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Isolation and Characterization of a Cisplatin-Resistant Strain of *Schizosaccharomyces pombe*

PAOLA PEREGO, GRETCHEN JIMENEZ, and STEPHEN B. HOWELL

Department of Medicine and the Cancer Center (P.P., S.B.H.) and the Department of Biology (G.J.), University of California, San Diego, La Jolla, California 92093

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SUMMARY

A *cis*-diamminedichloroplatinum [DDP (cisplatin)]-resistant population of *Schizosaccharomyces pombe* was developed through chronic exposure of the 972 h^- strain to increasing concentrations of the drug. The resulting cells, designated wtr2, were 5.25-fold resistant to DDP, and resistance was retained by clone isolated from this population in the absence of drug for ≥5 months. After backcrossing and isolation of a single clone, random spore analysis gave a segregation ratio close to 1:1 for DDP resistance and sensitivity. Tetrad analysis confirmed a mendelian 2:2 segregation, suggesting that a single nuclear gene was responsible for the DDP-resistant phenotype. Stable diploids obtained from the mating of a resistant spore carrying the ade6-216 marker with the mei2-102-ade6-210 meiosis-deficient mutant remained resistant, indicating that the resistant phenotype was expressed dominantly. There was no difference between the accumulation of the DDP analog [^{3}H]dichloro(ethylenediamine)-platinum(II) into whole cells derived from the sensitive and the resistant spores obtained from the last backcross. The resistant clones from a single tetrad did not have an increased level of glutathione and were collaterally sensitive to cadmium and arsenite. We conclude that in *S. pombe*, a stable and dominant DDP-resistant phenotype can be mediated by a single allele, that the phenotype is not accompanied by cross-resistance to cadmium or arsenite, and that the mechanism is not associated with a significant alteration in glutathione level or DDP uptake.

The platinum-containing compound DDP (cisplatin) is a cancer chemotherapeutic agent widely used in the treatment of bladder, testicular, lung, head and neck, and ovarian carcinomas (1). Despite the fact that many tumors initially respond well to this drug, resistance to DDP usually develops during the course of treatment (2, 3). Studies of human cells selected for DDP resistance *in vitro* have established that in many cases resistance is multifactorial. Impaired uptake (4, 5), increased thiol content (6), and increased DNA repair (7) have each been identified as being capable of contributing to the resistant phenotype; however, it remains unclear which if any of these is the most clinically relevant mechanism of resistance.

Many unicellular organisms have developed powerful defense mechanisms against environmental toxins, including metals (8, 9). Genes conferring resistance to cadmium, arsenite, antimonite, zinc, copper, and cobalt have been identified in both prokaryotes and lower eukaryotes, including some that code for transporters belonging to the ATP-binding cassette superfamily (10–13). Although reduced accumulation based on an active efflux of metal ions seems to be the primary mechanism of resistance utilized by prokaryotes, intracellular sequestration of the toxic metal ions through binding to metallothioneins or thioredoxin or conjugation with GSH is common in eukaryotes (6, 14, 15). Several of the metal ion transporters have been both functionally and structurally conserved throughout evolution. For example, the mammalian gene *Zn-T1*, which encodes a zinc transporter, has recently been shown to be homologous to the *zrc1* and *cot1* genes of the budding yeast *Saccharomyces cerevisiae* (16). The *zrc1* gene, which encodes a protein with six membrane-spanning domains, is involved specifically in defense against the group 12 metals zinc and cadmium. The *cot1* gene, which is similar in structure as well as in function to *zrc1*, confers increased tolerance to high levels of cobalt (17–19). In the fission yeast *Schizosaccharomyces pombe*, which lacks metallothioneins, the *hmt1* gene is able to confer resistance to cadmium through sequestration of the metal into vacuoles (20). It codes for an ATP-dependent pump located in the vacuolar membrane that has been recently shown to transport metal-chelated phytochelatins (21).

Based on the hypothesis that mammalian cells are likely to utilize mechanisms homologous to those developed by lower eukaryotes and with the goal of utilizing the power of yeast genetics to dissect the mechanisms that contribute to multifactorial DDP resistance, we sought to develop and charac-

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ABBREVIATIONS: DDP, *cis*-diamminedichloroplatinum; DEP, dichloro(ethylenediamine)platinum(II); GSH, γ-glutamylcystenylglycine.

terize a DDP-resistant strain of *S. pombe*. We report the successful isolation of a haploid strain in which stable DDP resistance is due to the action of a single dominant allele.

Materials and Methods

Yeast strains, growth conditions, and selection for DDP resistance. A DDP-resistant population was derived from the *S. pombe* strain 972 h^-, which was used as a wild-type control in this study, through chronic exposure to DDP starting at a concentration of 0.4 mM and increasing stepwise to 0.8, 1.6, and 2.8 mM. Cultures of 2×10^6 cells/ml were grown continuously in the presence of DDP until they reached the plateau phase, at which point they were diluted into fresh drug-containing medium. The wild-type strain, designated wt, was grown at 30° in YES medium (5 g/liter Difco yeast extract, 30 g/liter glucose, 0.075 g/liter adenine, 0.075 g/liter uracil); the DDP-resistant strains were grown in the same medium after the completion of drug selection.

Reagents. DDP was a gift from Bristol-Myers Squibb (Princeton, NJ). Transplatin, cadmium chloride, zinc chloride, cobalt chloride, and sodium arsenite were purchased from Sigma Chemical (St. Louis, MO).[^{3}H]DEP ($PtCl_2N_2C_2H_8$) was synthesized as described previously (22). Radioactive arsenate $^{73}AsO_4$ was purchased from Los Alamos National Laboratories (Los Alamos, NM).

Drug sensitivity studies. The toxicity of DDP and metal compounds was evaluated by measuring inhibition of the ability to form colonies on agar. Cultures containing 1×10^6 cells were exposed to the drug for 48 hr and then diluted into fresh medium. Aliquots were then seeded onto 60-mm agar plates at dilutions sufficient to yield 100–200 colonies/plate after a 4-day incubation at 30°. Each experiment was repeated a minimum of three times with triplicate cultures. A microtiter plate assay was used for the identification of sensitive and resistant spores from backcrosses. Cells were seeded in a volume of 100 μl and exposed to 0.25 mM DDP for 48 hr. An aliquot from each well was plated on agar, and growth was scored as either present or absent 4 days after plating. The relative resistance of different strains was also measured by determining growth rate during a continuous 48- or 72-hr period of exposure to a fixed DDP concentration. Cells were counted at the end of the drug exposure by using a hemocytometer. Growth rate assays were repeated a minimum of three times using triplicates cultures.

GSH content. The GSH level in exponentially growing cells was determined by conjugation with monobromobimane followed by quantification with high performance liquid chromatography as described by Miquel (23). Each experiment was performed three times with triplicate samples.

[^{3}H]DEP accumulation. Uptake was measured after a 4-hr exposure of 1×10^6 cells in a 3-ml culture to 5 mM [^{3}H]DEP (5 mCi/ml). After incubation with [^{3}H]DEP, the cells were washed four times with cold phosphate-buffered saline at 4°; an aliquot was removed for cell count; and the remaining cells were pelleted by centrifugation and lysed in 0.5 ml of 1 N NaOH overnight, after which radioactivity was determined by liquid scintillation counting. Each experiment was performed three times with triplicate cultures.

Genetic analysis. Standard procedures for genetic manipulation were followed according to Gutz *et al.* (24) and Moreno *et al.* (25). Briefly, random and tetrad spores were obtained by mating the DDP-sensitive h^+ ura4-d18leu1-32his3 and/or the h^+ *ade6-216* strain with the polyclonal DDP-resistant population. For the assessment of dominance or recessiveness, stable diploids were created by mating an *ade6-216*-resistant spore with the meiosis-deficient mutant *mat2-102-ade6-210*. The strains were mixed in 1% glucose/0.1% KH_2PO_4 on 3% agar plates. The mating mixture was subsequently checked for interallelic complementation through plating in low adenine medium on agar plates. Colonies were tested for resistance after a 48-hr exposure to DDP by cell counting.

Arsenite accumulation. Radioactive arsenate was chemically reduced to arsenite as reported previously (23). Uptake was measured after a 4-hr incubation using 1×10^6 cells in a 3-ml culture by the addition of 20 mM arsenite (5 mCi/ml). Immediately after completion of arsenite exposure, the cells were washed and lysed as for the [^{3}H]DEP accumulation experiments. An aliquot was removed for protein determination.

Northern blot analysis. Twenty milligrams of total RNA was electrophoresed on formaldehyde containing 1% agarose gel and transferred onto a nylon membrane. DNA probes were labeled with a Random Primer Kit (Amersham, Little Chalfont, UK). Hybridization was carried out as described previously (Ortiz 1992). The *hmt1* probe was kindly provided by Dr. David Ow (Albany, CA).

Results

Exposure of the wild-type 972 h^- strain of *S. pombe* to gradually increasing concentrations of DDP permitted the isolation of progressively more resistant populations of cells. Their sensitivity to a 48-hr exposure to DDP was assessed by measuring inhibition of the ability to form colonies in drug-free medium after seeding onto agar plates; this assay permits quantitative determination of the IC_{50} value. Fig. 1 shows survival as a function of DDP concentration for the wild-type parental cells and the wtr2 population that was capable of growing in 1.8 mM DDP. In this clonogenic assay, cells in the wtr2 population were 5.25-fold more resistant than the parental cells [for the wild-type cells, $IC_{50} = 0.08 \pm 0.005$ mM (mean ± standard deviation); for the wtr2 population, $IC_{50} = 0.42 \pm 0.02$ mM].

The stability of the DDP-resistant phenotype in individual clones isolated from the wtr2 population was determined after the entire population had been grown in the presence of 1.24 mM DDP for 5 months. Four individual clones (wtr2a, wtr2b, wtr2c, and wtr2d) were isolated and grown in the absence of DDP. Table 1 shows that even after growth in drug-free medium for 5 months, there was no loss of DDP resistance in any of the four clones as measured by growth rate during a 72-hr exposure to DDP. Thus, the DDP-resistant phenotype of four randomly chosen clones from the wtr2 population was very stable.

Genetic analysis was carried out to define the mechanisms underlying the resistant phenotype. The polyclonal wtr2 population was backcrossed to the wild-type 972 h^- strain, and after the first backcross, the resistant clone designated 5A5 was selected for further studies. Survival curves for this clone relative to the parental strain after exposure to DDP, cadmium, and arsenite are shown in Fig. 1, B, C, and D, respectively. The degree of resistance to DDP was similar to that of the original wtr2 population and the clones wtr2a, wtr2b, wtr2c, and wtr2d isolated from the wtr2 population; however, DDP resistance was not accompanied by a change in sensitivity to cadmium or arsenite. Therefore, this clone was identified as a suitable for further genetic analysis. Random spore analysis was undertaken on the products of the backcross of the 5A5 clone with a *S. pombe* strain carrying *ura4-d18*, *leu1-32*, and *his3-* as markers. This analysis demonstrated a resistance/sensitivity segregation ratio close to 1:1 because 36 of 80 spores that were analyzed were DDP resistant. The anticipated 1:1 segregation was obtained for two of the three control markers (1:0.95, *leu2*; 1:0.78, *his3*). The 1:0.66 segregation ratio observed for *ura4* is most readily explained by gene conversion phenomenon. Conventional tetrad analysis on 20 tetrads confirmed the mendelian segregation of 2:2. These observations demonstrate that despite the

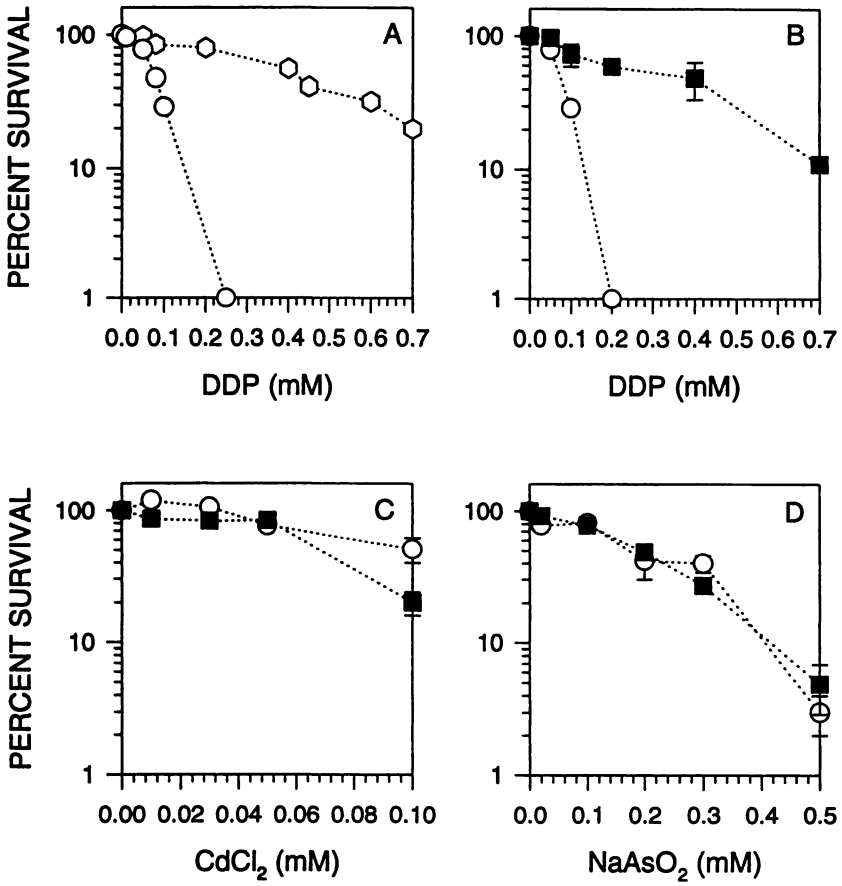

Fig. 1. Survival of *S. pombe* as a function of (A and B) DDP, (C) cadmium, and (D) arsenite concentration determined by clonogenic assay after a 48-hr exposure to drug. ○, Wild-type 972 h⁻ parental cells; ○, DDP-resistant wtr2 population; ■, clone 5A5. Each point represents the average of two or three experiments performed with triplicate cultures. *Vertical bars*, mean ± standard deviation; *bars* were smaller than the size of the symbols for many data points.

TABLE 1

Stability of the DDP-resistant phenotype in four clones isolated from the wtr2 DDP-resistant population of *S. pombe*

After 5 months of growth in drug-free medium, the growth rate of the wt 972 h- parental strain cells and four clones (wtr2a, wtr2b, wtr2c, and wtr2d) isolated from the wtr2 DDP-resistant population in the presence or absence of 1.24 mM DDP was determined over a 72-hr period.

Cell	Control cell growth
	%
wt	0.3 ± 0.1
wtr2	13.5 ± 1.0
wtr2a	12.0 ± 1.5
wtr2b	13.8 ± 3.5
wtr2c	13.0 ± 2.0
wtr2d	14.0 ± 2.0

Values are mean ± standard deviation of three independent experiments.

TABLE 2

GSH levels of DDP-sensitive and -resistant clones from the 7B1 tetrad

Cell	GSH
	μmol/10⁶ cells
wt	0.80 ± 0.045
wtr2	1.60 ± 0.095
7B1A	1.25 ± 0.055
7B1B	1.32 ± 0.090
7B1C	1.04 ± 0.040
7B1D	0.68 ± 0.009

Values are average ± standard deviation of three independent experiments.

fact that DDP selection may have produced multiple changes due to the mutagenic capacity of the drug, a single nuclear gene is responsible for the DDP-resistant phenotype of the haploid 5A5 clone.

Two further backcrosses were undertaken, and the 2:2 segregation was confirmed in an analysis of 20 tetrads. Four spores from one tetrad (7B1) were analyzed in more detail. They were designated 7B1A, 7B1B, 7B1C, and 7B1D; the A and D clones were DDP resistant, whereas the B and C were sensitive (see below). The GSH content of these clones is shown in Table 2. Increased GSH content did not cosegregate with the resistant phenotype. The relationship between resistance and DDP uptake was examined through accumulation of [^{3}H]DEP, a tritiatable analog of DDP. Fig. 2 shows the accumulation of [^{3}H]DEP in cultures grown from each of the four members of the 7B1 tetrad after exposure to 5 mM [^{3}H]DEP. Under the experimental conditions tested, resistance did not cosegregate with a change in either [^{3}H]DEP uptake, suggesting that a mechanism other than reduced drug accumulation accounted for resistance.

To prepare clones suitable for the assessment of the dominance or recessiveness of the DDP-resistant phenotype, a DDP-resistant *ade6-216 clone* (designated 7D) strain that was h⁻ at the mating type locus was built and crossed to the meiosis-deficient mutant *mei2-102-ade6-210*. Stable diploids, identified on the basis of the interallelic complementation at the *ade6* locus, were tested for sensitivity to DDP. Diploids obtained by mating a DDP-sensitive *ade6-216* h⁻ clone with the meiosis-deficient strain were included as a control. Diploids and the haploid strains used for matings were grown for 48 hr in the presence of 0.2 mM DDP, and growth rate was assessed. As shown in Fig. 3, four representative diploids obtained from the 7D clone were resistant to DDP, whereas

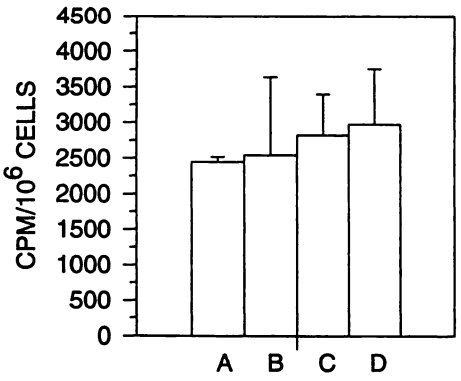

Fig. 2. [^{3}H]DEP accumulation in cells grown from the four spores of the 7B1 tetrad. Uptake was measured in 7B1A, 7B1B, 7B1C, and 7B1D cells after a 4-hr incubation with 5 μM [^{3}H]DEP. The mean of three independent experiments are reported. *Vertical bars*, mean ± standard deviation.

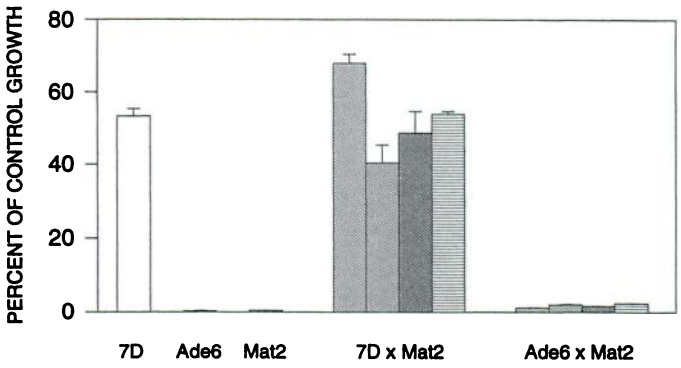

Fig. 3. DDP response of *S. pombe* haploid strains and four diploid clones resulting from the mating of 7D DDP-resistant *ade6-216* h$^-$ clone with the DDP-sensitive meiosis-deficient mutant *mat2-102-ade6-210* (*7D* × *Mat2*) as assessed by growth assay in 0.2 mM DDP for 48 hr. Four clones of control diploids [*mat2-102-ade6-210* × DDP-sensitive ade6-216 h$^-$ (*Ade6* × *Mat2*)] and the original haploid strains are shown. The reported percentage of cell growth is derived from a representative experiment performed with triplicate cultures. *Vertical bars*, mean ± standard deviation.

control diploids were DDP sensitive. Thus, the DDP-resistant phenotype was expressed in a dominant fashion.

DDP-resistant and DDP-sensitive clones from a single tetrad (7B1A, 7B1B, 7B1C, and 7B1D) were tested for sensitivity to DDP and for their pattern of cross-resistance to related metal salts with the use of colony-forming assays. Fig. 4 A shows that 7B1A and 7B1D were resistant to DDP, whereas 7B1B and 7B1C were sensitive. Fig. 4B shows that the two most resistant members of the tetrad were hypersensitive to arsenite and cadmium. Surprisingly, the 7B1A but not the 7B1D cells were hypersensitive to nickel (Fig. 4B). Thus, there seems to be cosegregation between DDP resistance and hypersensitivity to arsenite and cadmium. To determine whether there was any relation between the arsenite sensitivity of the four clones and arsenite accumulation, uptake was measured after a 4-hr exposure to 20 mM [^{73}As]arsenite. As shown in Table 3, the 7B1A clone had the highest accumulation of arsenite. However, the content of clone D, which was also hypersensitive, was comparable to that of the sensitive B clone. Therefore the observed differences in sensitivity were not directly related to the drug accumulation, suggesting predominance of some other mechanism.

Because the *hmt1* gene has been shown to confer resistance to cadmium in fission yeast and cadmium-resistant *S. pombe* contains increased *hmt1* mRNA (20), a Northern blot analysis was undertaken to determine whether this gene was differentially expressed at the mRNA level in the 7B1 tetrad. Fig. 5 shows that there was no marked difference in the *hmt1* mRNA level among the members of the 7B1 tetrad, providing one piece of evidence that the *hmt1* gene may not mediate either the hypersensitivity to cadmium or the resistance to DDP in this model.

Discussion

The apparent multifactorial nature of DDP resistance in mammalian and murine cell lines selected for DDP resistance has made it difficult to dissect the contribution made to the overall phenotype by any one mechanism. Because of the facility with which genetic manipulations can be carried out in yeast and because there is evidence from other systems that mechanisms of resistance are often conserved through evolution, we sought to develop a DDP-resistant strain of the fission yeast *S. pombe* for use in characterizing the mechanisms of resistance to this agent. The use of yeast as a model for studying drug resistance has many advantages. *S. pombe* is sufficiently similar to mammalian cells to allow, in many well-documented cases, human genes and their products to function homologously in the yeast, and vice versa (27, 28). In addition, *S. pombe* grows quickly, and the genome, which is similar in structure to that of higher eukaryotes, is conveniently small (29). However, the yeast does not exactly mimic the mammalian system in pharmacological studies, and one difficulty in using *S. pombe* for resistance studies is that fact that this yeast is relatively more resistant than mammalian cells to many to drugs due to the presence of the cell wall. Nevertheless, at sufficiently high concentrations, DDP does kill *S. pombe*, and the use of a colony-forming assay allows precise detection of differences in sensitivity.

We found that it was possible to isolate a population of DDP-resistant *S. pombe* through the use of chronic exposure to increasing concentrations of DDP, an approach that is similar to what is used for mammalian cells (2). Mammalian cell lines selected *in vitro* with DDP commonly demonstrate only low-level resistance when tested in clonogenic assays, and this pattern was also observed in the *S. pombe*. It is important to note that although modest in comparison to the levels of resistance to other classes of drug mediated by *mdr1* and *MRP* (30, 31), even a 2-fold change in sensitivity is sufficient to account for the loss of the therapeutic effectiveness of DDP (2).

Several other features of the resistant *S. pombe* strain also accurately reflect the characteristics reported for DDP-resistant mammalian cells. First, the resistant phenotype was very stable in *S. pombe*, as it is in mammalian cells (2). Even after growth for ~1500 doublings in the absence of drug, all four clones tested retained a constant level of resistance. Second, the resistant phenotype that remained after three backcrosses was expressed in a dominant fashion in the diploid yeast cells resulting from the mating of DDP-sensitive and -resistant haploid strains. Hybrids between parental and highly DDP-resistant L1210 murine leukemia cells have been shown to retain their resistance (32), and using the 2008 human ovarian carcinoma system, we also documented that low-level DDP resistance is expressed in a dominant

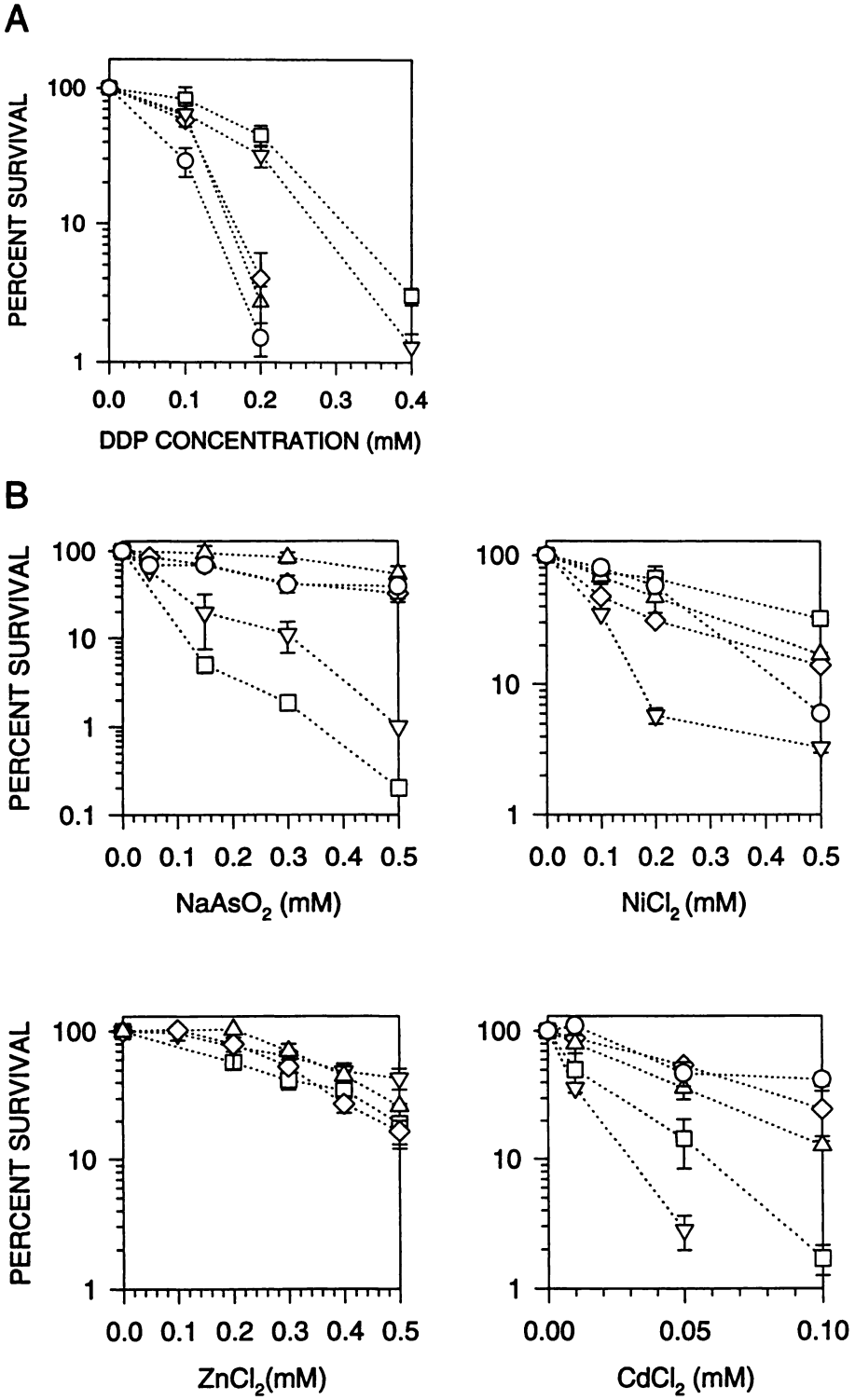

Fig. 4. Survival curves of the 7B1 tetrad clones after 48-hr exposure to DDP (A) and to cadmium, arsenite, zinc, and nickel (B). ○, 7B1A; ◇, 7B1B; △, 7B1C; □, 7B1D. Data are the mean of three independent experiments. *Vertical bars*, mean ± standard deviation.

manner.[1] The general similarity of the DDP-resistant phenotype in *S. pombe* and mammalian cells provides a further rationale for the use of the *S. pombe* system for detailed studies of mechanism.

The 7B1 tetrad exhibited a unique pattern of response to a variety of metal salts. No cross-resistance between DDP and any of the tested metal salts was found. This observation suggests that the mechanism involved in this DDP-resistant phenotype is likely not to be one already identified as mediating resistance to any of these metal compounds. However, hypersensitivity to cadmium and arsenite was detected in the DDP-resistant members of the 7B1 tetrad. Although is it possible that the same gene that mediates resistance to DDP also mediates hypersensitivity to these metal ions, documentation that these characteristics are truly linked must await the analysis of additional tetrads.

[1] S. B. Howell, unpublished observations.

TABLE 3

Arsenite accumulation in the 7B1A, 7B1B, 7B1C, and 7B1D clones

Clone	Accumulation
	cpm/μg protein
7B1A	1541.51 ± 83.76
7B1B	1215.46 ± 262.03
7B1C	761.62 ± 92.96
7B1D	1120.12 ± 188.49

Values are average ± standard deviation of three independent experiments.

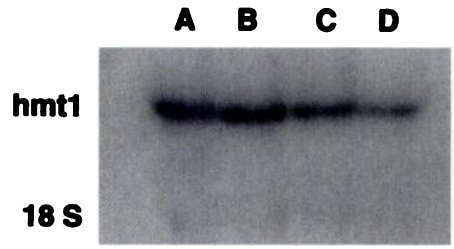

Fig. 5. Northern blot analysis of *hmt1* gene in 7B1A, 7B1B, 7B1C, and 7B1D clones. Twenty milligrams of total RNA was loaded for each clone. A representative experiment is shown.

The facts that *hmt1* mRNA was not overexpressed in the DDP-resistant clones of the 7B1 tetrad and that the DDP-resistant spores from this tetrad demonstrated hypersensitivity rather than resistance to cadmium argue strongly against the possibility that either increased expression or activity of the *hmt1* gene product mediates the DDP resistance in the 7B1A and 7B1D clones. Additional studies are in progress to further exclude involvement of *hmt1*. Because there was no increase in the GSH content in the resistant clones and the GSH content did not cosegregate with DDP resistance in the tetrad-derived clones, it seems that the resistance mechanism is not critically dependent on an elevated GSH content. This situation would be consistent with an increased capacity to remove DDP from DNA, and studies of DNA repair must now be undertaken to address this possibility.

The data from this study are consistent with the concept that a single nuclear gene mediates the DDP-resistant phenotype in the tetrad-derived clones. This conclusion is based on the fact that in the tetrad analysis, there was a clear 2:2 segregation of resistant and sensitive spores. It may be that a single genetic change can also produce resistance in mammalian cells, but other changes produced by repeated cellular injury that are not causally involved in resistance obscure the critical mechanism; indeed, it is possible that the 5A5 cells contain modifying mutations due to the mutagenizing effect of DDP as well. It is important to emphasize that there are other genes whose alteration can individually cause significant degrees of DDP resistance in both yeast and mammalian cells. Loss of the Ixr1 protein in *S. cerevisiae* results in low-level resistance to DDP (33). However, if the IXR1 homolog is involved in the *S. pombe* phenotype that we have identified, the IXR1 homolog would have to be functioning in a transdominant negative mode because the phenotype is dominant.

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Send reprint requests to: Dr. Stephen B. Howell, Department of Medicine 0058, University of CA, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0058. E-mail: showell@ucsd.edu