0026-895X/03/6402-259–268$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 64:259–268, 2003

Vol. 64, No. 2
2296/1083442
Printed in U.S.A.

Anticancer Drug Resistance Induced by Disruption of the *Saccharomyces cerevisiae NPR2* Gene: a Novel Component Involved in Cisplatin- and Doxorubicin-Provoked Cell Kill

PAUL W. SCHENK, MARIËL BROK, ANTONIUS W. M. BOERSMA, JOURICA A. BRANDSMA, HANS DEN DULK, HERMAN BURGER, GERRIT STOTER, JAAP BROUWER, and KEES NOOTER

Section of Experimental Chemotherapy, Department of Medical Oncology, Erasmus University Medical Center Rotterdam, Josephine Nefkens Institute, Rotterdam, the Netherlands (P.W.S., M.B., A.W.M.B., H.B., G.S., K.N.); and Medical Genetics Centre South-West Netherlands, Department of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Leiden, the Netherlands (J.A.B., H.D.D., J.B.)

Received November 20, 2002; accepted May 9, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The therapeutic potential of antitumor drugs is seriously limited by the manifestation of cellular drug resistance. We used the budding yeast *Saccharomyces cerevisiae* as a model system to identify novel mechanisms of resistance to one of the most active anticancer agents, cisplatin. We pinpointed *NPR2* (nitrogen permease regulator 2) as a gene whose disruption conferred resistance to cisplatin. In addition, we observed a 4-fold cross-resistance of yeast *npr2*Δ cells (i.e., cells from which the *NPR2* gene had been disrupted) to the anticancer drug doxorubicin, in combination with hypersensitivity to cadmium chloride. Furthermore, *npr2*Δ cells displayed unaltered cellular cisplatin and doxorubicin accumulation and showed an enhanced rate of spontaneous mutation compared with the isogenic parent. These data indicate that the *npr2*Δ phenotype overlaps that of the *sky1*Δ cells that we characterized previously (*Mol Pharmacol* **61:**659–666, 2002). Therefore, we generated yeast *npr2*Δ *sky1*Δ double-knockout cells and performed clonogenic survival assays for cisplatin and doxorubicin, which revealed that *NPR2* and *SKY1* (SR-protein-specific kinase from budding yeast) are epistatic. The double-knockout strain was just as resistant to cisplatin and doxorubicin as the single-knockout strain that was most resistant to either drug. In conclusion, we identified *NPR2* as a novel component involved in cell kill provoked by cisplatin and doxorubicin, and our data support the hypothesis that *NPR2* and *SKY1* may use mutual regulatory routes to mediate the cytotoxicity of these anticancer drugs.

The overall results of treatment of metastasized tumors with anticancer drugs are rather disappointing, because of intrinsic and acquired drug resistance. One of the most commonly applied anticancer agents is cisplatin, which forms platinum-DNA adducts that play an important role in the cytotoxicity of the drug. Platinum-based chemotherapy is curative for most patients with advanced testicular cancer (Einhorn, 1997) and active against ovarian, bladder, cervical, head and neck, and lung cancers (Perez, 1998). Regrettably, cellular resistance to cisplatin, either at the onset of treatment or at relapse, is frequently encountered and seriously limits its therapeutic potential (Perez, 1998; Niedner et al., 2001). In vitro studies have revealed numerous resistance mechanisms, including reduced intracellular accumulation, detoxification by glutathione or metallothioneins (resulting in decreased DNA platination), alterations in DNA repair, increased tolerance, and aberrations in pathways modulating programmed cell death (Perez, 1998; Niedner et al., 2001). Unfortunately, the mechanisms identified so far do not satisfactorily explain the unresponsiveness of particular tumors to platinum-based chemotherapy observed in the clinic. Therefore, additional cisplatin resistance pathways (for which the genes involved still need to be pinpointed) may exist.

We are using the budding yeast *Saccharomyces cerevisiae* as a model system for drug sensitivity and resistance in higher eukaryotes. Over the years, yeast has proven its value as an accessible tool to gain a better understanding of complicated and often integrated mechanisms such as recombi-

This work was supported in part by grant DDHK 97-1397 from the Dutch Cancer Society.

Preliminary parts of this work were presented in abstract form at the 92nd Annual Meeting of the American Association for Cancer Research; 2001 March 24–28; New Orleans, LA [Schenk PW, Boersma AWM, Brok M, Brandsma JA, Den Dulk H, Brouwer J, Burger H, Stoter G, and Nooter K (2001) Cisplatin resistance in vitro: possible sensitivity genes in translational research from yeast to man. *Proc Am Assoc Cancer Res* **42:**abstract 4998].

ABBREVIATIONS: NER, nucleotide excision repair; YNB, yeast nitrogen base; YPD, yeast extract/peptone/dextrose; PCR, polymerase chain reaction; *NPR2*, nitrogen permease regulator 2; *SKY1*, SR-protein-specific kinase from budding yeast; SRPK1, SR-protein-specific kinase; kb, kilobase(s); AAS, atomic absorption spectrometry; HPLC, high-performance liquid chromatography; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MMR, mismatch repair.

TABLE 1
Yeast strains used in this study
Strains CMY375 and BUH3 were kindly provided by Dr. G. Rousselet. BUH3 cells were derived from CMY375 parental cells by ethylmethylsulfonate mutagenesis. It was subsequently demonstrated that introduction of *NPR2* on a low-copy plasmid fully restores a wild-type phenotype in BUH3 cells (Rousselet et al., 1995).

Strain	*Saccharomyces cerevisiae* Genotype	Reference / Source
MGSC131	*MATα ho ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad4Δ::his*G-*URA3-his*G	Verhage et al. (1996)
BY4742	*MATα his3-1 leu2 lys2 ura3*	ATCC (Manassas, VA)
npr2Δ	BY4742 with YEL062W::*kanMX4*	ResGen Invitrogen Corp. (Huntsville, AL)
CMY375	*MATα CUP1 gal2 mal SUC2 ura3-52*	Rousselet et al. (1995)
BUH3	CMY375 *npr2*	Rousselet et al. (1995)
dur3Δ	BY4742 with YHL016C::*kanMX4*	ResGen Invitrogen Corp. (Huntsville, AL)
npr2Δ sky1Δ	*npr2Δ with sky1Δ::lox-URA3-lox*	This study
BY-*sky1Δ*	BY4742 with *sky1Δ::lox-URA3-lox*	This study

nation, DNA repair, and checkpoint control in mammalian cells. Human genetic defects can regularly be addressed directly in yeast because of the evolutionary conservation of genes and signal transduction pathways (Resnick and Cox, 2000). With regard to the cellular response to anticancer agents, there are many fine examples that illustrate the power of yeast as a model organism for mammalian cells. To mention a few, the *Saccharomyces cerevisiae* pleiotropic drug resistance network comprises homologs to the human ATP-binding cassette-type transporters P-glycoprotein and MRP and is an important laboratory model for the clinical problem of multidrug resistance (Kolaczowska and Goffeau, 1999). The yeast nucleotide excision repair (NER) system has been instrumental in the study of NER in mammalian cells (Prakash and Prakash, 2000; Resnick and Cox, 2000). It has long been known that *S. cerevisiae* NER mutants are hypersensitive to cisplatin (Abe et al., 1994). In line with this, the involvement of NER in cisplatin resistance in patient-derived material has been observed repeatedly (Crul et al., 1997), whereas decreased NER may contribute to the high cisplatin sensitivity of testis tumors (Köberle et al., 1999). The predictive value of yeast in the study of cellular drug resistance in humans is also illustrated by our own research. In previous studies (Schenk et al., 2001; Schenk et al., 2002), we identified *SKY1* (SR-protein-specific kinase from budding yeast) as a cisplatin sensitivity gene. Its abrogation conferred cisplatin resistance in yeast, and down-regulation of its human homolog SRPK1 (SR-protein-specific kinase) led to cisplatin resistance in an ovarian carcinoma cell line (Schenk et al., 2001). Importantly, we recently monitored SRPK1 protein expression in a series of testicular germ cell tumors, and found that the expression in tumors from chemorefractory patients was significantly lower than in tumors from patients responding to platinum-based chemotherapy (P. W. Schenk H. Stoop, F. Mayer, C. Bokemeyer, G. Stoter, J. W. Oosterhuis, L. H. J. Looijenga, and K. Nooter, manuscript in preparation[1]). This indicates that the identification of *SKY1* as a cisplatin sensitivity gene in *S. cerevisiae* may ultimately be of importance to predict the chemoresponsiveness of particular tumors in the clinic.

Our present data indicate that *NPR2* (nitrogen permease regulator 2) is a novel yeast drug sensitivity gene whose abrogation leads to resistance not only to cisplatin but also to another anticancer agent, doxorubicin. The overall phenotype that we show for *S. cerevisiae npr2Δ* cells (i.e., cells from which the *NPR2* gene had been disrupted) is highly reminiscent of our previous data for *sky1Δ* cells (Schenk et al., 2002). Therefore, we generated *npr2Δ sky1Δ* double-knockout cells and found that *NPR2* and *SKY1* are epistatic. The cisplatin and doxorubicin resistance of the double-knockout cells was neither additive nor synergistic compared with the single-knockout cells, suggesting that *NPR2* may act in mutual regulatory routes with *SKY1*.

Materials and Methods

Chemicals. Yeast nitrogen base (YNB), Bacto-agar and yeast extract/peptone/dextrose (YPD) broth were obtained from DIFCO Laboratories (Detroit, MI). Cisplatin [*cis*-diamminedichloroplatinum(II) (Platinol; Platosin)] and doxorubicin hydrochloride (Adriamycin; Doxorubin) were purchased from Pharmachemie (Haarlem, The Netherlands). Oxaliplatin [[*trans*-(L)-1,2-diaminocyclohexane]oxalatoplatinum(II) (Eloxatin)] was obtained from Sanofi-Synthelabo (Maassluis, The Netherlands). Etoposide (Vepesid) was from Bristol-Myers Squibb (Woerden, The Netherlands), 5-fluorouracil (Fluorouracil-TEVA; Adrucil) from TEVA Pharma (Mijdrecht, The Netherlands), and copper sulfate from Merck (Darmstadt, Germany). Other chemicals including amino acids and additional cytotoxic agents were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Yeast Strains and Culture Conditions. *S. cerevisiae* strains used in this study are listed in Table 1. All yeast strains were routinely maintained on selective synthetic YNB medium [0.67% YNB and 2% D-(+)-glucose]. Where appropriate, media were solidified by the addition of 2% Bacto-agar and supplemented with amino acids depending on the auxotrophic requirements. *S. cerevisiae* was grown at 30°C under vigorous shaking for liquid cultures. *Escherichia coli* strains MC1061 and DH5α were used as bacterial hosts for plasmids.

Yeast Random Transposon Insertion Library, Screening Strategy, and Characterization of Cisplatin-Resistant Strains. A yeast genomic mini-Tn*3*::*lacZ*::*LEU2* transposon insertion library (a kind gift from Dr. P. B. Ross-Macdonald) (Burns et al., 1994) was amplified in *E. coli* strain MC1061 and transformed into leucine-deficient yeast cells to randomly disrupt genes in the *S. cerevisiae* genome as described previously (Schenk et al., 2001). Homologous recombination between yeast DNA transposon flanking sequences and endogenous genomic sequences is thought to yield transformants in which the original genomic copy has been replaced by the mutagenized version (Rothstein, 1991). NER-deficient *rad4Δ* yeast strain MGSC131 (Verhage et al., 1996) was used as recipient, because it displays a steep dose-response curve to cisplatin. A total of

[1] The data on SRPK1 expression in germ cell tumors were presented in Plenary Session 2 of the 14th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics; 2002 Nov 19–22; Frankfurt, Germany [Schenk PW, Boersma AWM, Brok M, Brandsma JA, Den Dulk H, Burger H, Brouwer J, Stoter G and Nooter K (2002) Mechanisms of cisplatin resistance—role of yeast SKY1 and its human homolog SRPK1. *Eur J Cancer* **38**(Suppl. 7)**:**15.].

3×10^5 leucine-proficient *S. cerevisiae* MGSC131 transformants were replated at a density of 10^4 cells per 94-mm dish on selective YNB containing 4 μg/ml cisplatin, and colonies surviving this one-step drug selection were picked and retested in semiquantitative spot assays and quantitative clonogenic survival assays as described previously (Schenk et al., 2001). Upon confirmation of resistance phenotypes, the number of transposons per yeast strain was determined by Southern blotting. For the strains containing single insertions, inverse PCR (Ochman et al., 1988) employing *Bst*YI and outward-directed primers complementary to mini-Tn*3*::*lacZ*::*LEU2* was directly used to obtain sequences flanking the transposon elements. Suitable PCR products were purified and sequenced using transposon-specific primers, and sequences were finally analyzed employing public databases (Schenk et al., 2001).

Plasmid Construction and Transformation. The low-copy yeast expression vector pYCTEF111 containing the *LEU2* auxotrophic marker was obtained by ligating the 5.8-kb *Pvu*II backbone fragment from centromeric plasmid YCplac111 (Gietz and Sugino, 1988) to the 1.0-kb *Pvu*II fragment from pYCTEF (Schenk et al., 2001), containing the constitutive translation elongation factor 1α promoter. A 1.9-kb *NPR2* PCR product was obtained employing primers 5′NPR2-S (5′-GAC TAG CCC GGG CTC TAC TAA AGG GAA TGG TCA G-3′) and 3′NPR2-S (5′-GAC TTG AGT CGA CGA ATT TCT CTA ATT TTA ACT CAG C-3′). This fragment was restricted with *Sma*I and *Sal*I and cloned into *Sma*I/*Sal*I-digested pYCTEF111, yielding pYCTEF111-*NPR2*. After propagation in *E. coli* subcloning efficiency DH5α competent cells (Invitrogen, Breda, The Netherlands), the *NPR2* expression plasmid and the empty vector were transformed into the appropriate yeast strains.

Cytotoxicity Assays. Sensitivity to cytotoxic chemicals was determined by a semiquantitative spot assay as described previously (Burger et al., 2000). Briefly, *S. cerevisiae* cells from freshly streaked plates were inoculated and grown to mid-log phase in selective liquid YNB medium. Different aliquots of cells (as indicated in the figure legends) were then spotted onto selective YNB plates containing various compound concentrations and incubated at 30°C for 3 to 4 days. Sensitivity to ionizing radiation was tested by a similar assay, during which spotting of the yeast cells was immediately followed by irradiation with increasing doses of γ-rays using opposing ^{137}Cs sources (Gamma Cell 40; Atomic Energy of Canada, Ottawa, Canada) at a rate of 1.06 to 1.08 Gy/min. Sensitivity to ultraviolet light was also tested by a semiquantitative spot assay as described previously (Burger et al., 2000), using a germicidal lamp at 254 nm. Sensitivity to cisplatin and doxorubicin was analyzed in detail by a quantitative clonogenic survival assay. Serial dilutions of mid-log phase yeast cells were plated onto selective YNB containing various drug concentrations. Plates were incubated at 30°C for 3 to 4 days, colonies were counted, and percentage survival was calculated based on the number of colonies arising in the absence of cytotoxic agents (Burger et al., 2000; Schenk et al., 2002). Where appropriate, the slopes of the log-linear concentration-survival curves were determined by linear regression analysis using SPSS 10.1 (SPSS Inc., Chicago, IL).

Determination of Cellular Platinum and Doxorubicin Accumulation. Cellular platinum content was determined by atomic absorption spectrometry (AAS) using a flameless spectrometer (type 4110 ZL; PerkinElmer Analytical Instruments, Shelton, CT) as described previously (Burger et al., 2000). A total of 2×10^7 exponentially growing yeast cells were incubated in 5 ml of selective liquid YNB medium containing various cisplatin concentrations for 18 h. After drug exposure, cells were immediately washed three times, and 3×10^7 cells were pelleted and completely lysed in 100 μl of chloroform. After evaporation of residual chloroform, samples were dissolved in 0.2% nitric acid and subjected to AAS to determine total platinum content. Cellular doxorubicin content was measured by high-performance liquid chromatography (HPLC) as described previously (de Bruijn et al., 1999). A total of 8×10^6 exponentially growing yeast cells were incubated in 2 ml of selective liquid YNB medium per Vacutainer tube with Hemogard closure (BD Clinical Laboratory Solutions, Franklin Lakes, NJ) containing various doxorubicin concentrations for 18 h. After drug exposure, cells were immediately washed three times in ice-cold medium, and 1.5×10^7 cells were pelleted and dissolved in drug-free human plasma. Samples were finally subjected to pretreatment and HPLC as described previously (de Bruijn et al., 1999) to determine total doxorubicin content.

Determination of Mutation Rates. The frequency of spontaneous mutations was assessed by a forward mutation rate assay that detects genetic alterations inactivating the arginine permease gene (Tishkoff et al., 1997) [i.e., conversion of the *CAN1*$^+$ to the *can1* (canavanine resistance) mutant phenotype]. A total of 11 parallel cultures per strain were inoculated at low density from freshly streaked plates and grown to stationary phase in liquid YPD broth at 30°C. The viable titers and numbers of canavanine-resistant mutants were then determined by plating different aliquots on YPD broth and synthetic medium containing 40 μg/ml canavanine sulfate, respectively. Mutation rates were finally calculated from the resulting median mutant frequencies by iteration according to Drake (1991).

Generation of *npr2*Δ *sky1*Δ Cells. A *SKY1* disruption was generated in *npr2*Δ and BY4742 cells using a *sky1*Δ::*lox-URA3-lox* geneblaster (i.e., a 5′ *SKY1* flank-*lox-URA3-lox*-3′ *SKY1* flank fragment). First, the flanking sequences of the *SKY1* gene were amplified separately from yeast genomic DNA by PCR, employing primers dis1 (5′-CCG AAG CCA TTG TAG GGG AG-3′) and dis2 (5′-CAT **GGT GAC C**AA TTA TTT CTC AGC GCC AGG TG-3′) for the 5′-flank and dis3 (5′-CAT **GGT TAC C**TT TAT TTT GCC CTT GCC TTT T-3′) and dis4 (5′-GCC ACA ACG GTC GCA AAG TC-3′) for the 3′-flank. *Bst*EII-specific bases are indicated in bold. The resulting products, containing different *Bst*EII sites, were then digested with *Bst*EII and ligated to a 1.3-kb *Bst*EII fragment containing a *URA3* cassette flanked by *loxP* sequences (Jansen et al., 2002). Finally, the entire geneblaster was amplified by PCR using primers dis1 and dis4 and transformed into *S. cerevisiae npr2*Δ and BY4742 cells to obtain *npr2*Δ *sky1*Δ and BY-*sky1*Δ cells, respectively. In the resulting yeast cells, *SKY1* sequences were deleted from −72 to + 2242 relative to the 2229-bp open reading frame. Disruptions of *NPR2* and/or the *SKY1* gene in *npr2*Δ, *npr2*Δ *sky1*Δ, and BY-*sky1*Δ cells were checked by PCR.

Results

Isolation and Characterization of Cisplatin-Resistant Yeast Strains. To identify yeast cisplatin sensitivity genes (whose disruption confers resistance to the drug), we transformed haploid, NER-deficient, cisplatin-sensitive *S. cerevisiae* MGSC131 cells with a yeast genomic mini-Tn*3*::*lacZ*::*LEU2* transposon insertion library (Burns et al., 1994) and screened 3×10^5 transformants for cisplatin resistance as described previously (Schenk et al., 2001). In line with the low level of acquired resistance (1.5- to 3-fold) emerging in tumor samples or cell lines obtained before and after cisplatin treatment of patients (Andrews et al., 1990; Niedner et al., 2001), we selected nine library-derived yeast strains that were 2- to 4-fold cisplatin-resistant. These strains were characterized further. The sites flanking the transposon elements (i.e., the loci that had been disrupted in these strains) were identified by means of inverse PCR (Ochman et al., 1988) and sequencing, followed by comparison with public databases. As described previously (Schenk et al., 2001), the YMR216C locus corresponding to the *SKY1* gene had been disrupted in five transposon-containing yeast strains, which were all about 4-fold cisplatin-resistant compared with isogenic *SKY1*$^+$ cells.

In one of the remaining library-derived strains, the single

transposon insertion was located between the YEL063C and the YEL062W locus corresponding to the *CAN1* and the *NPR2* gene, respectively. In this region, *CAN1* and *NPR2* have a common 5′-untranscribed sequence that may represent a shared functional regulatory segment (Rousselet et al., 1995). Because the original MGSC131 strain carries a *can1-100* mutation (Verhage et al., 1996), it seems very unlikely that the cisplatin resistance in the transposon containing strain would have resulted from deregulation of *CAN1* instead of the *NPR2* gene. The Npr2p gene product is thought to be a regulatory protein for nitrogen permeases, which may act at both the transcriptional and post-transcriptional levels (Rousselet et al., 1995). We tested the transposon-derived mutant in a clonogenic survival assay (Fig. 1A) and found that it was 2-fold cisplatin-resistant compared with untransformed parental MGSC131 cells.

Characterization of Independent Mutant Cells. To exclude that the observed cisplatin-resistant phenotype of the transposon containing yeast strain might have arisen from unrelated mutations induced during the screening procedure (i.e., cisplatin-induced mutations instead of library-derived *NPR2* disruption leading to resistance) or that NER deficiency might be involved, we obtained an independent disruption mutant. *S. cerevisiae npr2*Δ cells were made by disruption of the *NPR2* gene in the repair-proficient *RAD*$^+$ strain BY4742, which also contains an intact *CAN1/NPR2* untranscribed regulatory segment and *CAN1* coding region (Table 1). Analogous to the original transposon-containing strain, *npr2*Δ cells were also cisplatin-resistant compared with isogenic BY4742 cells (Fig. 1B). In addition, we tested independent BUH3 *npr2* cells that had been generated in a different repair-proficient, *CAN1*$^+$ background (Table 1). These *npr2* mutant cells (Rousselet et al., 1995) were also cisplatin-resistant compared with their isogenic parent CMY375, with a similar level of resistance (Fig. 1C). The data thus clearly indicate that the observed cisplatin resistance was linked to disruption of the *S. cerevisiae NPR2* gene, without requirement for NER deficiency or deregulation of the *CAN1* gene.

Cross-Resistance of Yeast Cells Containing a Disrupted *NPR2* Gene. To learn more about the mechanisms that may underlie the cisplatin resistance phenotype and to determine its specificity, we monitored *npr2*Δ mutants for cross-resistance to other cytotoxic agents. Semiquantitative spot assays were performed using a range of different classes of chemicals (mostly anticancer drugs), including the cisplatin analog oxaliplatin, heavy metals (cadmium chloride, zinc chloride, and copper sulfate), the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), the antimetabolite 5-fluorouracil, and topoisomerase II inhibitors (doxorubicin and etoposide). In addition, we assessed possible cross-resistance to ionizing radiation and ultraviolet light. Interestingly, disruption of *NPR2* conferred resistance not only to cisplatin but also to the anthracycline doxorubicin (Table 2; Fig. 2), with a level of resistance in the 4-fold range. However, sensitivity toward the cisplatin analog oxaliplatin and another topoisomerase II inhibitor, etoposide, was unaltered. Moreover, we did not observe changes in sensitivity to zinc chloride, copper sulfate, MNNG, 5-fluorouracil, ionizing radiation, or ultraviolet light either. Notably, *npr2*Δ mutants were hypersensitive toward cadmium chloride. In Fig. 2, representative examples of the spot assays performed are shown.

Complementation of Yeast *npr2*Δ Cells with an Expression Plasmid Harboring NPR2. To further confirm that *NPR2* is involved in the cytotoxicity induced by anticancer agents, its coding region was cloned into the low-copy *S. cerevisiae* expression vector pYCTEF111. The resulting plasmid was transformed into *npr2*Δ cells, and the cisplatin and doxorubicin sensitivity of the transformed strain was determined versus the appropriate controls. Upon transformation of plasmid pYCTEF111-*NPR2* (harboring *NPR2* under the constitutive translation elongation factor 1α promoter) into *npr2*Δ cells, the cisplatin- and doxorubicin-resistant phenotype was fully complemented. The cells became as sensitive to cisplatin (Fig. 3A) and doxorubicin (Fig. 3B) as strain BY4742 transformed with the empty vector, whereas transformation with pYCTEF111 alone left the *npr2*Δ cells clearly drug-resistant. These data affirm that the observed cisplatin and doxorubicin resistance was linked to disruption of the *NPR2* gene, and indicate that the corresponding gene product may be actively involved in the cytotoxicity of these drugs (i.e., that *NPR2* is a cisplatin and doxorubicin sensitivity gene).

Cisplatin and Doxorubicin Sensitivity of Yeast Cells Containing a Disrupted *DUR3* Gene. The most likely downstream target of *NPR2* is the *S. cerevisiae DUR3* gene, encoding a putative transmembrane component required for active transport of urea (ElBerry et al., 1993). Rousselet et al. (1995) showed that the level of *DUR3* mRNA in BUH3 *npr2* cells is strongly increased, indicating that *NPR2* might code for a transcriptional regulator of *DUR3* (see *Discussion*). Furthermore, several lines of evidence suggest that the Npr2p protein is also involved in the regulation of the nitrogen permease Dur3p at the post-transcriptional level (Rousselet et al., 1995). Therefore, we initially hypothesized that *NPR2* might mediate cisplatin- and doxorubicin-induced cell kill through a *DUR3*-dependent pathway. To assess the possible role of *DUR3* in anticancer drug resistance, we obtained *dur3*Δ disruption mutant cells derived from strain BY4742 (Table 1) and determined their sensitivity to cisplatin and doxorubicin. In contrast to *npr2*Δ cells, *dur3*Δ cells did not show altered drug sensitivities, compared with their isogenic parent (Fig. 4). This finding implies that *DUR3* is not involved in the cytotoxic action of cisplatin or doxorubicin.

Platinum and Doxorubicin Accumulation in *npr2*Δ Mutants. To determine whether the resistant phenotype of *npr2*Δ cells might be linked to impaired drug accumulation, we monitored cellular platinum and doxorubicin content by AAS (Burger et al., 2000) and HPLC (de Bruijn et al., 1999), respectively. For both agents, we found a clear dose-effect relationship between drug exposure and cellular accumulation. However, *S. cerevisiae npr2*Δ cells did not display reduced platinum or doxorubicin accumulation compared with isogenic *NPR2*$^+$ cells (Fig. 5). Therefore, the observed cisplatin and doxorubicin resistance can probably not be explained by decreased drug import or increased drug export.

Yeast *npr2*Δ Cells Display a Mutator Phenotype. Interestingly, the pattern of drug resistance and hypersensitivity that we found for *S. cerevisiae npr2*Δ cells is highly reminiscent of our previous data for *sky1*Δ cells (Schenk et al., 2002). *Sky1*Δ cells also displayed cross-resistance to cisplatin (but not oxaliplatin) and doxorubicin, in combination with

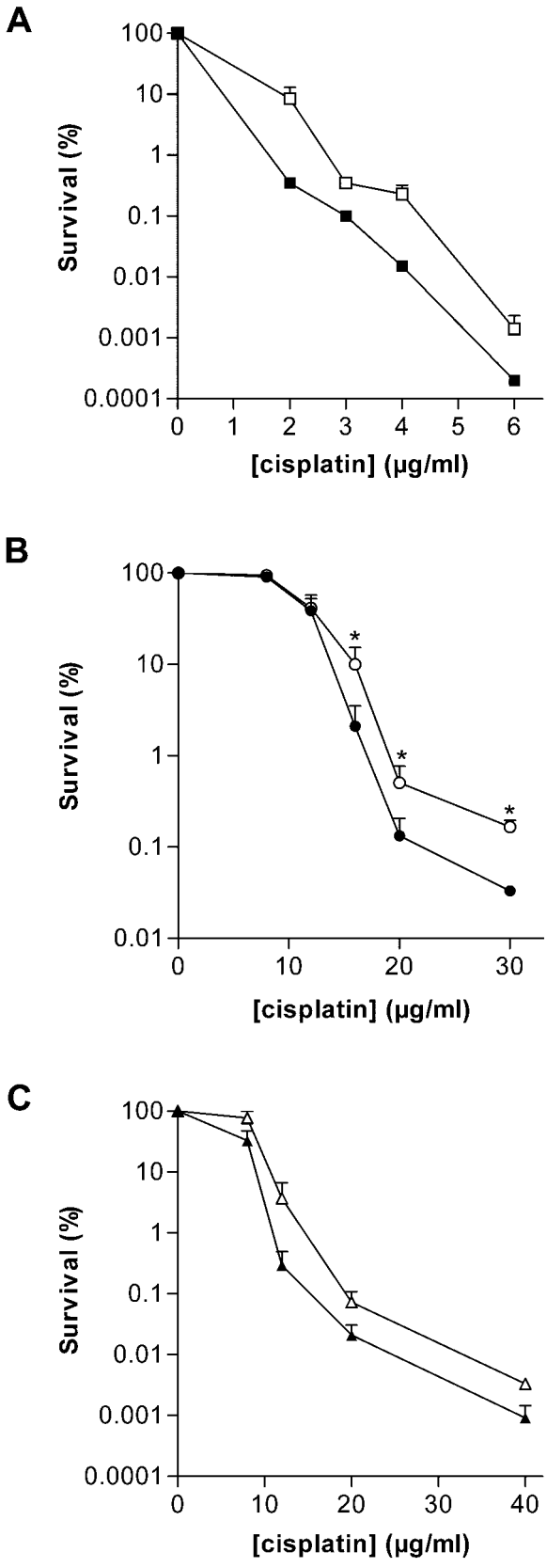

Fig. 1. Cisplatin sensitivity of yeast *npr2* mutants. Percentage survival-(colony formation) at each concentration of cisplatin is expressed relative to untreated control cells (100%). Each experiment was performed using duplicate plates at all drug concentrations, and the mean values from the separate experiments were averaged to obtain the data points and their S.D.s. The S.D.s are represented by bars, which are sometimes masked by the data point symbols. A, cisplatin sensitivity profiles of *S. cerevisiae* MGSC131 parental cells (■) and isogenic library-derived transformant cells harboring a transposon insertion in the *NPR2* 5′-regulatory region (□). The data shown are means and S.D. of two independent experiments. B, cisplatin sensitivity profiles of *S. cerevisiae* BY4742 parental cells (●) and isogenic specific *npr2*Δ disruption mutant cells (○). The data shown are means and S.D. of five independent experiments. *, $P < 0.05$; survival of the *npr2*Δ strain was significantly higher than that of the *NPR2*$^+$ parental strain (Student's *t* test). In addition, the slopes of the log-linear concentration-survival curves were determined for each strain for each experiment by means of linear regression analysis. The slopes were averaged across the experiments, and the difference between the slopes for the two yeast strains was statistically significant (Student's *t* test, $P < 0.05$). C, cisplatin sensitivity profiles of *S. cerevisiae* CMY375 parental cells (▲) and isogenic BUH3 *npr2* cells obtained by ethyl-methyl-sulfonate mutagenesis (△). The data shown are means and S.D. of two independent experiments. Please note that the axes are in different ranges, corresponding to the NER deficiency versus repair proficiency of the MGSC131 versus BY4742 and CMY375 parental cells (see *Characterization of Independent Mutant Cells*).

TABLE 2

Effect of *NPR2* disruption on yeast sensitivity to cytotoxic agents

Sensitivity was monitored by semiquantitative spot assays in *S. cerevisiae npr2*Δ cells (i.e., cells from which the *NPR2* gene had been disrupted) versus isogenic *NPR2*$^+$ cells. For each agent, these assays were performed at least three times, and consistent results were obtained each time. Different classes of chemicals are indicated by footnotes.

Resistance	Hypersensitivity	No Change
Cisplatin[a]	Cadmium chloride[c]	Oxaliplatin[a]
Doxorubicin[b]		Etoposide[b]
		Copper sulfate[c]
		5-Fluorouracil[e]
		Zinc chloride[c]
		MNNG[d]
		Ionizing radiation
		Ultraviolet light

[a] Platinum analog.
[b] Topoisomerase II inhibitor.
[c] Heavy metal.
[d] Methylating agent.
[e] Antimetabolite.

hypersensitivity to cadmium chloride. In addition, the cisplatin- and doxorubicin-resistant phenotype of *sky1*Δ cells was not linked to impaired drug accumulation either. *NPR2* and *SKY1* might thus be involved in common cellular pathways. As demonstrated before (Schenk et al., 2002), *sky1*Δ cells also showed a mutator phenotype (i.e., a 2.4-fold enhanced rate of spontaneous mutation compared with their isogenic parent). To further explore a possible relationship between *NPR2*- and *SKY1*-dependent cellular processes, we determined whether *npr2*Δ cells also display this phenotype. The rate of forward mutation at the *CAN1* locus was thus assessed for *npr2*Δ cells and isogenic BY4742 *NPR2*$^+$ cells. There was a 2-fold increase of the mutation rate per replication in the *npr2*Δ strain (rate, 4.8×10^{-8}) versus the *NPR2*$^+$ strain (rate, 2.5×10^{-8}), as shown in Fig. 6. These data indicate that, like disruption of the *SKY1* gene, loss of *NPR2* induces a mutator phenotype.

Cisplatin and Doxorubicin Resistance of *npr2*Δ *sky1*Δ Cells. To further explore whether *NPR2* and *SKY1* might play a role in common pathways, we generated a *SKY1* disruption in *npr2*Δ and BY4742 parental cells, to obtain an *npr2*Δ *sky1*Δ double-knockout strain and its isogenic control BY-*sky1*Δ, respectively. To determine whether *NPR2* and *SKY1* are epistatic, we tested the *npr2*Δ *sky1*Δ cells for their sensitivity toward cisplatin and doxorubicin. Because the double knockout strain showed diminished growth in the absence of cytotoxic agents, we assessed its possible drug resistance in semiquantitative spot assays (Fig. 7, A and B)

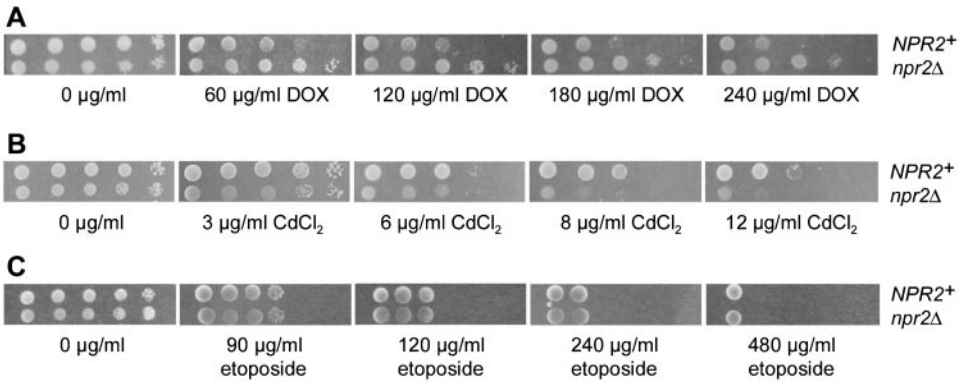

Fig. 2. Sensitivity to cytotoxic chemicals of *S. cerevisiae npr2*Δ versus *NPR2*$^+$ cells. A-C, yeast BY4742 *NPR2*$^+$ and isogenic *npr2*Δ cells were tested for their relative ability to grow on selective medium plates containing a range of doxorubicin (A, DOX), cadmium chloride (B, $CdCl_2$), or etoposide (C) concentrations as indicated. Decreasing aliquots of cells (i.e., 10-fold serial dilutions) were spotted from left to right (starting at 10^6 cells per spot on the left) and incubated at 30°C for 3 to 4 days. For each agent, these spot assays were performed at least three times, and consistent results were obtained each time.

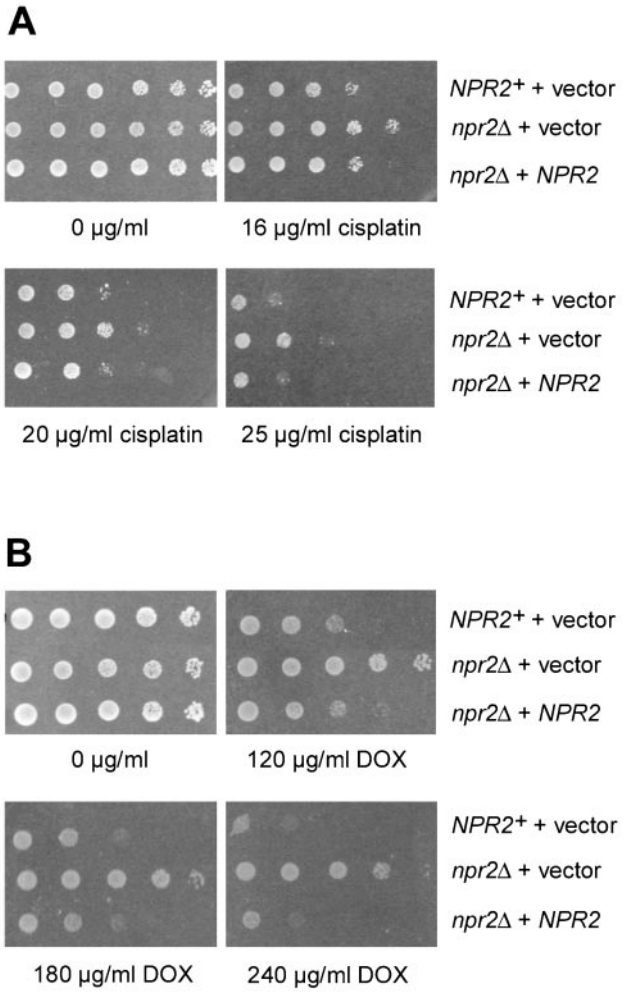

Fig. 3. Complementation of yeast *npr2*Δ disruption mutant cells. A and B, cisplatin and doxorubicin sensitivity of *S. cerevisiae* BY4742 parental cells transformed with empty vector pYCTEF111 (top rows), isogenic disruption mutant *npr2*Δ cells transformed with vector pYCTEF111 alone (middle rows), and *npr2*Δ cells transformed with plasmid pYCTEF111-*NPR2* (bottom rows). Yeasts were tested for their relative ability to grow on selective medium plates containing cisplatin (A) or doxorubicin (B, DOX). A, decreasing aliquots of cells (i.e., 3-fold serial dilutions) were spotted from left to right (starting at 3×10^4 cells per spot on the left) and incubated on plates containing different concentrations of cisplatin as indicated at 30°C for 4 days. B, decreasing aliquots of cells (i.e., 5-fold serial dilutions) were spotted from left to right (starting at 10^5 cells per spot on the left) and incubated on plates containing different concentrations of doxorubicin as indicated at 30°C for 3 days. All spot assays were performed at least two times, and consistent results were obtained each time.

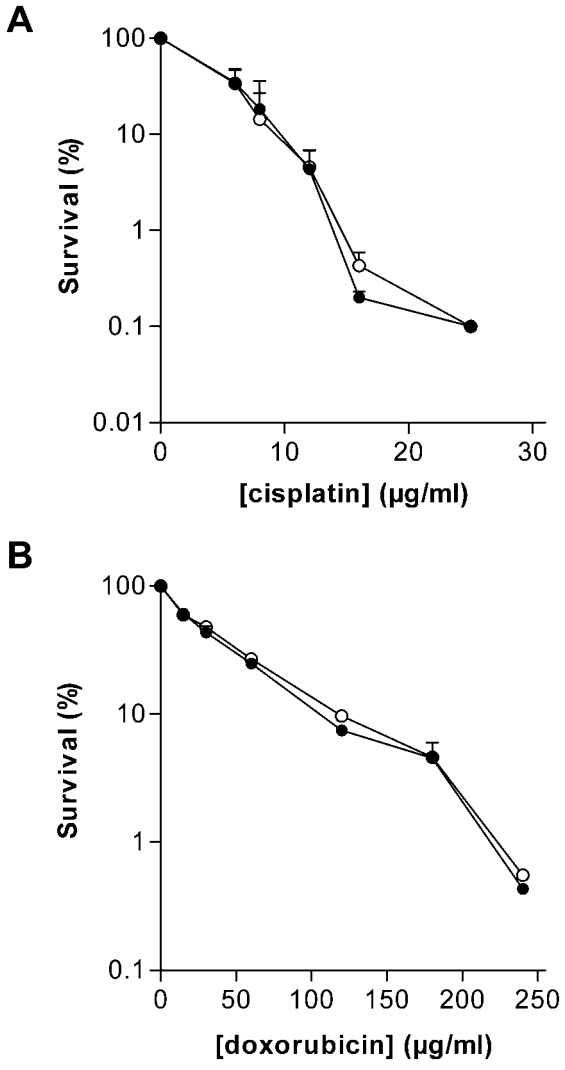

Fig. 4. Cisplatin and doxorubicin sensitivity of yeast *dur3*Δ disruption mutants. Percentage survival (colony formation) at each drug concentration is expressed relative to untreated control cells (100%). Each experiment was performed using duplicate plates at all drug concentrations, and the mean values from the separate experiments were averaged to obtain the data points and S.D. (bars). A, cisplatin sensitivity profiles of *S. cerevisiae* BY4742 parental cells (●) and isogenic *dur3*Δ cells (○). The data shown are means and S.D.s of three independent experiments. None of the differences in cisplatin sensitivity between the *dur3*Δ strain and its *DUR3*$^+$ parent were statistically significant (Student's *t* test, $P > 0.1$). B, doxorubicin sensitivity profiles of *S. cerevisiae* BY4742 cells (●) and *dur3*Δ cells (○). The data shown are means and S.D.s of two independent experiments.

as well as in quantitative clonogenic assays (Fig. 7, C and D). From our experiments, it is clear that neither the cisplatin nor the doxorubicin resistance displayed by the *npr2*Δ *sky1*Δ cells was additive or synergistic, compared with the single-knockout strains. We conclude that the double-knockout cells were as resistant to cisplatin as the single-knockout cell that was most resistant to this drug (i.e., strain BY-*sky1*Δ) (Fig. 7, A and C) and as resistant to doxorubicin as the *npr2*Δ strain, which was the most resistant single-knockout strain for the anthracycline (Fig. 7, B and D). In summary, our data indicate that *NPR2* and *SKY1* are epistatic. In the case of parallel pathways, the drug resistance of the double knockout strain would have been additive or greater than additive. Given the phenotype observed, *NPR2* may thus act in mutual regulatory routes with *SKY1* to mediate the cytotoxicity of anticancer agents.

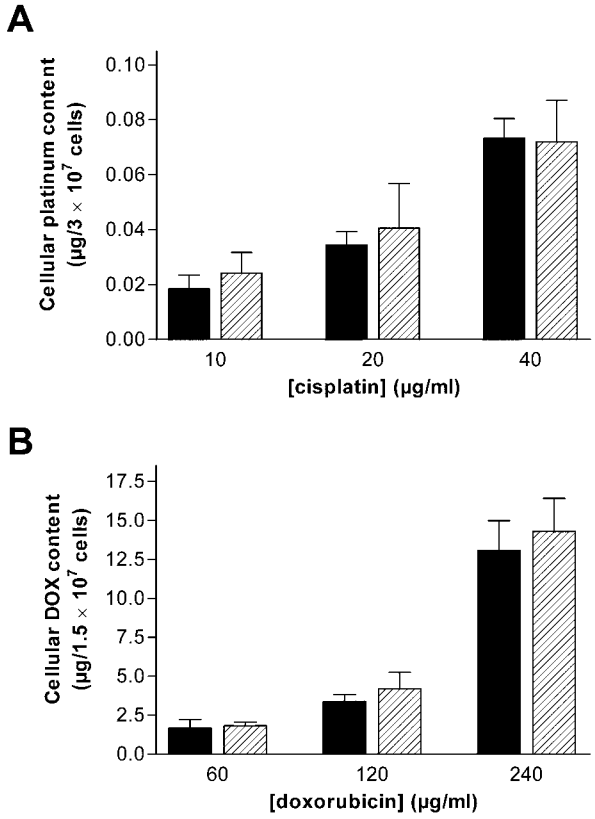

Fig. 5. Cellular cisplatin and doxorubicin accumulation in *S. cerevisiae* *npr2*Δ versus *NPR2*$^+$ cells. A-B, yeast BY4742 *NPR2*$^+$ cells (■) and isogenic *npr2*Δ cells (▨) were incubated in selective liquid medium containing 10, 20, and 40 μg/ml cisplatin (A) or 60, 120, and 240 μg/ml doxorubicin (B) at 30°C for 18 h. A, a total of 3×10^7 cells was pelleted and lyzed, and platinum content was measured by AAS. B, a total of 1.5×10^7 cells was pelleted, and doxorubicin (DOX) content was assessed by HPLC. For each drug, the accumulation data and the S.D.s shown were derived from a typical experiment performed with triplicate cultures.

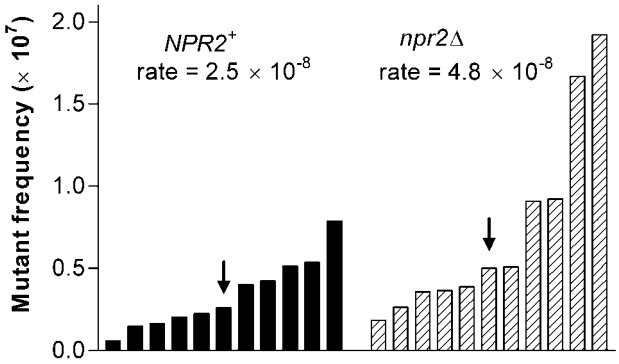

Fig. 6. Spontaneous mutation rates in yeast *npr2*Δ versus *NPR2*$^+$ cells. Mutant frequencies at the *CAN1* locus were determined in 11 individual parallel cultures of *S. cerevisiae* BY4742 *NPR2*$^+$ cells (■) and isogenic *npr2*Δ cells (▨). For each strain, bars are grouped according to the data obtained, with frequencies increasing from left to right. The mutation rates per replication were calculated from the median mutant frequencies indicated by vertical arrows.

Discussion

In the present study, we aimed to identify and characterize novel cisplatin sensitivity genes whose disruption renders *S. cerevisiae* cisplatin-resistant. We found that different yeast strains lacking a functional *NPR2* gene were all cisplatin-resistant compared with their isogenic parents. In cross-resistance studies, *npr2*Δ cells were resistant to cisplatin and the anthracycline drug doxorubicin and hypersensitive to cadmium chloride. Whereas Rousselet et al. (1995) previously reported that *npr2* mutants are affected in their urea and proline transport capacities, the observed cisplatin and doxorubicin resistance is probably not a direct result of decreased drug import or increased drug export. *Npr2*Δ cells did not display reduced platinum accumulation compared with isogenic *NPR2*$^+$ cells. This seems to exclude a role for the high-affinity copper transporter Ctr1p, which was very recently identified as a regulator of cisplatin uptake and sensitivity (Ishida et al., 2002; Lin et al., 2002). In line with this, *npr2*Δ cells did not show altered sensitivity to copper sulfate. Although the combination of cisplatin resistance and hypersensitivity to cadmium chloride may be poorly understood, it is not unprecedented in either yeast (Perego et al., 1997) or mammalian cells (Farnworth et al., 1990), as discussed previously (Schenk et al., 2002). Our finding that *NPR2* disruption led to a 4-fold doxorubicin resistance is an important observation, as unresponsiveness to anthracycline treatment constitutes a tremendous clinical problem (Monneret, 2001). Doxorubicin is thought to inhibit topoisomerase II through DNA intercalation (Pratt et al., 1994). Topoisomerase II is, however, probably not involved in the doxorubicin resistance of *npr2*Δ cells, because we did not see cross-resistance to etoposide, another well known inhibitor of this enzyme (Pratt et al., 1994). Because the observed resistance cannot immediately be explained by altered doxorubicin accumulation either, a role for the yeast pleiotropic drug resistance network involved in anthracycline efflux (Kolaczowska and Goffeau, 1999) seems unlikely as well. Because *DUR3* and its gene product have been regarded as the most likely downstream targets of *NPR2* (Rousselet et al., 1995), we assessed a possible role of this putative transmembrane active urea transporter (ElBerry et al., 1993) in anticancer drug resistance. In contrast to yeast *npr2*Δ cells, *dur3*Δ cells were neither resistant nor hypersensitive to cisplatin or doxorubicin compared with their isogenic parent. Our data thus indicate that *NPR2* exerts its cytotoxic effects in response to either cisplatin or doxorubicin independent of the nitrogen permease *DUR3*. Although we can not dismiss a tentative role of nitrogen transport in the drug-resistant phenotype of the *npr2*Δ cells, this suggests that a novel function of *NPR2* (unrelated to nitrogen transport) might be involved.

A possible mechanistic lead can be obtained by evaluation of the overall phenotypes induced by disruption of *NPR2* and *SKY1*. The pattern of cross-resistance to cisplatin (but not oxaliplatin) and doxorubicin, in combination with hypersensitivity to cadmium chloride in *npr2*Δ cells, is highly reminiscent of our previous data for *sky1*Δ cells (Schenk et al., 2002). In addition, the *npr2*Δ strain did not show reduced platinum or doxorubicin accumulation and displayed an enhanced rate of spontaneous mutation compared with the isogenic parent, similar to the *sky1*Δ strain that we characterized earlier (Schenk et al., 2002). We tested *npr2*Δ *sky1*Δ double-knockout cells for cisplatin and doxorubicin resistance, and concluded that *NPR2* and *SKY1* are epistatic. These data suggest that *NPR2* may act in mutual regulatory routes with *SKY1*. The Sky1p protein is believed to phosphorylate several serine-rich proteins, including its well established in vivo *S. cerevisiae* substrate Npl3p (Yun and Fu, 2000). Although Rousselet et al. (1995) correctly noted that Npr2p (SWISS-PROT accession number P39923) is a serine-rich protein as well, it does not comprise a true consensus site for serine phosphorylation by Sky1p (Yun and Fu, 2000). It is, therefore, not very likely that Sky1p might directly regulate Npr2p through straightforward phosphorylation. According to the *Saccharomyces* Genome Database (http://www.yeastgenome.org/), Npr2p may be a transcription factor, as inferred from electronic annotation. It is thus conceivable that Npr2p functions as a transcriptional modulator for specific downstream components within a tentative *SKY1* network. Although we can not dismiss a possible role of Npr2p in the transcriptional regulation of *SKY1*, the transcription of *SKY1* is probably not regulated by cisplatin. In previous experiments, we did not detect alterations in *SKY1* RNA levels upon cisplatin treatment of yeast cells (Schenk et al., 2001). In fact, our data imply that *NPR2* and *SKY1* are connected in a shared regulatory network instead of just a single hierarchic cascade. Because *npr2*Δ cells were more resistant to doxorubicin, whereas BY-*sky1*Δ cells were more resistant to cisplatin, it seems unlikely that one component would simply act directly upstream from the other to mediate the same response (i.e., cell death) to different stimuli (i.e., cisplatin and doxorubicin). Based on the drug sensitivity profile and mutator phenotype of *S. cerevisiae sky1*Δ cells, we previously proposed that Sky1p might play a significant role in mismatch repair (MMR), base excision repair, and/or Rev3p-dependent pathways (Schenk et al., 2002). By analogy, Npr2p might also be involved in such processes. Because cisplatin and doxorubicin resistance (Drummond et al., 1996; Durant et al., 1999) and an elevated mutation rate (Branch et al., 1995) have been associated with loss of MMR in several cell types, MMR deficiency could, for instance, underlie these

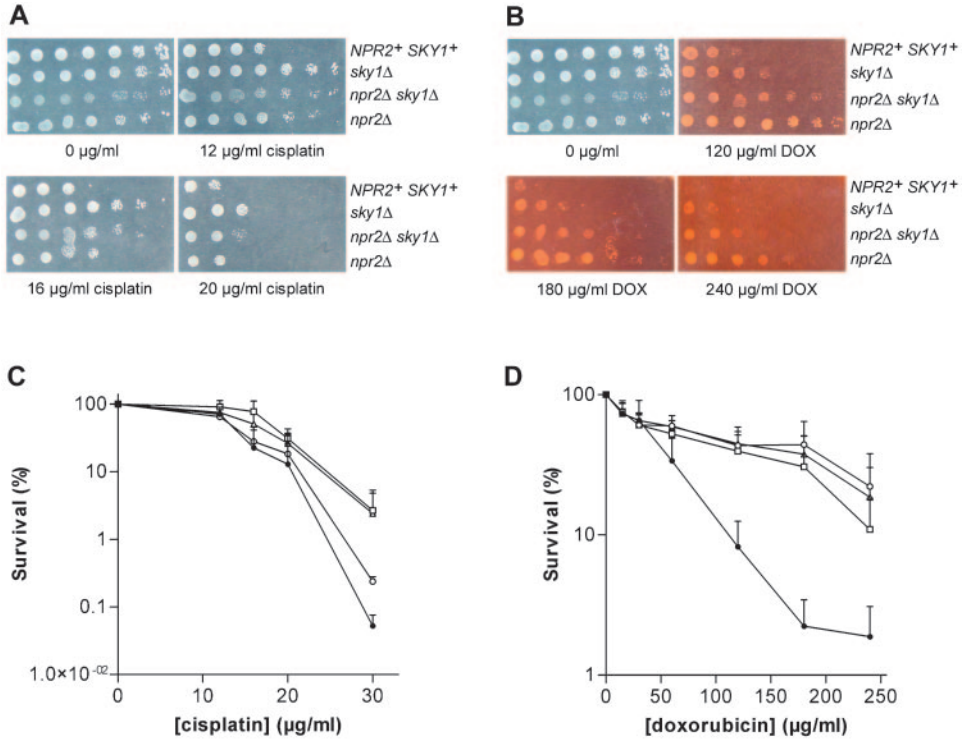

Fig. 7. Cisplatin and doxorubicin sensitivity of *S. cerevisiae npr2*Δ *sky1*Δ versus isogenic wild-type, *sky1*Δ, and *npr2*Δ cells. A and B, yeast BY4742 *NPR2*$^+$ *SKY1*$^+$, BY-*sky1*Δ, *npr2*Δ *sky1*Δ, and *npr2*Δ cells were tested for their relative ability to grow on selective medium plates containing a range of cisplatin (A) or doxorubicin (B, DOX) concentrations as indicated. Decreasing aliquots of cells (i.e., 3-fold serial dilutions) were spotted from left to right (starting at 10^5 cells per spot on the left) and incubated at 30°C for 4 days. The double-knockout strain was as resistant to cisplatin and doxorubicin as the single-knockout strain that was most resistant to either drug. Where growth of the *npr2*Δ *sky1*Δ cells in the presence of cisplatin or doxorubicin was decreased compared with BY-*sky1*Δ or *npr2*Δ cells, respectively, it should be noted that the growth of the double-knockout cells in the absence of cytotoxic chemicals was equally reduced. This probably reflects the fact that *npr2*Δ *sky1*Δ cells displayed a 2-fold increase in doubling time in liquid selective medium compared with the isogenic controls (data not shown). C and D, given the diminished growth of the double knockout in the absence of both compounds, resistance to cisplatin (C) and doxorubicin (D) was also assessed in clonogenic assays. Sensitivity profiles of yeast BY4742 *NPR2*$^+$ *SKY1*$^+$ cells (●), BY-*sky1*Δ cells (□), *npr2*Δ *sky1*Δ cells (△), and *npr2*Δ cells (○) are shown. Percentage survival (colony formation) at each drug concentration is expressed relative to untreated control cells (100%). Each experiment was performed using duplicate plates at all drug concentrations, and the mean values from three separate experiments were averaged to obtain the data points and their S.D.s (bars) for each compound.

phenomena in *npr2*Δ cells. Because MMR deficiency has also been linked with tolerance to DNA methylation damage in human cancer cells (Branch et al., 1995), one might argue that the absence of cross-resistance to the methylator MNNG contradicts a role for MMR. However, in *S. cerevisiae*, mutations in MMR genes do not necessarily render the cells more tolerant to MNNG (Bawa and Xiao, 1997), in line with our present data.

Irrespective of the underlying mechanisms, our data identify *S. cerevisiae NPR2* as a novel gene involved in cell kill provoked by the anticancer drugs cisplatin and doxorubicin. Based on primary sequence conservation, we found *NPRL2/Gene21* as the most credible candidate human *NPR2* homolog (using http://www.ncbi.nlm.gov/blast/Blast.cgi). There are three regions of high similarity between the predicted polypeptide sequence encoded by the human *NPRL2/Gene21* cDNA (RefSeq NP_006536.1) and Npr2p, with 32% identity (53% similarity) over 152 amino acids, 36% identity (50% similarity) over 119 amino acids, and 36% identity (54% similarity) over 55 amino acids. Strikingly, *NPRL2/Gene21* is one of the candidate tumor suppressor genes residing on a 120-kb critical tumor homozygous deletion region of human chromosome 3p21.3 found in lung and breast cancers (Lerman and Minna, 2000). Recently, it was reported that *NPRL2/Gene21* is, indeed, one of the candidates whose adenovirus vector-mediated expression results in tumor suppressor activity in vitro and in vivo (Ji et al., 2002). Our next aim will be to see whether this human tumor suppressor gene might also be involved in processes altering sensitivity to anticancer drugs. In addition, it will be highly interesting to assess the possible interplay between *NPRL2/Gene21* and other components controlling drug sensitivity, such as the *SKY1* homolog *SRPK1*. The finding that *NPRL2/Gene21* resides on human chromosome 3 is of special interest with respect to a tentative role in MMR. Chauhan et al. (2000) recently tested the hypothesis that a single wild-type copy of the key MMR gene *hMLH1* on chromosome 3 might be exclusively responsible for the restoration of MMR function in MMR-deficient HCT116 human colon cancer cells, upon transfer of a whole copy of the chromosome. However, profound inhibition of hMLH1 expression did not abrogate DNA MMR activity in the corrected cells, suggesting either that hMLH1 is expressed in large excess compared with that required for functional MMR, or that chromosome 3 contains another uncharacterized MMR gene. Given our data for yeast *npr2*Δ cells, it is tempting to speculate that the human Npr2p homolog is somehow involved in MMR, and that the introduction of an extra copy of *NPRL2/Gene21* on human 3p21.3 thus aids in the restoration of MMR function in HCT116 cells upon whole chromosome transfer.

Acknowledgments

We thank Dr. Petra Ross-Macdonald (Yale University, New Haven, CT) for providing us with the random mini-Tn*3*::*lacZ*::*LEU2* insertion library, and Dr. Germain Rousselet (Service de Biologie Cellulaire, CEA, Centre d'Etudes de Saclay, Gif-sur-Yvette Cedex, France) for the CMY375 and BUH3 yeast strains. Furthermore, Robert Oostrum, Peter de Bruijn, and Dr. Walter Loos are acknowledged for expert technical assistance. Finally, we are grateful to Maarten Niemantsverdriet for creating the *sky1*Δ::*lox-URA3-lox* geneblaster.

References

Abe H, Wada M, Kohno K, and Kuwano M (1994) Altered drug sensitivities to anticancer agents in radiation-sensitive DNA repair deficient yeast mutants. *Anticancer Res* **14:**1807–1810.
Andrews PA, Jones JA, Varki NM, and Howell SB (1990) Rapid emergence of acquired *cis*-diamminedichloroplatinum(II) resistance in an in vivo model of human ovarian carcinoma. *Cancer Commun* **2:**93–100.
Bawa S and Xiao W (1997) A mutation in the *MSH5* gene results in alkylation tolerance. *Cancer Res* **57:**2715–2720.
Branch P, Hampson R, and Karran P (1995) DNA mismatch binding defects, DNA damage tolerance, and mutator phenotypes in human colorectal carcinoma cell lines. *Cancer Res* **55:**2304–2309.
Burger H, Capello A, Schenk PW, Stoter G, Brouwer J, and Nooter K (2000) A genome-wide screening in *Saccharomyces cerevisiae* for genes that confer resistance to the anticancer agent cisplatin. *Biochem Biophys Res Commun* **269:**767–774.
Burns N, Grimwade B, Ross-Macdonald PB, Choi E-Y, Finberg K, Roeder GS, and Snyder M (1994) Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. *Genes Dev* **8:**1087–1105.
Chauhan DP, Yang Q, Carethers JM, Marra G, Chang CL, Chamberlain SM, and Boland CR (2000) Antisense inhibition of hMLH1 is not sufficient for loss of DNA mismatch repair function in the HCT116+chromosome 3 cell line. *Clin Cancer Res* **6:**3827–3831.
Crul M, Schellens JHM, Beijnen JH, and Maliepaard M (1997) Cisplatin resistance and DNA repair. *Cancer Treat Rev* **23:**341–366.
de Bruijn P, Verweij J, Loos WJ, Kolker HJ, Planting AS, Nooter K, Stoter G, and Sparreboom A (1999) Determination of doxorubicin and doxorubicinol in plasma of cancer patients by high-performance liquid chromatography. *Anal Biochem* **266:**216–221.
Drake JW (1991) A constant rate of spontaneous mutation in DNA-based microbes. *Proc Natl Acad Sci USA* **88:**7160–7164.
Drummond JT, Anthoney A, Brown R, and Modrich P (1996) Cisplatin and adriamycin resistance are associated with MutLα and mismatch repair deficiency in an ovarian tumor cell line. *J Biol Chem* **271:**19645–19648.
Durant ST, Morris MM, Illand M, McKay HJ, McCormick C, Hirst GL, Borts RH, and Brown R (1999) Dependence on *RAD52* and *RAD1* for anticancer drug resistance mediated by inactivation of mismatch repair genes. *Curr Biol* **9:**51–54.
Einhorn LH (1997) Testicular cancer: an oncological success story. *Clin Cancer Res* **3:**2630–2632.
ElBerry HM, Majumdar ML, Cunningham TS, Sumrada RA, and Cooper TG (1993) Regulation of the urea active transporter gene (*DUR3*) in *Saccharomyces cerevisiae*. *J Bacteriol* **175:**4688–4698.
Farnworth P, Hillcoat B, and Roos I (1990) Metallothionein-like proteins and cell resistance to *cis*-dichlorodiammineplatinum(II) in L1210 cells. *Cancer Chemother Pharmacol* **25:**411–417.
Gietz RD and Sugino A (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:**527–534.
Ishida S, Lee J, Thiele DJ, and Herskowitz I (2002) Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals. *Proc Natl Acad Sci USA* **99:**14298–14302.
Jansen LE, Belo AI, Hulsker R, and Brouwer J (2002) Transcription elongation factor Spt4 mediates loss of phosphorylated RNA polymerase II transcription in response to DNA damage. *Nucleic Acids Res* **30:**3532–3539.
Ji L, Nishizaki M, Gao B, Burbee D, Kondo M, Kamibayashi C, Xu K, Yen N, Atkinson EN, Fang B, et al. (2002) Expression of several genes in the human chromosome 3p21.3 homozygous deletion region by an adenovirus vector results in tumor suppressor activities *in vitro* and *in vivo*. *Cancer Res* **62:**2715–2720.
Köberle B, Masters JRW, Hartley JA, and Wood RD (1999) Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. *Curr Biol* **9:**273–276.
Kolaczowska A and Goffeau A (1999) Regulation of pleiotropic drug resistance in yeast. *Drug Resist Updates* **2:**403–414.
Lerman MI and Minna JD (2000) The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. *Cancer Res* **60:**6116–6133.
Lin X, Okuda T, Holzer A, and Howell SB (2002) The copper transporter *CTR1* regulates cisplatin uptake in *Saccharomyces cerevisiae*. *Mol Pharmacol* **62:**1154–1159.
Monneret C (2001) Recent developments in the field of antitumour anthracyclines. *Eur J Med Chem* **36:**483–493.
Niedner H, Christen R, Lin X, Kondo A, and Howell SB (2001) Identification of genes that mediate sensitivity to cisplatin. *Mol Pharmacol* **60:**1153–1160.
Ochman H, Gerber AS, and Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. *Genetics* **120:**621–623.
Perego P, Vande Weghe J, Ow DW, and Howell SB (1997) Role of determinants of cadmium sensitivity in the tolerance of *Schizosaccharomyces pombe* to cisplatin. *Mol Pharmacol* **51:**12–18.
Perez RP (1998) Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer* **34:**1535–1542.
Prakash S and Prakash L (2000) Nucleotide excision repair in yeast. *Mutat Res* **451:**13–24.
Pratt WB, Ruddon RW, Ensminger WD, and Maybaum J (1994) *The Anticancer Drugs*. Oxford University Press, New York, NY.
Resnick MA and Cox BS (2000) Yeast as an honorary mammal. *Mutat Res* **451:**1–11.
Rothstein R (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* **194:**281–301.
Rousselet G, Simon M, Ripoche P, and Buhler J-M (1995) A second nitrogen permease regulator in *Saccharomyces cerevisiae*. *FEBS Lett* **359:**215–219.
Schenk PW, Boersma AWM, Brandsma JA, den Dulk H, Burger H, Stoter G,

Brouwer J, and Nooter K (2001) *SKY1* is involved in cisplatin-induced cell kill in *Saccharomyces cerevisiae*, and inactivation of its human homologue, *SRPK1*, induces cisplatin resistance in a human ovarian carcinoma cell line. *Cancer Res* **61:**6982–6986.

Schenk PW, Boersma AWM, Brok M, Burger H, Stoter G, and Nooter K (2002) Inactivation of the *Saccharomyces cerevisiae SKY1* gene induces a specific modification of the yeast anticancer drug sensitivity profile accompanied by a mutator phenotype. *Mol Pharmacol* **61:**659–666.

Tishkoff DX, Filosi N, Gaida GM, and Kolodner RD (1997) A novel mutation avoidance mechanism dependent on *S. cerevisiae RAD27* is distinct from DNA mismatch repair. *Cell* **88:**253–263.

Verhage RA, Van de Putte P, and Brouwer J (1996) Repair of rDNA in *Saccharomyces cerevisiae*: *RAD4*-independent strand-specific nucleotide excision repair of RNA polymerase I transcribed genes. *Nucleic Acids Res* **24:**1020–1025.

Yun CY and Fu X-D (2000) Conserved SR protein kinase functions in nuclear import and its action is counteracted by arginine methylation in *Saccharomyces cerevisiae*. *J Cell Biol* **150:**707–718.

Address correspondence to: Dr. K. Nooter, Department of Medical Oncology, Erasmus University Medical Center Rotterdam, Josephine Nefkens Building room Be422, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: k.nooter@erasmusmc.nl