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27. Single-letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Ixr1, a Yeast Protein That Binds to Platinated DNA and Confers Sensitivity to Cisplatin

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Structure-specific recognition proteins (SSRPs) bind to DNA containing intrastrand cross-links formed by the anticancer drug cisplatin. A yeast gene encoding an SSRP, designated *IXR1*, was cloned and sequenced. The Ixr1 protein, a member of the high mobility group–box protein family, bound specifically to DNA modified with cisplatin but not inactive platinum compounds. A yeast strain with an inactivated *IXR1* gene was half as sensitive to cisplatin and accumulated one-third as many platinum-DNA lesions after treatment with cisplatin as the parental strain. These findings suggest that SSRPs play a role in mediating the cytotoxicity of cisplatin.

The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) is widely used in cancer chemotherapy and is especially effective in the treatment of testicular and ovarian cancer (*1*). We have identified SSRPs that bind to the major cisplatin-DNA adducts, 1,2-intrastrand d(GpG) and d(ApG) cross-links, but not to DNA damaged by inactive platinum compounds such as *trans*-diamminedichloroplatinum(II) (*trans*-DDP) (*2*). The SSRPs contain the recently identified high mobility group (HMG)–box motif (*3*). Several HMG-box proteins bend DNA (*4*, *5*), and we have suggested that HMG-box proteins recognize and bind specifically to locally bent and unwound DNA, such as DNA that has been modified by cisplatin (*6*).

Elucidation of a role for SSRPs in the mechanism of action of platinum antitumor drugs could greatly facilitate the design of new cancer therapies. Several models for the modulation of cisplatin cytotoxicity by SSRPs have been proposed (*7*). One model postulates that when SSRPs bind to platinated DNA they shield the DNA lesions from repair. As a result, the platinum-DNA adducts persist, block DNA replication, and ultimately lead to cell death. In this model, removal of the SSRPs would confer cisplatin resistance to the cell but would have no effect on the cytotoxicity of inactive platinum compounds.

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We have used the yeast *Saccharomyces cerevisiae* to study the role of SSRPs in mediating cisplatin cytotoxicity. We screened a λgt11 yeast expression library with radiolabeled, platinated DNA and isolated one partial clone, λyPt (*8*, *9*). A 3.0-kb Sac I DNA fragment containing the full-length *IXR1* gene was obtained by screening a yeast DNA library (*10*). Sequence analysis revealed a single, large open reading frame (ORF) encoding 591 amino acids. The deduced Ixr1 protein contains several long stretches of Asn and Gln residues (Fig. 1), a feature that is common in transcription factors (*11*, *12*). A database search revealed that Ixr1 is a member of the HMG-box protein family (*3*) and contains two repeated HMG-box motifs within residues 347 to 493. The best alignment of the HMG-box domain of Ixr1 occurs with ABF2 (*ARS*-binding factor 2) from *S. cerevisiae* (*13*), which shares 56 identical residues with Ixr1 over a 151–amino acid region.

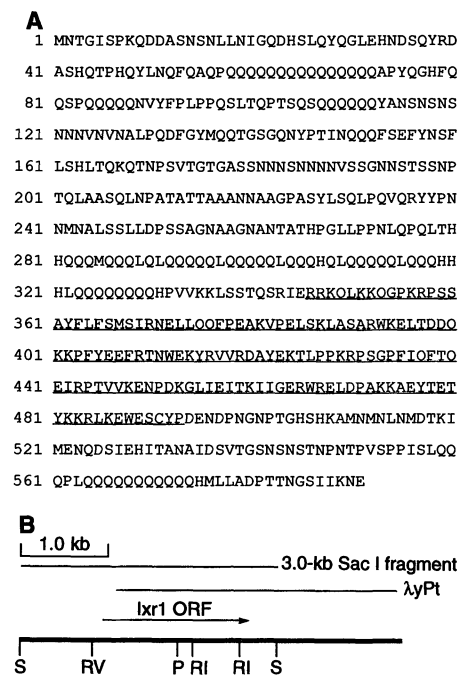

Fig. 1. (**A**) Deduced sequence of the Ixr1 protein. The two consecutive HMG boxes are underlined. Double-stranded plasmid DNA was alkaline denatured for the sequencing reactions. We performed sequencing by the dideoxy chain termination method with T7 DNA polymerase (United States Biochemical). Database homology searches were performed with the BLAST network service provided by the National Center for Biotechnology Information. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The DNA sequence has been submitted to GenBank (accession number L16900). (**B**) Restriction map of the *IXR1* gene. RI, Eco RI; P, Pst I; RV, Eco RV; and S, Sac I.

Ixr1 protein is a single-copy gene localized on yeast chromosome XI (*14*), and Northern (RNA) blot analysis revealed that it produces a 2.1-kb transcript. We disrupted the *IXR1* gene by replacing the Eco RV–Pst I region of the 3.0-kb Sac I fragment with a copy of the *LEU2* gene. A diploid strain (*15*) with a null allele of *IXR1* was obtained, and tetrad analysis demonstrated that the *IXR1* gene product is not essential for cell growth. The *ixr1* haploid cells were not obviously different in phenotype from *IXR1* cells. For example, they displayed similar growth rates at temperatures ranging from 20° to 40°C, and homozygous *ixr1* diploid cells sporulated normally (*16*, *17*).

To investigate the DNA binding properties of Ixr1, we analyzed λyPt *Escherichia coli* lysogen and whole-cell extracts prepared from the wild-type (*IXR1*) and mutant (*ixr1*) yeast strains (*18*) by a modified Western analysis in which the protein blot is probed with radiolabeled DNA (*8*). The λyPt β-galactosidase fusion protein bound to *cis*-DDP–modified DNA and had an apparent molecular mass of 176 kD (Fig. 2). Extracts from wild-type yeast contained several proteins that bound specifically to *cis*-DDP–modified DNA; these proteins were ~100, 80, 55, and 20 kD in size. The 80-kD protein was absent from the *ixr1* strain and, thus, most likely represents the *IXR1* gene product. The λyPt β-galacto-

sidase fusion protein and Ixr1 did not bind to unmodified DNA or to DNA modified with *trans*-DDP (*19*). The ~55-kD protein is probably a degradation product of Ixr1 because its relative abundance varied among different extracts and was inversely correlated with the amount of the 80-kD protein. Moreover, the ~55-kD protein was absent from the *ixr1* extracts. The 20-kD protein is tentatively assigned as ABF2, which also has an HMG-box domain and migrates with an apparent molecular mass of 20 kD (*13*).

We next examined the cytotoxicity of *cis*-DDP in haploid *IXR1* and *ixr1* yeast strains. Over a concentration range of 50 to 1000 μM *cis*-DDP, the *IXR1* strain was twice as sensitive to the drug as the *ixr1* strain lacking the Ixr1 protein (Fig. 3A). In contrast, *trans*-DDP was equally toxic to both strains (Fig. 3B); at the solubility limit of this compound (~3.3 mM), about one-third of the cells survived. As an additional control, we examined the sensitivity of *ixr1* and *IXR1* cells to ultraviolet irradiation. Absence of the Ixr1 protein did not alter the response of yeast to ultraviolet-induced damage (*19*). Disruption of the *IXR1* gene, therefore, did not sensitize yeast to all DNA damaging agents but was specific for cisplatin.

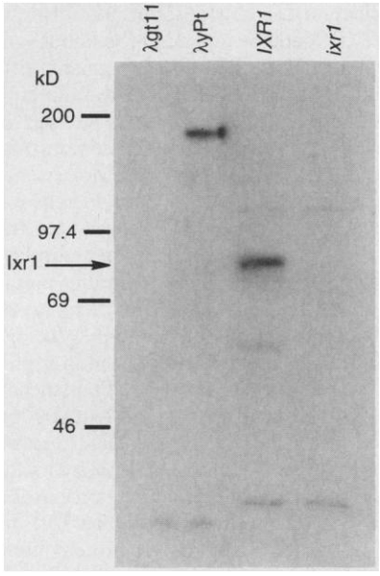

Fig. 2. Modified Western analysis of proteins that bind to *cis*-DDP–modified DNA. Lanes labeled λgt11 and λyPt contain protein (10 μg) from *E. coli* Y1090 phage lysogens induced with isopropyl β-D-thiogalactopyranoside. Lanes labeled *IXR1* and *ixr1* contain protein (50 μg) from yeast whole cell extracts. The samples were resolved by 8% SDS–polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose, and treated as in (*8*). The probe was a 123-bp DNA fragment modified with *cis*-DDP (*23*) at a bound platinum to nucleotide ratio of 0.021.

We also investigated the effect of Ixr1 on the ratio of bound platinum per DNA nucleotide (r_b) in yeast cells treated with cisplatin for 4 hours (*20, 21*). The r_b values for *ixr1* and *IXR1* cells treated with cisplatin were 0.0008 and 0.0024, respectively. The r_b values for yeast treated similarly with *trans*-DDP were 0.0020 for *ixr1* cells and 0.0024 for *IXR1*. Thus, although the absence of the Ixr1 protein had no effect on *trans*-DDP binding to DNA, it diminished the amount of bound cisplatin. The lesser amounts of bound platinum on DNA in the *ixr1* cells correlated with their reduced sensitivity to the drug. These findings support the notion that SSRPs play a role in mediating cisplatin cytotoxicity. The normal function of Ixr1 in yeast is unknown.

The most prevalent cisplatin-DNA lesions are 1,2-intrastrand d(ApG) and d(GpG) cross-links (*22*), both of which are bound specifically by HMG1 (*23, 24*). Interestingly, human cell extracts that are competent for excision repair do not repair a platinated plasmid containing a single d(GpG) intrastrand cross-link (*25*). Similarly, yeast extracts do not remove lesions from cisplatin-modified plasmid DNA (*26*). The inability of eukaryotic cell extracts to repair the cisplatin intrastrand d(GpG) cross-link, and the present observations that *ixr1* cells are more resistant to cisplatin and contain fewer platinum-DNA lesions than *IXR1* cells, are consistent with a model in which SSRPs shield intrastrand cross-links from DNA repair enzymes. We speculate that in mutant cells lacking the Ixr1 protein the shield is absent, thereby allowing repair to occur, and the cells become less sensitive to the drug.

Other interpretations are possible, however. For example, SSRPs may facilitate the formation of toxic cisplatin-DNA lesions, or perhaps the SSRP-cisplatin-DNA ternary complex provides a stronger block to replication or transcription or both than do cisplatin-DNA adducts alone. Although the levels of SSRP1 mRNA in human tissues do not correlate with the tissue specificity of the drug (*6*), the platinated DNA binding properties of SSRP1 might be modulated in various tissues, for example, by post-translational modification or by the formation of complexes with other DNA binding proteins. In addition, other SSRPs may contribute to the activity of cisplatin.

Finally, these results predict that one could sensitize cells to cisplatin by increasing the concentration of the SSRPs. Indeed, a two- to threefold increase in sensitivity would be of clinical significance for cisplatin-resistant human tumors (*27*).

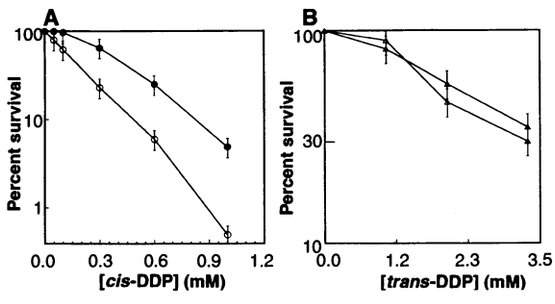

Fig. 3. Cytotoxicity of (**A**) *cis*-DDP on *IXR1* (○) and *ixr1* (●) yeast strains, and (**B**) *trans*-DDP on *IXR1* (△) and *ixr1* (▲) yeast strains. Yeast strains SB2 (*ixr1*) and SB3 (*IXR1*) were grown to saturation in complete media (YPD). The cells were washed twice and resuspended in synthetic minimal (SD) media. Approximately 2×10^7 cells in 1.0 ml of SD media were treated with *cis*- or *trans*-DDP at the indicated concentration. The cells were incubated in the drug solution for 2 hours at 30°C with constant shaking, washed once with SD media, and plated on solid YPD media. We incubated the plates for 2 days at 30°C before scoring them for colony formation. Each point is an average ± SD of three plates.

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9. The λgt11 yeast expression library was screened with radiolabeled *cis*-DDP–modified DNA as in (*8*) except that duplicate lifts were taken in the second and third rounds of screening and the second set of filters was not treated with the guanidinium chloride denaturation buffer. After the primary round of screening, all protein replica filters were prepared in parallel and screened with either unmodified or *cis*-DDP–modified DNA. We picked 25 positive clones in the first round. After three rounds of screening, only two clones remained positive for binding to *cis*-DDP–modified probe DNA, and these were plaque purified to homogeneity in a fourth round of screening. Restriction digestion analysis revealed them to be identical clones, which we refer to as λyPt. Digestion with Eco RI of λyPt DNA released three fragments of 1.7, 1.1, and 0.6 kb; these fragments were subcloned into the Eco RI site of pBluescript II SK(+), yielding plasmids pSB1, pSB2, and pSB3, respectively. The relative orientation of the fragments was determined by Southern (DNA) blot analysis and direct sequencing of λyPt DNA.
10. We prepared a DNA library by cloning a Sac I digest of yeast genomic DNA into the Sac I site of pBluescript II SK(+). We screened this library with ^{32}P-labeled DNA from the 0.6-kb Eco RI fragment of pSB3 and isolated a plasmid (pSB4) with a 3.0-kb insert containing the full-length *IXR1* gene. "IXR" denotes intrastrand cross-link recognition.
11. The sequence was assembled with the UWGCG

program package [J. Devereux, P. Haberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984)].
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14. A yeast chromosome filter (Clontech) was screened with a radiolabeled probe made from the 0.6-kb Eco RI fragment.
15. Genotypes of yeast strains used in this study are the following: AA295, *MATa/MATα ura3-52/ura3-52 ade2/ade2 +/trp1-1 lys2Δ201/lys2Δ201 his3-200/his3-200 leu2-3,112/leu2-3,112;* SB1, *MATa/MATα ixr1-1::LEU2/+ ura3-52/ura3-52 ade2/ade2 +/trp1-1 lys2Δ201/lys2Δ201 his3-200/his3-200 leu2-3,112/leu2-3, 112;* SB2, *MATa ixr1-1::LEU2 ura3-52 ade2 trp1-1 lys2Δ201 his3-200;* and SB3, *MATa ura3-52 ade2 trp1-1 lys2Δ201 his3-200 leu2-3,112.*
16. Media were prepared and sporulation and tetrad dissections carried out as in (*17*). Yeast transformations were performed by the lithium acetate method [H. Ito, Y. Fudada, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)]. To obtain strain SB1, strain AA295 [H. K. Rudolph *et al.*, *Cell* **58**, 133 (1989)] was transformed to leucine prototrophy with a disrupted copy of the *IXR1* gene. We disrupted the *IXR1* gene by replacing by Eco RV–Pst I insert present in the 3.0-kb Sac I fragment carrying the *IXR1* gene with a DNA fragment containing a copy of the *LEU2* gene. Southern blot analysis confirmed the *IXR1* gene deletion. Strains SB2 and SB3 were grown from spores from dissected tetrads.
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18. Yeast extracts were prepared from cells grown to mid-logarithmic phase. The pelleted cells were resuspended in 3.5 volumes of lysis buffer [50 mM tris HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM potassium acetate, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride] and lysed in a French pressure cell at ~15,000 psi. An equal volume of extraction buffer [lysis buffer + 0.8 M $(NH_4)_2SO_4$] was added to the lysate and the mixture rocked gently for 15 min. After centrifugation at 35,000 rpm for 30 min in a Sorvall 45Ti rotor, the supernatant was dialyzed against several changes of storage buffer [20 mM tris-HCl (pH 7.5), 100 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride] and flash frozen in liquid nitrogen. During the extraction, all materials and samples were kept at 4°C.
19. S. J. Brown, P. J. Kellet, S. J. Lippard, unpublished data.
20. Cells grown to saturation in synthetic complete media were treated for 4 hours at 30°C with 10 μM *cis-* or *trans*-DDP and then harvested. We prepared DNA as in (*21*). Platinum concentrations were determined by flameless atomic absorption spectroscopy with a Varian AA1475 instrument equipped with a GTA95 graphite furnace. Concentrations of DNA were measured by ultraviolet spectroscopy.
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A Detailed Genetic Map for the X Chromosome of the Malaria Vector, *Anopheles gambiae*

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***Anopheles gambiae*, the primary vector of human malaria in Africa, is responsible for approximately a million deaths per year, mostly of children. Despite its significance in disease transmission, this mosquito has not been studied extensively by genetic or molecular techniques. To facilitate studies on this vector, a genetic map has been developed that covers the X chromosome at an average resolution of 2 centimorgans. This map has been integrated with the chromosome banding pattern and used to localize a recessive, sex-linked mutation (*white eye*) to within 1 centimorgan of flanking markers.**

We describe the development of a detailed genetic map of the principal malaria vector, *Anopheles gambiae*, using microsatellite markers (*1*). Microsatellites are tandem arrays of simple sequence repeats and have the advantage of being abundant, widely dispersed in the genomes of humans and other higher organisms, extensively polymorphic, and easily assayed by the polymerase chain reaction (PCR) (*1*). Despite the difficulties in maintaining inbred strains and performing crosses in anopheline mosquitoes, the advantages of microsatellites greatly facilitated construction of a map consisting of 24 sex-linked microsatellite markers. This map is anchored to the *white eye* (*w*) mutation (*2*) and is usable with any of the *An. gambiae* strains examined to date (*3*).

To generate the map, we isolated clones containing dinucleotide repeats from libraries of total adult mosquito genomic DNA (*4*) or chromosomal division-specific DNA pools (*5*) by screening with oligonucleotide probes: $(GT)_{15}$, or more rarely $(CA)_{15}$ or $(GA)_{15}$. Tandem arrays of 5 to 48 repeats were revealed by sequencing in 125 $(GT)_{15}$-positive clones, some of which contained interrupted arrays. For selected clones, pairs of primers, usually 20 nucleotides in length and based on unique sequences flanking the repeat array, were designed, synthesized, and used to amplify by PCR the corresponding genomic DNA fragment (*6*) from individual mosquitoes of five *An. gambiae* strains (*3*). The sizes of the amplified fragments were evaluated by electrophoresis in a sequencing gel. Markers encompassing as few as 6 dinucleotide repeats were found to be polymorphic between strains, the size difference ranging from 1 to more than 30 repeats. The inter-strain polymorphism between two strains, Suakoko and WE, was on the order of 85%, and within-strain polymorphism was also observed for many markers.

We constructed the genetic map and simultaneously mapped the *w* mutation by performing crosses involving the WE strain (homozygous for *w*) (*3*) and the Suakoko strain (homozygous for w^+, the dominant allele for the wild-type dark purple eye color). These strains are largely homosequential, that is, they do not seem to differ in terms of gross chromosomal inversions on the X. F_1 females were generated by mass mating between WE males and Suakoko females and were individually backcrossed with WE males by forcible pair mating (*7*). From such pairs five families (A to E) totaling 248 progeny were obtained; they contained 45, 63, 76, 13, and 51 progeny, respectively. Chromosomal DNA was prepared from each mosquito (*8*), and small portions (typically corresponding to 1/200 of a mosquito) were amplified with a pair of primers (*6*) and scored for the corresponding marker on a sequencing gel. All alleles (electrophoretic bands) behaved as codominant Mendelian factors. For 24 markers, the male progeny received one of the maternal (F_1) alleles and no paternal (WE) allele, indicating linkage to the X chromosome. These markers were designated AGXH (*An. gambiae* X chromosome, Harvard) followed by a clone number; they are identified in Table 1. The AGXH prefix will be omitted hereafter for convenience. Genotypic scoring for one of the markers is shown in Fig. 1 and the results of scoring family A for all loci are summarized in Table 2.

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