxicity Assay

strain	slope (*k*)
JM43+pCD43	11.7 ± 0.9
JM43Δixr1+pCD43	4.0 ± 0.9
Ixr1-1+pCD43	10.9 ± 1.0
Ixr1-1+pCox5a	10.8 ± 1.2

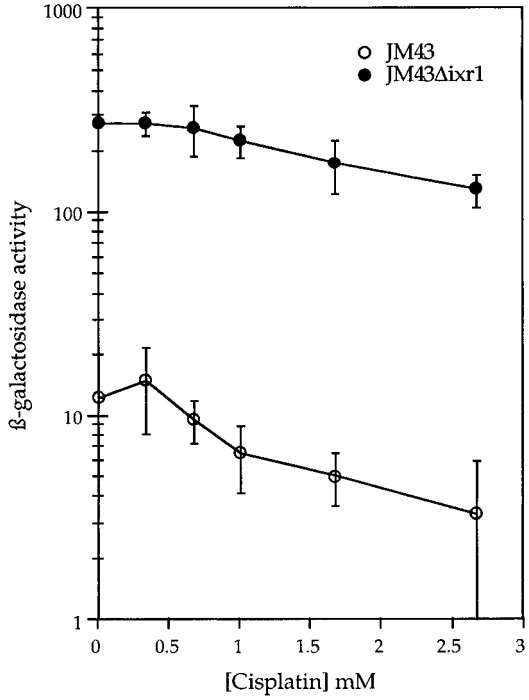

FIGURE 9: β-Galactosidase activity of JM43 and JM43Δixr1 strains treated with cisplatin. Cells were grown to exponential phase before cisplatin treatment.

protein known not to bind to cisplatin−DNA adducts. *rox1* cells express Cox5b, yet they are no less sensitive than wild-type cells, so the diminished sensitivity of the *ixr1* strains is not due to increased expression of Cox5b.

Cytotoxicity Assays on Ixr1 Mutants. There are several strains of yeast isolated by the Cumsky laboratory which contain mutant forms of Ixr1. These mutants act like *ixr1* strains in that they express CoxVb in the presence of oxygen. The best characterized is Ixr1-1, which contains most of the sequence of Ixr1, including the HMG-domains, but lacks the C-terminal tail (Okamoto & Cumsky, 1995). *Ixr1-1* is in a JM43Δcox5a background, so a plasmid that expresses CoxVa, pCox5a, was introduced into the cells for subsequent cytotoxicity assays. A control plasmid, pCD43, was used in the other strains. CoxVa, expressed from its genomic promoter, may be more stable in cells than Ixr1, and thus more readily expressed. Four strains, JM43+pCD43, JM43Δixr1+pCD43, Ixr1-1+pCD43, and Ixr1-1+pCox5a, were grown to saturation in SCΔUra (dextrose) media. The difference in cisplatin cytotoxicity between JM43+pCD43 and JM43Δixr1+pCD43 was significant (Figure 8, Table 4), but the two *Ixr1-1* strains were just as sensitive as the JM43+pCD43 strain. The Ixr1-1 mutant protein does not cause the cells to become less sensitive to cisplatin even though strains with that protein express CoxVb. These experiments demonstrate that it is the HMG-domain of Ixr1 which is crucial to the cisplatin sensitivity of the strain, not the ability of the protein to suppress CoxVb expression.

Anaerobic Cytotoxicity Assays. Since Ixr1 regulates the expression of a protein used only under hypoxic or anaerobic conditions, cytotoxicity assays were performed under the latter conditions. Cells and plates grown without oxygen were monitored with oxygen indicator strips. The cisplatin treatments themselves were performed in an anaerobic chamber at room temperature. The difference in cisplatin sensitivity between the JM43 and JM43Δixr1 cells was still present (Table 3). The difference was a bit less pronounced than in cells grown aerobically, with a slope ratio of 2.4 instead of 3.9, but this result may be more of an indication of differences in cell growth conditions than anything else. This result indicates that the increased expression of CoxVb in *ixr1* cells does not cause the cisplatin cytotoxicity difference and that the latter is not a function of oxygen metabolism.

Titration Hypothesis Experiments. Plasmid-Based Assay. The titration hypothesis was first tested by using a plasmid, pYep, which contains the Cox5b promoter in front of the β-galactosidase gene. β-Galactosidase activity was easily assayed in crude cell extracts. These experiments were all performed with cells in exponential phase growth because β-galactosidase expression only occurs under these conditions. JM43 and JM43Δixr1 cells were treated with various amounts of cisplatin, and the level of β-galactosidase activity was measured (Figure 9). As expected, when no cisplatin was present, the JM43Δixr1 cells produced much more β-galactosidase because there was no Ixr1 to inhibit transcription from the Cox5b promoter. As cisplatin was added, the level of β-galactosidase in the JM43Δixr1 cells dropped, presumably because cisplatin binding to the pYep plasmid blocked transcription. If the titration hypothesis were correct, the level of β-galactosidase expression in the JM43 cells should increase upon cisplatin treatment because the adducts would titrate the Ixr1 away from the Cox5b promoter. No significant increase in β-galactosidase expression was seen in the experiment reported in Figure 9 or in any similar experiments. This assay was performed five times using these conditions, and three times when cisplatin levels between 0 and 0.5 mM were investigated. The small peak visible in the JM43 data in Figure 9 was no greater in any other experiment and did not occur in all experiments. These data therefore do not support the titration hypothesis.

Several other treatments of the cells were investigated to probe further for evidence of titration activity. Cells were treated with cisplatin for 2 h instead of 6 h, which made no

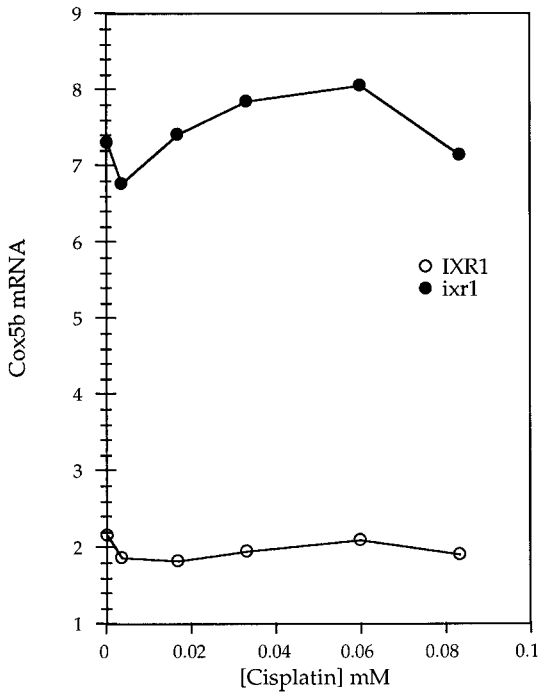

FIGURE 10: mRNA analysis of IXR1 and ixr1 strains treated with low levels of cisplatin. Quantitation of the Northern blot after loading levels were taken into account by probing for actin mRNA. Cells were grown to exponential phase before cisplatin treatment.

difference. In some experiments, cells were incubated in fresh media after the cisplatin treatment to see whether the cells needed time to respond to the cisplatin treatment. Neither a 16 nor a 2 h post-incubation treatment showed any increase in the level of β-galactosidase in JM43 as cisplatin was added. The JM43Δixr1 strain did not lose β-galactosidase activity with increasing cisplatin levels in the 16 h recovery experiment, probably because the cells repaired cisplatin from the plasmid. Cells were also treated with the same amount of cisplatin for various time periods to determine whether there would be any increase in the β-galactosidase activity in the JM43 strain. The results for this experiment were similar to those reported above. None of these experiments supports the titration hypothesis.

mRNA-Based Assay. Experiments investigating the titration hypothesis were also performed by examining the level of Cox5b mRNA. These experiments do not suffer from the drawbacks of using a plasmid based system, such as the need to rely on a small circle of DNA that is not necessarily processed in the same manner as genomic DNA. As in the β-galactosidase activity experiments, cells were treated with cisplatin and then lysed. Total RNA was extracted and Northern blots were probed with anti-Cox5b probe. The loading was controlled in all experiments by subsequently probing the blots for actin. Several experiments were performed in which exponential phase cells were treated with various levels of cisplatin. Quantitation of the Cox5b bands from a typical Northern blot is shown in Figure 10. When the cells were not treated with cisplatin, *ixr1* produced a higher level of Cox5b mRNA than *IXR1* cells because Ixr1 was not present to inhibit transcription. Low levels of cisplatin did not produce any appreciable increase in Cox5b mRNA in the *IXR1* strain (Figure 10). Higher levels of cisplatin caused the level of Cox5b mRNA to decrease in both the *IXR1* and *ixr1* strains; there was no significant transient increase in the *IXR1* strain (data not shown). Both of these experiments were repeated several times.

Different conditions of cisplatin treatment were also investigated in this assay. In one experiment, the cells were permitted to recover for 16 h in fresh media following cisplatin treatment. A similar experiment was performed in which the cells were treated with cisplatin for 3 h and permitted to recover for 3 h. Cells grown to saturation were also investigated by this method. Blots from these experiments gave no indication to support the titration hypothesis. One experiment was performed by using the JM43 and JM43Δixr1 strains, which yielded no different results. The *IXR1* and *ixr1* cells were also treated with cisplatin for various time periods. Again, this experiment did not show any increase in Cox5b mRNA expression in the *IXR1* cells with increasing levels of cisplatin treatment.

DISCUSSION

The discovery that Ixr1 alters the sensitivity of *S. cerevisiae* to cisplatin was the first demonstration that an HMG-domain protein could affect cisplatin cytoxicity (Brown et al., 1993; McA'Nulty & Lippard, 1996). Several hypotheses have been discussed which could account for this behavior, the most attractive being either that the protein shields platinum adducts from excision repair or that diversion of Ixr1 from its natural complexes on the genome through binding to cisplatin−DNA adducts indirectly affects the viability of the cell (Donahue et al., 1990; Lippard, 1994). It was subsequently determined that the desensitization of yeast in an *ixr1* strain depended upon the ability of Ixr1 to block excision repair of cisplatin−DNA adducts (McA'Nulty & Lippard, 1996), an activity independently demonstrated for HMG-domain proteins in human cell extracts (Huang et al., 1994). Since the hypotheses for how HMG-domain proteins sensitize cells to cisplatin are not mutually exclusive, other mechanisms might also be operative.

In the present work, we expressed Ixr1 from a clone in *E. coli*, purified the protein to near homogeneity, demonstrated by hydroxyl radical footprinting that it covers a 15-base pair region of a site-specificially modified DNA centered at the platinum adduct, and quantitated its binding to cisplatin−DNA adducts. Ixr1 binds to cisplatin-modified DNA at least an order of magnitude better than it binds to unmodified DNA. Such increased affinity is sufficient to titrate another HMG-domain protein, hUBF, away from its natural binding target sequence on DNA in vitro (Treiber et al., 1994). We therefore wondered whether the binding of Ixr1 to cisplatin−DNA adducts in yeast could divert the protein from its natural binding site, one of which is now known to be the Cox5b promoter (Lambert et al., 1994). Two sets of experiments were therefore performed which demonstrate that Ixr1 is not titrated away from the Cox5b promoter sufficiently to affect transcription in vivo. Thus the data obtained in the present investigation reveal that the HMG domains of Ixr1 are responsible for its ability to affect the sensitivity of *S. cerevisiae* to cisplatin, and not an activity related to the natural function of the protein to serve as a repressor of Cox5b expression.

Binding of Ixr1 to Cisplatin−DNA Adducts. We have demonstrated in the present work that Ixr1, expressed in *E. coli* and substantially purified by using heparin and DNA affinity columns, binds with good affinity and specificity to the major cisplatin adduct on DNA, the intrastrand d(GpG) cross-link. Such specificity of binding for cisplatin−DNA adducts has been observed for several HMG-domain proteins,

including HMG1, SSRP1, mtTFA, and hUBF (Toney et al., 1989; Pil & Lippard, 1992; Brown et al., 1993; Chow et al., 1994; Lambert et al., 1994; Treiber et al., 1994). Gel mobility shift assays revealed the presence of multiple bands following incubation of Ixr1 with globally platinated 123-bp DNA probes, as well as for a site-specifically modified 92-bp DNA probe. The low mobility of these complexes suggests that they arise either from multiple protein complexes binding to the probes or from an aggregate of DNA probes and protein molecules (Hu et al., 1994).

The dissociation constant for Ixr1 binding to the platinum site of an oligonucleotide containing a single cisplatin adduct was determined to be 2.5×10^{-7} M, the same order of magnitude as that of HMG1 bound to a similar DNA probe (4×10^{-7} M). The binding constant for HMG1 was previously estimated to be approximately 100-fold greater for the cisplatin adduct than for unmodified DNA (Pil & Lippard, 1992). Such a large difference was not seen for Ixr1, which binds with only 10-fold greater affinity. A recent study of bend angles induced by HMG domains and proteins containing these bending elements revealed that the 100-fold difference estimated for HMG1 may be high, since the specific-to-nonspecific binding ratios for the HMG domains of HMG1, SRY, and LEF-1 are in the 4−10-fold range (Chow et al., 1994). This study also revealed the presence of a "ladder" of bands in the gel mobility shift assays, which was not previously reported for HMG1. In the present work, the bands of differing mobility were further investigated through competition experiments in which unlabeled DNA probes were added to compete the protein−DNA complexes. The band of highest mobility, resulting from specific interactions of Ixr1 with the cisplatin−DNA lesion, is the only band competed by addition of unlabeled 92Pt. These results support the previous conclusion, based on an analysis of bending induced by Ixr1 (Chow et al., 1994), that only the fastest migrating band corresponds to a specific complex between protein and platinated DNA.

Binding of Ixr1 to the 92-bp probes afforded distinctive hydroxyl radical digestion patterns for both platinated and unplatinated DNA. Addition of Ixr1 to the platinated 92-bp probe yielded hypersensitive bands similar to those seen for the DNase I footprint of 100-bp DNA containing the *cis*-[$Pt(NH_3)_2${d(GpG)-N7(1)-N7(2)}] cross-link bound to hUBF (Treiber et al., 1994). The most striking similarity to the hUBF result is the size of the protected fragments spanning the hypersensitive sites. hUBF produces a 14-bp fragment centered on the cisplatin−GG adduct (Treiber et al., 1994), and Ixr1 has a 15-bp protection pattern, similarly centered. Hydroxyl radical digestion of the control 92-bp probe in the presence of Ixr1 also shows a 15-bp protection pattern centered around the area of the same guanine residues. A recent study of the effects of pH on HMG1 binding to DNA by fluorescence quenching revealed that the bite size of HMG1 on an unmodified dsDNA probe was 13 bp at pH 7.5 (Kohlstaedt & Cole, 1994), the conditions most similar to those used in the present footprinting study.

The distinct pattern of the hypersensitive bands in the hydroxyl radical footprint of Ixr1 bound to the platinated and unplatinated 92-bp probes indicates a different mode of protein binding of Ixr1. This hypothesis is supported by the results of a recent study involving the use of circularly permuted DNA probes (Chow et al., 1994). This work revealed that the binding of HMG-domain proteins increases the bend already present in Pt−DNA intrastrand cross-links from 34° to values ranging from 68° ± 6° for Ixr1 to 86 ± 2° HMG1 (Chow et al., 1994). In a control experiment performed with HMG1 binding to the 92Ct circularly permuted oligonucleotides, no measurable bend of the unmodified DNA probe could be detected. These data are consistent with the present hydroxyl radical footprint analysis of Ixr1 complexes indicating that HMG-domain proteins bind in a distinct manner to platinated and unplatinated DNA probes.

The HMG Domains of Ixr1 Are Responsible for Its Ability To Affect the Sensitivity of S. cerevisiae Cells to Cisplatin. Experiments were performed in order to determine whether the natural function of the protein as a repressor of Cox5b might contribute to its ability to sensitize cells to cisplatin. Overexpression of Ixr1 in the *ixr1* strain JM43Δixr1 yielded cells that were more sensitive to cisplatin than JM43Δixr1 to which control, unplatinated plasmid has been added. This result indicated that the difference in cytotoxicity between *IXR1* and *ixr1* strains is caused by the lack of the Ixr1 protein.

Several experiments were performed which are consistent with the conclusion that increased expression of CoxVb in the *ixr1* strain was not the cause of its decreased sensitivity to cisplatin. A strain with a mutant Ixr1 protein, *Ixr1-1*, was examined. This strain expresses CoxVb, so if CoxVb expression were able to affect cisplatin cytotoxicity, these cells would be less sensitive than the corresponding wild-type strain. Such was not the case, further confirming that a change in CoxVb expression does not affect cisplatin cytotoxicity. Rox1 is also a transcriptional repressor of CoxVb expression; CoxVb is expressed when either Ixr1 or Rox1 is missing. JM43Δrox1 was as sensitive to cisplatin as JM43. Finally, the cytotoxicity assay was performed under anaerobic conditions. CoxVb is expressed under such hypoxic conditions in both *IXR1* and *ixr1* cells because Rox1 is no longer repressed. The JM43Δixr1 strain remained less sensitive than the JM43 strain in these experiments, a result indicating that no protein differentially expressed in anaerobic cells is responsible for the cisplatin cytotoxicity difference. These results conclusively demonstrate that the increased CoxVb expression in the *ixr1* strains does not affect cisplatin cytotoxicity.

The conclusion that the ability of Ixr1 to affect cisplatin cytotoxicity derives from its HMG domains receives support from several experimental results. Ixr1 binds to cisplatin DNA adducts, as can be seen in the Southwestern blots, and has been quantitated and mapped by footprint analysis. Ixr1 binds to cisplatin−DNA adducts, but not to DNA modified by *trans*-DDP, $[Pt(dien)Cl]^+$, or UV adducts; *IXR1* and *ixr1* strains are equally sensitive to *trans*-DDP and UV light damage (data not shown) (Brown et al., 1993). A homolog of cisplatin which forms similar bifunctional DNA adducts, $[Pt(en)Cl_2]$, is similarly more toxic to *IXR1* than to *ixr1* cells (data not shown). If the mere act of damaging DNA were sufficient to cause the differential cytotoxicity, then the *ixr1* strain should have been less sensitive than the *IXR1* strain to either *trans*-DDP or UV light. Only damaging agents that form adducts to which HMG-domain proteins bind cause *ixr1* strains to be less sensitive than *IXR1* strains. Furthermore, studies with cells containing Ixr1-1 revealed no less sensitivity to cisplatin than the corresponding wild-type cells, JM43. The Ixr1-1 protein lacks the C-terminal domain (Okamoto & Cumsky, 1995) which contains a long stretch of glutamine residues thought to be involved in protein−protein interactions (Latchman, 1991). Since the HMG domains are intact, however, Ixr1-1 can still bind to the major

cisplatin−DNA adducts. Therefore, it is the absence of the HMG domains, not the absence of a putative protein−protein interaction domain, which diminishes cisplatin cytotoxicity in the *ixr1* strains.

The titration hypothesis theorizes that cisplatin−DNA adducts may divert HMG domain proteins such as Ixr1 from their natural binding sites, thus disturbing the transcription of the genes they regulate (Donahue et al., 1990; Lippard, 1994; Treiber et al., 1994). Ixr1 inhibits transcription from the Cox5b promoter. The effect of cisplatin−DNA adducts on transcription from the Cox5b promoter was measured both in a plasmid-based system and by examining Cox5b mRNA levels. Neither experiment yielded any result suggesting that Ixr1 could be removed from its binding site sufficiently to affect transcription from the promoter. Different amounts and types of cisplatin treatment were investigated. Two different sets of yeast strains were examined. Cells were treated in their exponential growth phase for most experiments, but some of the mRNA assays were performed with cells grown to saturation. None of the different cisplatin treatment protocols led to substantially increased expression from the Cox5b promoter in the *IXR1* strains. These experiments demonstrate that the titration hypothesis does not apply for Ixr1 and the Cox5b promoter in vivo. The shielding of cisplatin−DNA adducts from excision repair by Ixr1 thus remains the most likely cause of the difference in cisplatin cytotoxicity of *IXR1* and *ixr1* cells.

CONCLUSIONS

The specific binding of Ixr1 to cisplatin−DNA adducts was determined to be an order of magnitude better than to unplatinated DNA. The titration hypothesis was tested by examining the effects of cisplatin on transcription from the Ixr1-repressed Cox5b promoter. Ixr1 was not titrated away from the Cox5b promoter sufficiently to affect transcription. It is still possible that such behavior might occur for other HMG-domain proteins such as hUBF. The increased expression of CoxVb did not cause decreased sensitivity of the *ixr1* cells. These experiments help define the parameters for devising new cisplatin analogs. Compounds which are bound tightly by HMG-domain proteins or related constructs could be more effective against tumor cells.

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