

A Yeast Homologue of the Human Phosphotyrosyl Phosphatase Activator PTPA Is Implicated in Protection against Oxidative DNA Damage Induced by the Model Carcinogen 4-Nitroquinoline 1-Oxide*

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The model carcinogen 4-nitroquinoline 1-oxide (4-NQO) has historically been characterized as “UV-mimetic” with respect to its genotoxic properties. However, recent evidence indicates that 4-NQO, unlike 254-nm UV light, may exert significant cytotoxic and/or mutagenic potential via the generation of reactive oxygen species. To elucidate the response of eukaryotic cells to 4-NQO-induced oxidative stress, we isolated *Saccharomyces cerevisiae* mutants exhibiting hypersensitivity to the cytotoxic effects of this mutagen. One such mutant, EBY1, was cross-sensitive to the oxidative agents UVA and diamide while retaining parental sensitivities to 254-nm UV light, methyl methanesulfonate, and ionizing radiation. A complementing gene (designated *yPTPA1*), restoring full UVA and 4-NQO resistance to EBY1 and encoding a protein that shares 40% identity with the human phosphotyrosyl phosphatase activator hPTPA, has been isolated. Targeted deletion of *yPTPA1* in wild type yeast engendered the identical pattern of mutagen hypersensitivity as that manifested by EBY1, in addition to a spontaneous mutator phenotype that was markedly enhanced upon exposure to either UVA or 4-NQO but not to 254-nm UV or methyl methanesulfonate. Moreover, the *ytpa1* deletion mutant exhibited a marked deficiency in the recovery of high molecular weight DNA following 4-NQO exposure, revealing a defect at the level of DNA repair. These data (i) strongly support a role for active oxygen intermediates in determining the genotoxic outcome of 4-NQO exposure and (ii) suggest a novel mechanism in yeast involving yPTPA1-mediated activation of a phosphatase that participates in the repair of oxidative DNA damage, implying that hPTPA may exert a similar function in humans.

Treatment with the agent 4-nitroquinoline 1-oxide (4-NQO)¹ has been widely employed in mammalian systems as a paradigm for DNA damage-induced carcinogenesis. To exert its neoplastic effect, 4-NQO must first undergo metabolic activation to the proximate carcinogen 4-hydroxyaminoquinoline 1-oxide, which, following acylation, reacts with DNA to form

stable quinoline-purine monoadducts, *i.e.* at the exocyclic N-2 and N-6 positions of guanine and adenine, respectively (1, 2). In bacteria, yeast, and mammalian cells, these genotoxic “bulky” DNA lesions are processed largely by the nucleotide excision repair (NER) pathway in a manner analogous to classical dipyrimidine photoproducts (*viz.* cyclobutane pyrimidine dimers and (6–4) pyrimidine-pyrimidone photoproducts) generated by the model DNA-damaging agent 254-nm UV light (3, 4). As such, mutants that are deficient in NER are hypersensitive to the genotoxic effects of 254-nm UV light, as well as 4-NQO (3–6). The apparent strong similarity in modes of cellular processing for 254-nm UV light- and 4-NQO-induced DNA damage in diverse prokaryotic and eukaryotic systems has often resulted in categorization of the latter agent as “UV-mimetic” (7).

However, this designation may be inappropriate, because several recent investigations have clearly demonstrated that 4-NQO, unlike 254-nm UV light, can generate a substantial degree of intracellular oxidative stress. This may herald significant consequences for 4-NQO-exposed cells at the level of cell killing and mutagenesis. Indeed, it was shown that 4-NQO is a potent inducer of the *Escherichia coli* soxR/S regulon that responds to intracellular superoxide imbalances (8). It is believed that 4-NQO undergoes redox cycling to produce superoxide anion, which can be further converted into genotoxic reactive oxygen species (*e.g.* singlet oxygen and hydroxyl radicals) that engender modified bases and DNA strand breaks (8, 9). In fact, it has been shown *in vitro* that activated 4-hydroxyaminoquinoline 1-oxide can generate the highly premutagenic product 8-oxo-guanine, as well as strand breaks, in the presence of Cu(II) (9, 10). In *E. coli*, 8-oxoguanine is removed by the formamidopyrimidine-DNA glycosylase (Fpg), which in turn generates apurinic (AP) sites as secondary lesions (11). AP sites are also highly premutagenic but can be efficiently removed from cellular DNA by AP endonucleases (12, 13). It is therefore not surprising that bacterial mutants lacking either Fpg or AP endonucleases are hypersensitive to the mutagenic effects of 4-NQO (but not of 254-nm UV light) (8, 14).

Based on the demonstrated ability of 4-NQO to induce oxidative stress that contributes to cell killing and mutagenesis in bacteria, we postulated that this agent may act similarly in eukaryotic cells. As such, using the model organism *Saccharomyces cerevisiae*, the aim of the present investigation was to identify novel eukaryotic genes implicated in the cellular response to 4-NQO-induced oxidative DNA damage. Our strategy involved, sequentially (i) isolation of a large panel of yeast mutants exhibiting hypersensitivity to the cytotoxic effects of 4-NQO, (ii) categorical preclusion from further analysis of any mutants showing full cross-sensitivity to 254-nm UV light,

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¹ The abbreviations used are: 4-NQO, 4-nitroquinoline 1-oxide; NER, nucleotide excision repair; AP, apurinic; MMS, methyl methanesulfonate; kb, kilobase(s).

because these are likely to carry mutations in previously identified DNA repair genes, *e.g.* comprising the NER pathway known to participate in the processing of UV-type dipyrimidine photoproducts, as well as classical 4-NQO-induced bulky DNA adducts, and (iii) appropriate characterization of any remaining mutants expressing putative defects specifically in the processing of 4-NQO-induced oxidative DNA damage. In this manner, we identified a novel 4-NQO-sensitive yeast mutant that retains parental resistance to 254-nm UV light, while displaying certain hallmarks consistent with a deficiency in the repair of oxidative DNA lesions. A gene complementing this defective phenotype was isolated from a yeast genomic library, and was shown to correspond to a previously identified, but functionally uncharacterized, yeast gene with a predicted amino acid sequence that manifests significant identity with the human phosphotyrosyl phosphatase activator hPTPA. Our data strongly support a genotoxic role for reactive oxygen species in 4-NQO-exposed yeast, and provide novel *in vivo* evidence for the participation of cellular phosphatases in the repair of oxidative DNA damage in eukaryotic cells.

MATERIALS AND METHODS

Strains, Media, Genetic Analysis, and Transformation—The wild type *S. cerevisiae* strains used in this study were DBY747 (*MAT α* , *leu2-3, 112, his3 Δ 1, trp1-289a, ura3-52*, laboratory stock), DBY747-1 (isogenic to DBY747, except *trp1::hisG*), W303a (*MAT α* , *ade2-1, his3-11, leu2-3, 112, trp1-1, ura3-1*, provided by Dr. M. Stark, University of Dundee, UK), FY86 (*MAT α* ; *his3 Δ 200, ura3-52, leu2 Δ 1*, provided by Dr. Fred Winston, Harvard University), and MKp-o (*MAT α* , *can1-1000, ade2-1, lys2-1, ura3-52, leu2-3, 112, his3 Δ 200, trp1- Δ 901*, provided by Dr. Bernard Kunz, Deakin University, Australia). The mutant strain EBY1 is isogenic to DBY747, except that it bears a mutated copy of the *yPTPA1* gene (see below). EBY2, EBY3, and EBY4 were derived, respectively, from DBY747-1, MKp-o, and W303a by deleting a portion of the *yPTPA1* coding region and replacing it with the selective marker *LEU2* (see below). Likewise, CHY26 and CHY27 were derived, respectively, from DBY747-1 and EBY2 by deleting a portion of the coding region of the *yPTPA2* gene and replacing it with the selective marker *TRP1* (see below). The yeast strains DEY10-2C (isogenic to W303a, except *pph22 Δ 1::URA3*), DEY142-4D (isogenic to W303a, except *MAT α* , *pph21 Δ 1::HIS3*), and DEY22-12 (isogenic to W303a, except *pph21 Δ 1::HIS3, pph22-12*) were all generously provided by Dr. M. Stark (University of Dundee, UK). Yeast cells were grown in either complete yeast peptone dextrose (YPD) or minimal synthetic (SC) medium, to which nutritional supplements were added at 20 μ g/ml (15). Standard genetic analysis and transformation were carried out as described previously (16, 17). The *E. coli* strain used for plasmid maintenance was HB101.

Isolation of Mutants Hypersensitive to 4-NQO—Exponentially growing wild type yeast (strain DBY747) were treated with 0.1% MMS for 1 h, and surviving colonies were streaked onto YPD agar plates containing 0.4 μ g/ml of 4-NQO. This drug concentration permits 90–95% growth of wild type, and strains that did not sustain growth at this drug concentration were scored as mutant.

Construction of the *ytpa1* and *ytpa2* Deletion Mutants—A 1.5-kb *SmaI/HindIII* yeast DNA fragment bearing the *yPTPA1* coding region and flanking 5'- and 3'-untranslated regions was derived from pDR1022 (see below) and subcloned into the pBluescript vector K/S to produce the plasmid pTV3. This plasmid was digested with *SalI/PstI* to release a 125-base pair fragment from within the coding region of the *yPTPA1* gene. The released fragment was substituted with the *S. cerevisiae* *LEU2* selective marker, which was obtained as a 2.5-kb *SalI/PstI* fragment from the plasmid YEp13 (16), to produce plasmid pTV4. A linear 3.2-kb fragment, *ytpa1 Δ ::LEU2*, was obtained from pTV4 by digestion with *HindIII* and *ScaI* and transformed directly into the wild type haploid strains DBY747, FY86, and MKp-o using the lithium acetate procedure (17). *Leu2*⁺ transformants bearing a chromosomal deletion of the *yPTPA1* gene were verified by Southern blot analysis (16), using a ³²P-labeled 1.0-kb fragment of the *yPTPA1* coding region as probe. To construct a *ytpa2* deletion mutant, the entire *yPTPA2* gene was first isolated from strain DBY747 by polymerase chain reaction amplification using the upstream (5'-TCTTTTAAAGCTTAA-GAAAATCG-3') and downstream (5'-GGAAAAAGTACGTACCG-3') primers containing the restriction sites *HindIII* and *SnaBI* (18). These

primers amplified a 1.9-kb *HindIII/SnaBI* DNA fragment, which was then subcloned into pBluescript digested with *HindIII* and *SmaI* to produce pCH1. pCH1 was in turn digested with *EcoRI*, which removes 160 nucleotides from the N-terminal end of the *yPTPA2* coding region, and a 1.2-kb *EcoRI* fragment containing the *TRP1* selective marker gene was inserted at the *EcoRI* site to produce pCH2. Digestion of pCH2 with *HindIII* and *BamHI* produced the *ytpa2 Δ ::TRP1* 3.0-kb DNA fragment, which was transformed into strains DBY747 and EBY2. *ytpa2* deletion mutants were confirmed by Southern blot analysis.

Survival Curves—The sensitivities of the various yeast strains to 4-NQO, diamide, and methyl methanesulfonate (all purchased from Sigma) were measured using exponential phase cultures. Overnight cultures grown to saturation at 30 °C in YPD were diluted into fresh medium at an A₆₀₀ of 0.2 (~2 × 10⁶ cells/ml) and incubated to an A₆₀₀ of 1.0. Aliquots were then treated with various concentrations of drugs at 30 °C with shaking (250 rpm) for 1 h. Relative survival was determined by immediately diluting the samples in sterile 20 mM potassium phosphate buffer (pH 7.0) and plating onto YPD agar. Colonies were counted after 3–4 days of growth at 30 °C. In the case of γ -irradiation treatment, exponential cells were irradiated in cold YPD medium using a Co⁶⁰ source at a dose rate of 55.5 rad/s (experiments carried out in the laboratory of Dr. Michael Resnick, NIEHS, NIH).

For exposure to defined regions of the UV wavelength spectrum, mid-log phase yeast were irradiated in sterile distilled water using fluorescent 25-W lamps emitting either UVA (GE model F25T8-BL), UVB (Spectroline model XX25B), or 254-nm UV light (GE model G25T8). Cells were treated at relatively low density (10⁵ cells/ml in 35-mm Petri dishes) and with constant shaking to avoid cellular shielding. The incident light was rigorously purified using 2-mm-thick glass filters (Schott, Mainz, Germany) to virtually eliminate contaminating wavelengths below 290 nm in the case of UVB (filter WG 305) and below 320 nm for UVA (filter WG 345). The incident UVB ($\lambda > 290$ nm), UVA ($\lambda > 320$ nm), and 254-nm UV light dose rates were measured using a Spectroline DRC 100X digital radiometer equipped with DIX 300, DIX 365, and DIX 254 sensors, respectively.

Gradient Plate Assay—This assay was performed as described previously (19). Briefly, cells were replicated as a thin line along the drug gradient, and after 2 days of growth at 30 °C the distance of growth of each strain is expressed as a percentage of the wild type. Growth all along the gradient is considered to be 100%.

Mutation Rate Assay—Spontaneous mutation rates were determined using a fluctuation test as described previously (20). The measurement of the drug-induced mutation rate was done by directly adding the appropriate concentration of drug to the selective medium. For UVA treatment, a 24-well plate containing the culture was irradiated with UVA 2 days after the cells were exhausted of adenine, by which time they had grown from an initial inoculum of ~5,000 to a final density of ~1.0 × 10⁶ cells.

Alkaline Sucrose Density Gradient—Chromosomal DNA from either untreated or 4-NQO-treated cells were isolated and analyzed as described previously (21).

Plasmids—The plasmids pEB1 and pEB2 were constructed by subcloning the 1.5-kb *SmaI/HindIII* DNA fragment carrying the *yPTPA1* gene into the single copy vector YCplac33 and the 2 μ multicopy vector YEplac195, respectively, cut with *SmaI* and *HindIII* (22). Similarly, the plasmids pEB3 and pEB4 were constructed by subcloning the 1.9-kb *HindIII/SnaBI* polymerase chain reaction fragment of the *yPTPA2* gene into YCplac33 and YEplac195, respectively, also cut with *HindIII* and *SmaI*. The multicopy plasmids pPPH21 and pPPH22, respectively, bearing the entire *PPH21* and *PPH22* genes were provided by Dr. M. Stark.

Northern Blot—Total RNA was prepared by the rapid glass bead method (23). The nitrocellulose blot was probed with a random primed ³²P-labeled 1.1-kb *HindIII/SnaBI* fragment derived from the coding region of the *yPTPA1* gene.

Construction of *yPTPA1* Promoter Fusion to *lacZ*—The primers, 5'-CCCTGTGGCCGAATTCATCTGCTC-3' and 5'-ATCTACAGGATCCAGAGACAT-3' containing, respectively, the restriction sites *EcoRI* and *BamHI* (underlined), were used to amplify a 632-base pair fragment consisting of the promoter region of *yPTPA1* gene from -615 to +17 base pairs. The *EcoRI/BamHI* fragment was subcloned into the multicopy vector YEP356R (provided by Dr. Fred Winston, Harvard Medical School, Boston, MA), such that the ATG start codon of the *yPTPA1* gene forms the initiation codon for the *E. coli* reporter *lacZ* gene encoding β -galactosidase.

DNA Sequence Analysis—To determine the location of the mutation in the *yPTPA1* gene derived from the mutant EBY1, the primers 5'-CCCTGTGGCCGAATTCATCTGCTC-3' and 5'-TAATGCTTGGGAT-

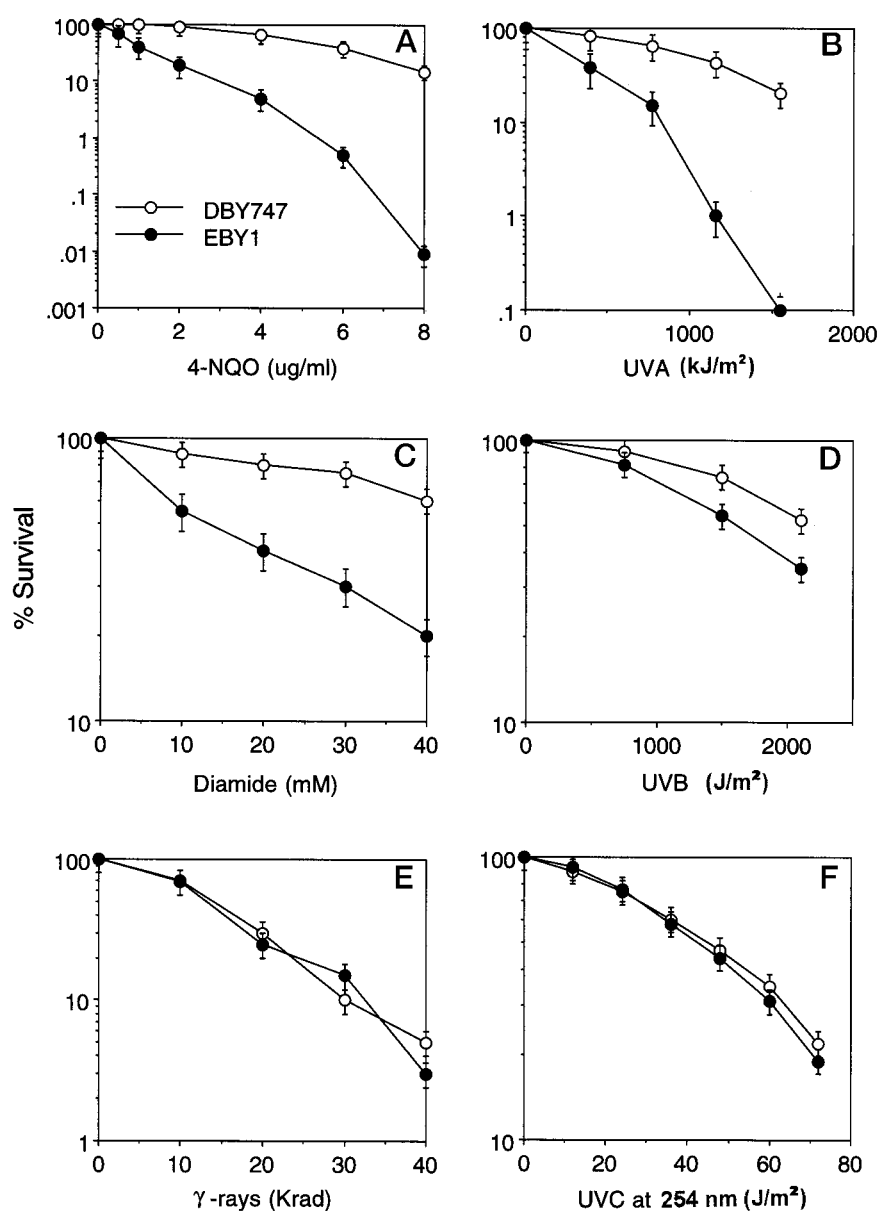


FIG. 1. Sensitivities of *S. cerevisiae* wild type and mutant strains to various mutagens. Points represent the mean of two independent determinations. Open circle, DBY747 (wild type); closed circle, EBY1 (*ytpa1* mutant).

CCACATTTATA-3' were used to isolate a 1.5-kb DNA fragment of the *yPTPA1* gene, with the underlined *EcoRI/BamHI* sites, respectively, from strain EBY1 carrying the mutant allele. The fragment was subcloned into pBluescript, and only one strand of the mutant allele of the *yPTPA1* gene was entirely sequenced by the dideoxy chain termination method (24). All additional DNA fragments isolated by polymerase chain reaction were also sequenced by this method.

RESULTS

Characterization of Yeast Mutants Hypersensitive to 4-NQO—In an attempt to identify novel eukaryotic genes implicated in the repair of 4-NQO-induced oxidative DNA damage, a panel of 55 *S. cerevisiae* mutants exhibiting hypersensitivity to the cytotoxic effects of this agent was initially isolated. Fifty of the mutants among this collection were excluded from further analysis, because they displayed strong cross-sensitivity to 254-nm UV light, *i.e.* reflecting the UV-mimetic character of 4-NQO, therefore indicating the involvement in the observed 4-NQO hypersensitivity of previously identified genes comprising, *e.g.* the NER pathway (3–5). The remaining five 4-NQO-sensitive mutants all retained parental resistance to 254-nm UV light and MMS and were therefore deemed potentially deficient in the processing of oxidative DNA damage. One of these 4-NQO-sensitive, 254-nm UV light-resistant mutants,

designated EBY1, was shown to be highly cross-sensitive to the oxidizing agents UVA (320–400 nm) and diamide (Fig. 1). Moreover, EBY1 displayed only marginal sensitivity to UVB (290–320 nm), which has a much smaller oxidative component relative to UVA (Fig. 1), as well as to hydrogen peroxide (data not shown). Finally, EBY1 also manifested parental resistance to ionizing radiation (Fig. 1) and bleomycin (data not shown), providing evidence that it is not defective in the Rad52 recombinational/double-strand break repair pathway (25).

The mutation conferring 4-NQO and UVA sensitivity to EBY1 was recessive, because a diploid strain constructed by crossing EBY1 (*MATa*) with a wild type strain FY86 (*MATα*) showed wild type resistance to 4-NQO (data not shown). Dissection of eight tetrads derived by sporulating the diploid EBY1 X FY86 produced 2:2 segregation of the 4-NQO sensitivity in the progeny, indicating that EBY1 carries a single mutation.

The EBY1-complementing Gene, Designated *yPTPA1*, Is a Yeast Homologue of the Human Phosphatase Activator *hPTPA*—Because UVA, 4-NQO, and diamide all generate free radicals, we reasoned that the hypersensitivity of EBY1 to these agents may be because of a defect in a pathway that signals the repair of, or itself repairs, oxidatively damaged

FIG. 2. Comparison of the predicted amino acid sequence between *S. cerevisiae* yPTPA1p and human PTPA. The four highly conserved regions (I, II, III, and IV) shown in boxes are also present in yPtpa2p and in proteins predicted from related genes found in other species (see text).

yPtpa1	1MSLDRVDWPHATFSTPVKRIQFDQTTLDFQSSLAHRIKY	40
		:	
hPTPA	1	MAEGERQPPPDSSSEAPPATQNFII PKKEI...HTVPDMGKWKR SQAYAD	47
		
	41	HLHKYTTLISHCSDPDPHATASSIAMVNGMLGVLDKLAHLIDETPPPLPGP	90
		::	
	48	YIGFILTNEGKVGKGLTFEYRVSEAEKLLALLNTLDRWIDETPPVDQF	97
		
	91	RRYGNLACREWHHKLDERLPQWLQEMLPSEYHEVVPQLYYLGNSFGSST	140
		:	
	98	SRFGNKAYRTWYAKLDEEAENLVATVVPVTHLAAVPEVAVYLKESVGNST	147
		
		Region I	
	141	RLDYGTTGHELSFMATVAALDMLGMF.PHMRGADVFLFNKYYTIMRRLIL	189
		:	
	148	RIDYGTGHEAAFAAFLCCLCKIGVLRVDDQIAIVFKVFNRYLEVMRKLQK	197
		
		Region II	
	190	TYTLEPAGSHGVWGLDDHFHLVYILGSSQWQLLDAQAPLQPREILDKSLV	239
		:	
	198	TYRMEPAGSQGVWGLDDFQFLPFIWGSS..QLID.HPYLEPRHFVDEKAV	244
		
		Region III	
	240	REYKDTNFYCQGINFINEVKMGPFEEHSPILYDIAVTPRWSKVCKGLLK	289
		: :	
	245	NENHKDYMFLCILFITEMKTGPF AEHSNQLWNIS.AVPSWSKVNQGLIR	293
		
		Region IV	
	290	MYSVEVLKFFVQHFVFGTGFPPWVNIQNGTDLVPFEEKEEESIEQANA	339
	294	MYKAECLKFFVQHFVFGTGFPPWVNIQNGTDLVPFEEKEEESIEQANA	324
		

DNA. Furthermore, the fact that the mutagen-hypersensitive phenotype of EBY1 was clearly distinct from that of other known yeast DNA repair-deficient mutants suggested that this strain may harbor a mutation at an uncharacterized locus. To isolate the complementing gene, a yeast genomic library constructed in the single copy vector YCp50, and bearing the *URA3* selective marker, was introduced into strain EBY1. At least 10,000 *Ura*⁺ colonies were replica plated onto YPD agar containing 0.4 μ g/ml of 4-NQO, a drug concentration that completely suppressed growth of the mutant but not the parental strain. Two *Ura*⁺ colonies, EBY1/pDR1022 and EBY1/pDR1023, were found to be resistant to 4-NQO and UVA (data not shown). The plasmids isolated from the two independent *Ura*⁺ colonies were identical, because each contained a 12-kb DNA fragment bearing the same restriction enzyme pattern. The portion of the pDR1022 plasmid that conferred 4-NQO or UVA resistance to strain EBY1 was traced to a 1.5-kb *Sma*I/*Hind*III DNA fragment derived from chromosome IX. The fragment contained a single open reading frame (designated *YIL153w* in accordance with the *S. cerevisiae* gene data base nomenclature) and predicted to encode a polypeptide of 394 amino acids in length. Comparison with protein sequences in the GenBankTM data base revealed that *YIL153w* encodes a protein that shares 31, 54, 38, and 40% identity with proteins encoded by, respectively, a *S. cerevisiae* gene *yPL152w* (accession number z73508), a fission *Schizosaccharomyces pombe* gene (accession number z98980), a *Drosophila* gene (accession number x98401), and the human PTPA (hPTPA) gene (accession number x73478) (26). This latter gene encodes the human phosphotyrosyl phosphatase activator PTPA that modulates the weak tyrosyl phosphatase activity of PP2A (26, 27). For simplicity, we refer to the yeast genes *YIL153w* and *YPL152w* as *yPTPA1* and *yPTPA2* and the encoded proteins as yPtpa1p and yPtpa2p, respectively. No homologue of yPtpa1p has yet been isolated from prokaryotic cells. A detailed comparison between *S. cerevisiae* yPtpa1p and human hPTPA1 is shown in Fig. 2. These proteins share four highly conserved regions (I, II, III, and IV) that are also present in yPtpa2p and in the proteins

predicted by the *S. pombe* and *Drosophila* genes, suggesting that they play an important role in cellular metabolism. A computer search for functional motifs revealed that only region II contains the consensus sequence of an ATP binding site (28).

Because yPtpa2p shares significant identity with yPtpa1p, it is possible that the observed phenotype of strain EBY1 might also arise from a genetic defect in the *yPTPA2* gene. To test this, we isolated the *yPTPA1* and *yPTPA2* genes from strain EBY1 using polymerase chain reaction and subjected both to DNA sequence analysis (18). No alteration was found in the nucleotide sequence of the *yPTPA2* gene, as compared with DNA sequences in the *S. cerevisiae* data base. In contrast, a single base pair substitution was found in the *yPTPA1* gene derived from strain EBY1. The mutation was located at codon 60, where TAC was mutated to the TAA stop codon. Thus, the phenotypes associated with EBY1 are because of a defective *yPTPA1* gene and not its homologue *yPTPA2*.

yptpa1 Null Mutants Are Hypersensitive to UVA and 4-NQO—To directly confirm that the *yPTPA1* gene is responsible for the observed phenotypes in strain EBY1, we replaced a portion of the *yPTPA1* coding region, with the selective marker *LEU2* in the parental strain DBY747. The resulting mutant EBY2 (*yptpa1 Δ ::LEU2*) showed identical sensitivity to UVA and 4-NQO, as compared with EBY1 (Fig. 3, A and B). Deletion of the *yPTPA1* gene in three additional parent strains FY86, MKp-o, and W303 also produced mutants with marked sensitivities to UVA and 4-NQO (data not shown), eliminating the possibility that the observed phenotypes were strain-specific. Like EBY1, none of the deletion mutants showed sensitivities to other DNA-damaging agents including MMS, γ -rays, and 254-nm UV light. Full parental UVA and 4-NQO resistance was restored to EBY2 by the single copy centromeric plasmid, pEB1, carrying the entire *yPTPA1* gene (positions -615 to +1400), including its putative promoter, the entire coding region, and 200 nucleotides downstream from the stop codon (Fig. 3, A and B). Increasing *yPTPA1* gene dosage by using a multicopy plasmid pEB2 conferred no further mutagen resistance, suggesting that yPtpa1p is not limiting in the cell

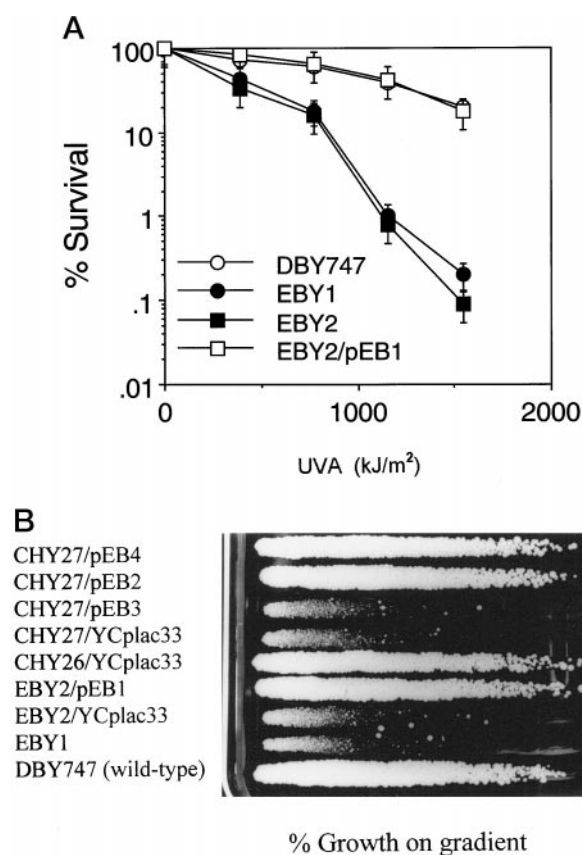


FIG. 3. Resistance of yeast strains to UVA and 4-NQO. **A**, UVA treatment of the indicated strains. **B**, 4-NQO gradient plate assay of the following strains: DBY747 (wild type), EBY1 (mutant), EBY2 (*ytpa1Δ::LEU2*)/YCplac33, EBY2/pEB1, HCY26 (*ytpa2Δ::TRP1*)/YCplac33, HCY27 (*ytpa1Δ::LEU2*; *ytpa2Δ::TRP1*)/YCplac33, HCY27/pEB3, HCY27/pEB2, and HCY27/pEB4. The plasmids pEB1 and pEB3 contained, respectively, the entire *yPTPA1* and *yPTPA2* genes inserted into the single copy vector YCplac33, and pEB2 and pEB4 contained, respectively, the entire *yPTPA1* and *yPTPA2* genes inserted into the multicopy vector YEplac195. Growth all along the gradient is taken to be 100% (see "Materials and Methods"). The bottom layer of the gradient contained 15 μg of 4-NQO.

(Fig. 3B).

Although the above data confirm that *yPtpa1p* is essential for cellular resistance to UVA and 4-NQO, they do not completely exclude a role for *yPtpa2p*. To directly test this, a mutant was constructed by deleting the *yPTPA2* gene from the parent strain DBY747 by replacing it with the auxotrophic marker gene *TRP1*. The resulting mutant CHY26 retained parental resistance to UVA, to 4-NQO, and to other DNA-damaging agents such as MMS (Fig. 3B; data shown only for 4-NQO). Furthermore, a *ytpa1 ytpa2* double mutant CHY27 was no more sensitive to 4-NQO than the single *ytpa1* mutant EBY2 (Fig. 3B). These data confirm that *yPTPA1* and not *yPTPA2* is required for UVA and 4-NQO tolerance. It should also be noted that 4-NQO resistance could not be restored to strain CHY27 by the plasmid pEB3, which bears a single copy of the *yPTPA2* gene (Fig. 3B). Interestingly, however, if the *yPTPA2* gene was inserted into the multicopy vector YEplac195, and the resulting plasmid pEB4 was introduced into strain CHY27, full parental 4-NQO resistance was restored (Fig. 3B). This latter observation may not be surprising in view of the high degree of homology between *yPtpa1p* and *yPtpa2p*, but nonetheless confirms that the endogenous level or activity of *yPtpa2p* is insufficient to compensate for complete loss of *yPtpa1p*.

yPtpa1p Expression Is Not Inducible—To determine whether

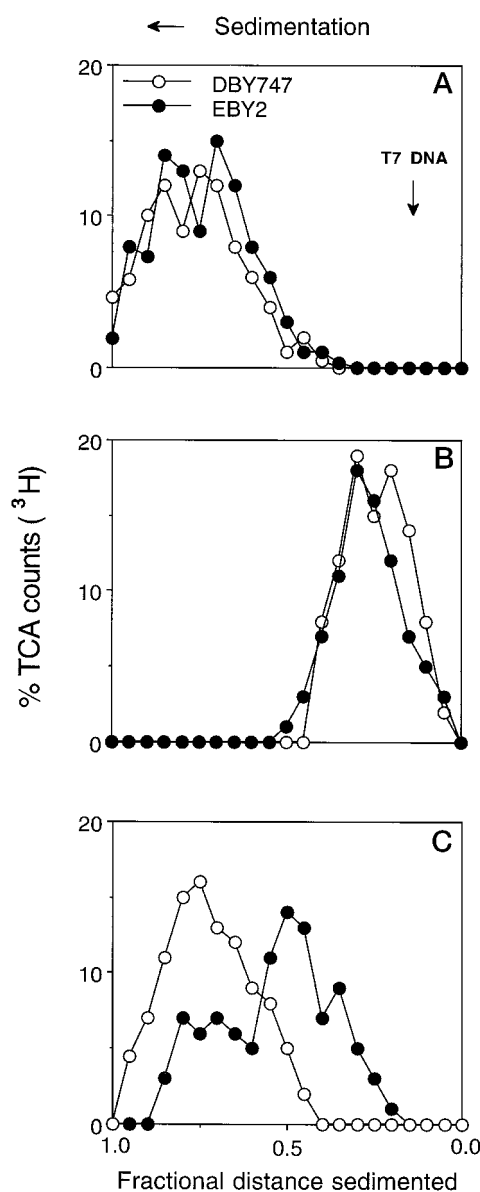


FIG. 4. Analysis of chromosomal DNA damage from 4-NQO-treated *S. cerevisiae* on an alkaline sucrose density gradient. **A**, untreated. **B**, treated with 2.5 μg/ml of 4-NQO for 1 h. **C**, treated with 4-NQO, washed, and recovered for 8 h in YPD medium. Sedimentation positions of bacteriophage T7 DNA are shown by vertical arrows. TCA, trichloroacetic acid.

yPtpa1p is inducible by cellular stress, the putative *yPTPA1* promoter region (spanning positions −615 to +1) was fused to the reporter gene *lacZ*, encoding β-galactosidase. Upon introduction of the fusion plasmid construct into either EBY2 or the wild type strain, each expressed the same basal level of β-galactosidase activity (120 units/mg protein). This activity could not be induced by exposure to either UVA or 4-NQO nor by stress conditions including heat and osmotic shock. It would therefore appear that the endogenous level of *yPtpa1p* is sufficient for its biological function.

The ytpa1 Null Mutant Is Defective in the Recovery of High Molecular Weight DNA Following Treatment with 4-NQO—Mutants deficient in any of the three major DNA repair pathways, i.e. the nucleotide excision, post-replicative, and recombinational pathways, show hypermutability in response to a wide spectrum of DNA-damaging agents including 4-NQO. We therefore tested whether the hypersensitivity of EBY2 to 4-NQO might be attributable to a defect in DNA repair. The

TABLE I
Spontaneous and induced mutation rates in the parent and the *ytpa1* Δ mutant

Spontaneous mutation rates were measured at the *ade2-1* allele as described previously (20). The values are the average of three independent determinations.

Treatment	MKP-o	EBY3	Fold increase
	mutation rate / 10^8 cell/generation		
None	3.4 ± 0.8	10.9 ± 1.6	3
UVA (150 kJ/m ²)	4.6 ± 0.7	43.8 ± 3.1	9
UVA (300 kJ/m ²)	6.6 ± 0.9	110.8 ± 7.1	17
4-NQO (0.005 μ g/ml)	5.1 ± 1.1	39.8 ± 3.5	8
4-NQO (0.01 μ g/ml)	7.3 ± 1.7	95.2 ± 8.5	13
MMS (0.001%)	2.4 ± 0.5	8.4 ± 1.5	3
MMS (0.05%)	5.3 ± 0.9	13.4 ± 2.8	2
254-nm UV light (10 J/m ²)	4.1 ± 0.7	7.9 ± 1.6	2

parental and mutant strains were challenged, or not, with 0.5 μ g/ml of 4-NQO for 1 h, followed by isolation of chromosomal DNA and quantitation of strand breaks by alkaline sucrose density gradient analysis (21). Undamaged DNA isolated from either the parent or the mutant were indistinguishable with respect to size sedimentation (Fig. 4A). In response to 4-NQO treatment, however, chromosomal DNA from both strains were fragmented and in each case appeared to sustain the same extent of initial damage (Fig. 4B). When the cells were washed free of 4-NQO and allowed to recover for 8 h in fresh growth medium, the parental DNA was completely restored to high molecular weight status, whereas only approximately 40% of the EBY2-derived DNA was fully restored to its native size (Fig. 4C). Higher doses of 4-NQO (1.0 μ g/ml for 1 h) resulted in further accumulation of fragmented DNA only in the mutant, and at 2 μ g/ml of 4-NQO for 1 h, no restoration of high molecular weight DNA was observed in the mutant, whereas 55% of the parental DNA had recovered (data not shown). These data are consistent with the notion that 4-NQO induces two types of DNA lesion, only one of which is repaired less efficiently by the *ytpa1* mutant.

ytpa1 Null Mutants Exhibit a Hypermutable Phenotype—Because our data are consistent with the notion that yPtpa1p plays a role in the repair of 4-NQO-induced oxidative DNA lesions, this protein may be expected to act similarly for spontaneously occurring oxidative DNA lesions generated during aerobic growth. We therefore measured the reversion mutation rate at the *ade-2* locus in a wild type strain MKP-o, and in the isogenic *ytpa1* null mutant EBY3. The *ade-2* allele bears an ochre stop codon that results in a truncated protein unable to support adenine biosynthesis, *i.e.* the strain depends on exogenous adenine for growth. Under normal growth conditions, EBY3 showed a 3-fold increased rate of reversion to Ade⁺, as compared with MKP-o (Table I). This suggests that yPtpa1 may participate in the repair of endogenously generated DNA lesions. The reversion rate to Ade⁺ was increased by as much as 9-fold when the mutant was irradiated with 150 kJ/m² of UVA light and up to 17-fold with 300 kJ/m², whereas this rate was increased only approximately 2-fold in wild type cells irradiated with 300 kJ/m² UVA. Exposure to sublethal doses of 4-NQO, *i.e.* 0.005 and 0.01 μ g/ml, also dramatically increased the mutation rate in the *ytpa1* mutant by 8- and 13-fold, respectively, as compared with an increase of only 2-fold in parental cells treated with 0.01 μ g/ml of 4-NQO. There was no striking difference in the mutation rate between MKP-o and EBY3 upon exposure to MMS or 254-nm UV light, relative to untreated cells (Table I).

yPtpa1p Does Not Mediate Its Effect via the Pph21p or Pph22p Phosphatase—To explore the role of specific yeast protein phosphatases in the cellular response to 4-NQO-induced

DNA damage, we examined whether yeast mutants bearing mutations in two redundant PP2A phosphatase genes *PPH21* and *PPH22* (encoding proteins that display nearly 80% amino acid identity to the catalytic subunit of mammalian PP2A) are hypersensitive to 4-NQO (29, 30). Single mutants lacking either Pph21p or Pph22p protein phosphatase were not sensitive to 4-NQO (Fig. 5). The absence of drug sensitivity in either single mutant could be explained if there is functional redundancy between Pph21p and Pph22p. However, it is unlikely that one phosphatase substituted for the other, because a *pph21 pph22* double mutant was also found to be resistant to 4-NQO (Fig. 5). Furthermore, overproduction of either Pph21p or Pph22p conferred no 4-NQO or UVA resistance to the *ytpa1* mutant (Fig. 5). This finding suggests that yPtpa1p may not activate either Pph21p or Pph22p.

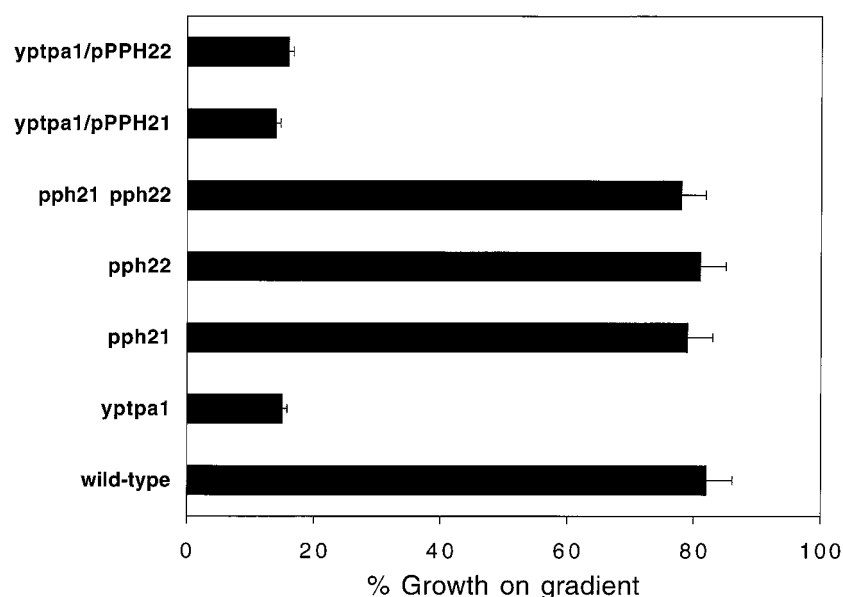
DISCUSSION

The results presented here provide novel evidence that the previously identified but functionally uncharacterized *S. cerevisiae* gene, herein designated *yPTPA1*, plays a significant role in cellular protection against the model DNA-damaging agent 4-NQO. Moreover, *ytpa1* null mutants exhibit prominent phenotypic characteristics that are consistent with a defect in the repair of 4-NQO-induced oxidative DNA lesions rather than of classical 4-NQO-induced bulky adducts, including (i) wild type resistance to 254-nm UV light, which shares considerable genotoxic properties with 4-NQO but does not significantly alter the redox state of the cell with respect to cytotoxicity and mutagenesis (31); (ii) cross-sensitivity to the cytotoxic and mutagenic effects of UVA, an agent that acts virtually exclusively through the generation of reactive oxygen species (32, 33); (iii) only marginal sensitivity to killing by UVB, which has an oxidative component but nonetheless closely resembles 254-nm UV light in genotoxic properties (34, 35); (iv) a spontaneous mutator phenotype, which is characteristic of prokaryotic and eukaryotic mutants deficient in the cellular response to oxidative DNA damage (36); and (v) impaired recovery of high molecular weight DNA following 4-NQO exposure, strongly indicating a defect at the level of DNA repair. We note that the *ytpa1* mutant is not sensitive to the powerful oxidant ionizing radiation, which acts principally through the formation of hydroxyl radicals, rather than via singlet oxygen that is implicated in the genotoxic effect of UVA (32, 33). The overall data strongly support the notion that 4-NQO-induced oxidative DNA damage, in addition to the well characterized N-2 and N-6 adducts of guanine and adenine, respectively, can exert considerable cytotoxic and premutagenic potential in eukaryotic cells.

The fact that *ytpa1* mutants are cross-sensitive to the cytotoxic and mutagenic effects 4-NQO and UVA presumably reflects the production of a common oxidative DNA lesion(s). This lesion would not appear to be 8-oxoguanine or AP sites, because unlike the case for *E. coli*, yeast mutants lacking either the enzyme Ogg1p (corresponding to the bacterial Fpg protein) or the major AP endonuclease Apn1, are not sensitive to either UVA or 4-NQO.² This difference between bacteria and yeast may be explained if the latter retains “backup” proteins to repair 4-NQO-induced oxidative DNA lesions. In support of this assumption, it has been shown that yeast expresses the protein Ogg2p, which displays similar activities to Ogg1, as well as another AP endonuclease, Pde1p (38, 39). In any case, the culpable premutagenic oxidative lesion(s) common to 4-NQO- and UVA-exposed cells and the yPtpa1p-mediated pathway that alleviates its (their) genotoxic potential remain to be characterized.

² D. Ramotar, unpublished data.

FIG. 5. **Resistance of yeast strains to 4-NQO.** Bars indicate wild type (W303a) and the following isogenic mutant strains: EBY4 (*ytpa1Δ::LEU2*), DEY142-4D (*pph21Δ1::HIS3*), DEY10-2C (*pph22Δ1::URA3*), DEY22-12 (*pph21Δ1::HIS3*, *pph22-12*), and EBY4 carrying the multicopy plasmid pPPH21 or pPPH22. For simplicity, mutants are indicated by their deleted genes. Growth all along the gradient is taken to be 100% (see "Materials and Methods"). The bottom layer of the gradient contained 15 μ g of 4-NQO.



We have noted, however, that yPtpa1p manifests significant homology with human hPTPA, which was shown to stimulate the weak phosphotyrosine phosphatase activity of PP2A in an ATP- and Mg^{2+} -dependent manner (26, 27). However, the biological consequences of this phosphatase activation step in human cells remain unclear. Based on our present findings that reveal a novel role for yPtpa1p in the repair of 4-NQO-induced oxidative DNA damage in yeast, we predict that hPTPA could perform a similar function in human cells. Indeed, considering the ubiquitous participation of reversible protein phosphorylation in diverse cellular processes (40, 41), including the overall response to genotoxic agents, *e.g.* cell cycle arrest (42) and apoptosis (43), it is reasonable to anticipate that protein phosphatases also play an essential role in DNA repair. Preliminary evidence for the involvement of cellular PP2A phosphatases in NER has been obtained in human cells *in vitro* (37). In this study, specific inhibitors of PP2A were shown to interfere with the NER activity of human Hela cell extracts, an effect that could be fully reversed by the addition of purified PP2A protein (37). However, the exact component of the NER pathway that is dephosphorylated by PP2A to allow efficient repair *in vitro* is unknown, and certainly, any precise *in vivo* roles for specific protein phosphatases in the DNA repair process remain to be elucidated.

It will therefore be crucial to identify the cellular targets, presumably (but not necessarily) corresponding to a protein phosphatase(s), that interact with yPtpa1p to protect against the genotoxic effects of 4-NQO/UVA exposure. In *S. cerevisiae*, there are two distinct families of serine/threonine protein phosphatases, PPP and PP2C (30). The PPP family consists of at least 12 members, Glc7, Pph21, Pph22, Cna1, Cna2, Ppz1, Ppz2, Sal6, Pph3, Sit4, Ppg1, and Ppt1 (30), which are further subdivided on the basis of enzymatic properties into three classes, PP2A, PP2B, and PP1. The PP2A members, including Pph3, Pph21, Pph22, Sit4, and Ppg1, are trimeric holoenzymes consisting of a 35-kDa catalytic subunit, a 65-kDa constant regulatory subunit, and a variable regulatory subunit ranging from 54 to 74 kDa in size (30). The constant and variable regulatory subunits are important for controlling phosphatase activity, substrate specificity, and cellular localization.

In view of the aforementioned putative role of PP2A-type phosphatases in human NER and the fact that hPTPA is capable of activating these phosphatases *in vitro* (26–27), we hypothesized that certain PP2A-related enzymes in yeast

might play a role in the cellular response to 4-NQO. However, it is unlikely that yPtpa1p mediates its biological effect via Pph21 or Pph22, because mutants lacking these serine/threonine phosphatases did not manifest hypersensitivity to either UVA or 4-NQO. Furthermore, overproduction of these same phosphatases in the *ytpa1* mutant did not confer increased resistance to UVA or 4-NQO. Full characterization of additional 4-NQO/UVA-sensitive mutants, coupled with direct investigation of proteins that interact with yPtpa1p, may be expected to shed significant light on the precise protective pathway mediated by this protein in yeast.

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A Yeast Homologue of the Human Phosphotyrosyl Phosphatase Activator PTPA Is Implicated in Protection against Oxidative DNA Damage Induced by the Model Carcinogen 4-Nitroquinoline 1-Oxide

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