Functional genomics reveals a family of eukaryotic oxidation protection genes

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Reactive oxygen species (ROS) are toxic compounds produced by normal metabolic processes. Their reactivity with cellular components is a major stress for aerobic cells that results in lipid, protein, and DNA damage. ROS-mediated DNA damage contributes to spontaneous mutagenesis, and cells deficient in repair and protective mechanisms have elevated levels of spontaneous mutations. In *Escherichia coli* a large number of genes are involved in the repair of oxidative DNA damage and its prevention by detoxification of ROS. In humans, the genes required for these processes are not well defined. In this report we describe the human *OXR1* (oxidation resistance) gene discovered in a search for human genes that function in protection against oxidative damage. *OXR1* is a member of a conserved family of genes found in eukaryotes but not in prokaryotes. We also outline the procedures developed to identify human genes involved in the prevention and repair of oxidative damage that were used to identify the human *OXR1* gene. This procedure makes use of the spontaneous mutator phenotype of *E. coli* oxidative repair-deficient mutants and identifies genes of interest by screening for antimutator activity resulting from cDNA expression.

Reactive oxygen species (ROS) are formed as by-products of normal metabolism of aerobic organisms and react with DNA to produce damage (1). Cells protect themselves from ROS by detoxification mechanisms and by mechanisms that repair the damage ROS produce (2–6). In humans oxidative damage results in mutagenesis, triggers apoptosis, and has been implicated as a contributing cause of a number of human diseases, including cancer and neurodegenerative diseases. Oxidative damage has also been implicated as a contributing factor to the aging process (3, 7, 8). For example, mutations in genes affecting the cell's ability to repair oxidative damage, such as BRCA1 and ATM, have been shown to predispose patients to cancer (9) and mutations in the superoxide dismutase gene, which affects the cell's ability to detoxify reactive oxygen species, predisposes patients to amyotrophic lateral sclerosis (10). Protective mechanisms also interfere with cancer therapies, preventing or repairing oxidative DNA damage produced by radiation treatments and other therapies (11). Our understanding of ROS in human cells is limited, and the biological consequences of oxidative damage are complex. The mechanisms that provide protection from ROS are more clearly understood in *Escherichia coli*.

In wild-type prokaryotic and eukaryotic cells spontaneous mutagenesis by reactive oxygen species is held in check by enzymes that detoxify ROS and by enzymes that repair ROS damage to DNA. Imbalances in these processes can increase the spontaneous levels of mutation and increase sensitivity to exogenous sources of ROS (2–6). We have constructed a series of mutant strains of *E. coli* defective in repair pathways acting on oxidative DNA damage for use in searches for human oxidation protection genes. These *E. coli* mutants carry various combinations of mutations in *fpg*, *mutY*, *nth*, *nei*, and *mutH*. All of these mutations confer sensitivity to exogenous peroxide treatments or oxidative mutagenesis, except *nei*, which increases the peroxide sensitivity of *nth*, *fpg*, and *mutH* strains but has no detectable effect in an otherwise wild-type cell (refs. 12–15 and M.R.V., J. Wyrzykowski, and L. Fan, unpublished data). Most of these mutant strains also exhibit a spontaneous mutator phenotype that results largely, or exclusively, from their inability to repair spontaneous oxidative damage (refs. 12–14 and M.R.V., J. Wyrzykowski, and L. Fan, unpublished data). Thus, by screening cDNA libraries for genes that counteract the spontaneous oxidation-dependent mutator phenotype of the above *E. coli* mutants, it is possible to identify genes that either prevent or repair oxidative DNA damage.

Materials and Methods

Bacterial Strains. MV3884 is a *mutH472*::*Tn*10 *nth-1*::*kan,ble* derivative of MV1161 (16). It was constructed by sequential introduction of the *mutH* allele from strain CGSC7254 (Coli Genetic Stock Center, Yale University) and the *nth* allele from strain BW372 (17) by P1 transduction selecting for the appropriate drug resistance, and testing for the mutator phenotype and peroxide sensitivity resulting from each mutation. The cDNA library used to transform the test strain was a gift from E. Perkins and M. Resnick (National Institute of Environmental Sciences, Research Triangle Park, NC; ref. 18).

Culture Media. LB ampicillin plates were standard LB medium (16) containing 100 μg/ml ampicillin. Semi-enriched medium containing E salts (ref. 19; ESEM) plates are standard SEM plates in which the salts solution has been replaced by E salts (16, 19). The low level of arginine supplied is sufficient to allow a background growth of arginine-requiring cells to reach a growth ceiling of approximately 5×10^9 cells per plate. Once the arginine is exhausted, only Arg^+ revertants will continue to grow to form colonies (20). Standard yeast extract/peptone/dextrose (YEPD) plates and broth were used for routine growth of yeast. Minimal drop-out medium lacking uracil was used to select for $Ura3^+$ recombinants (21). All yeast incubations were performed at 30°C; all bacterial incubations were performed at 37°C.

Screening for Human Antimutator Genes. Competent MV3884 cells were transformed with 600 ng of cDNA present in the pSE380 vector, which contains an isopropyl-β-D-thiogalactoside (IPTG)-inducible synthetic promoter that functions in *E. coli* (18). Transformants were selected on LB ampicillin plates. Transformants were then picked, inoculated into 96-well microtiter trays containing 250 μl LB ampicillin, and grown overnight for subsequent testing. Trays were spotted onto two ESEM-ampicillin plates with a multiprong device; one plate contained IPTG (2 mM) for induction of cDNA transcription. Spontaneous mutation frequen-

Abbreviations: ROS, reactive oxygen species; YEPD, yeast extract/peptone/dextrose; IPTG, isopropyl-β-D-thiogalactoside.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF309387).

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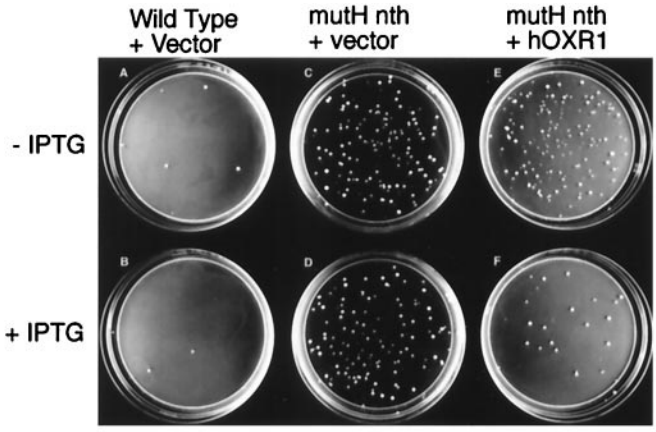

Fig. 1. Effect of h*OXR1* expression on Arg^+ mutagenesis in *E. coli*. (*A* and *B*) MV3626 (wild type/pTrc99A; Amersham Pharmacia). (*C* and *D*) *MV4174 (mutH nth*/pTrc99A). (*E* and *F*) MV4300 [*mutH nth*/pMV420 (h*OXR1*)]. (*A, C,* and *E*) No IPTG induction. (*B, D,* and *F*) IPTG (1 mM) induction.

cies were estimated, and spots showing an IPTG-inducible decrease in spontaneous mutagenesis were identified, purified, and tested further. Quantitative levels of Arg^- mutations in the presence or absence of IPTG induction were determined (Fig. 1), and clones showing a clear decrease in mutagenesis were selected, and their plasmids were purified and retransformed into a fresh isolate of the MV3884 mutant strain to confirm that the antimutator phenotype was due to the presence of the cDNA. To eliminate clones that either interfered with the Arg reversion assay or had nonspecific effects on mutagenesis, cDNA clones were transformed into either an *ung* or a *dnaQ* mutant strain, two mutator strains that also have an increased spontaneous mutation frequency similar to that of MV3884, but for reasons other than oxidative repair deficiencies. Clones showing antimutator activities in the *ung* or *dnaQ* mutants similar to those seen in MV3884 were presumed to affect steps in the Arg^+ mutagenesis process subsequent to the production and processing of the initial DNA damage and were eliminated from the screen.

Cloning of *Saccharomyces cerevisiae OXR1*. The *S. cerevisiae OXR1* gene (sc*OXR1*) was cloned via a PCR approach. Primers 1 and 2 (see below) were used to amplify the sc*OXR1* coding sequence and 300 bp of flanking DNA on each side of the wild-type yeast R117 (22). These primers also included new restriction sites that allowed cloning of the amplified sequence into the *Eco*RI and *Bam*HI sites of the vector pTrc99A (Amersham Pharmacia) to produce plasmid pMV600. A second PCR reaction was performed using the pMV600 plasmid as a template to clone the two flanking regions in separate reactions, using primers 1 and 4 to clone the upstream flanking region and primers 5 and 6 to clone the downstream flanking region. Each flanking region contained either the first or last three codons of sc*OXR1* and introduced restriction sites compatible with the *Sac*I *Xba*I sites needed to insert the $Ura3^+$ cassette between the two flanking DNA regions. The three fragments were assembled to produce the plasmid pMV605, in which the $Ura3^+$ DNA sequences replaced all but the first three and last three codons of sc*OXR1* and are flanked on each side by 300 bp of sc*OXR1* flanking DNA. This fragment (shown in *Results*, Fig. 4) was then purified as a single *Pvu*II fragment. Competent *ura3*$^-$ mutant yeast cells (strain R117) were then transformed by Li acetate transformation with the *URA3*$^+$-carrying *Pvu*II fragment purified from the pMV605 plasmid, and *URA*$^+$ colonies were selected by growth in the absence of uracil. Two *URA*$^+$ transformants were purified and designated strains N1 and N2. N1 and N2 were sporulated in acetate medium, and tetrads were dissected. Two haploid strains N1–9 (*URA3*$^+$) and N1–4 (*ura3*$^-$) were selected for further study. Genetic structures of the mutants were confirmed by Southern blotting. The primers used were: primer 1, ATCATCGAATTCATATGACCGGACTCGTAAT; primer 2, ATCATCGGATCCTTTTTTTTCACATTGGGAG-3′; primer 4, ATCATCGAGCTCTCCAAACATTGTCGCTCC; primer 5, ATCATCCCCGGGGTAGGATAGTGTCATCTA, and primer 6, ATCATCCTGCAGTTTTTTTTCACATTGGGAG.

Southern Hybridizations. Yeast genomic DNA was prepared as described by Adam *et al.* (21). Standard hybridization methods were used to measure the size of the *OXR1* or *URA3* replacement allele carrying DNA fragments (23). The 300-bp *Eco*RI–*Sac*I fragment from pMV603, corresponding to the upstream *OXR1* flanking DNA (shown in *Results*, Fig. 4), was used as a probe.

Yeast Strains. Yeast strains used in this study are derivatives of R117 (22), a strain congenic to 381G (24). Strains were grown in standard YEPD medium at 30°C. Additional strains constructed in this study were derivatives of R117 and carry the following additional genetic markers: N1, MATa/MATα Δ*oxr1*::*URA3*/*OXR1*; N1–9, MATa Δ*oxr1*::*URA3*; and N1–4, MATa *OXR1*.

Peroxide Sensitivity Testing. Peroxide sensitivity tests were performed as described by Ramotar *et al.* (1). Briefly, overnight cultures of yeast strains were diluted to an OD_{600} of approximately 0.3 in standard YEPD medium and grown with aeration to an OD_{600} of approximately 0.8–1. Cells were harvested, washed once in sterile water, and resuspended in PBS (pH 7.4). Samples were treated with H_2O_2 at the indicated concentrations for 1 h at 30°C with aeration. After treatment, cells were diluted in PBS and titered on YEPD plates. Experiments were repeated at least three times; representative data are shown.

DNA Sequencing. DNA sequencing was performed by the MIT Center for Cancer Research, the University of Massachusetts Medical Center, or the Iowa State University DNA sequencing facilities. DNA and predicted protein sequences were analyzed using Blast sequence searches (25).

Results

Screening for Oxidation Protection Genes. To identify DNA oxidation protection genes, we transformed *E. coli* oxidation repair-defective spontaneous mutator strains with a human cDNA library and screened transformants for a reduction in mutator activity. Genes exhibiting antimutator activity were then subjected to a variety of tests to confirm that the reduction in mutagenesis was a consequence of reduced oxidative mutagenesis, rather than nonspecific effects on the mutagenesis assay system (see *Materials and Methods*). Of the approximately 10,000 cDNAs tested in the initial screen, several reduced spontaneous mutagenesis in the oxidation-specific mutator strains. Of particular interest was the oxidation resistance gene, which we named *OXR1*. This gene was initially identified using the *E. coli* mutH *n*th double mutant strain as the mutagenesis indicator strain. This strain is highly sensitive to peroxide treatments, and both mutations contribute to this phenotype (refs. 12 and 15, and M.R.V., J. Wyrzykowski, and L. Fan, unpublished data). Fig. 1 compares spontaneous Arg^+ mutagenesis in wild-type and *mutH nth* double-mutant strains and shows that IPTG induction of the vector has no effect. However, when the human *OXR1* (h*OXR1*) gene is induced by IPTG it causes about a 5-fold reduction in spontaneous Arg^+ mutagenesis in the *mutH nth* strain (Fig. 1*F*) without a detectable effect on growth (data not shown). Thus, h*OXR1* functions as an antimutator in this *E. coli* genetic background.

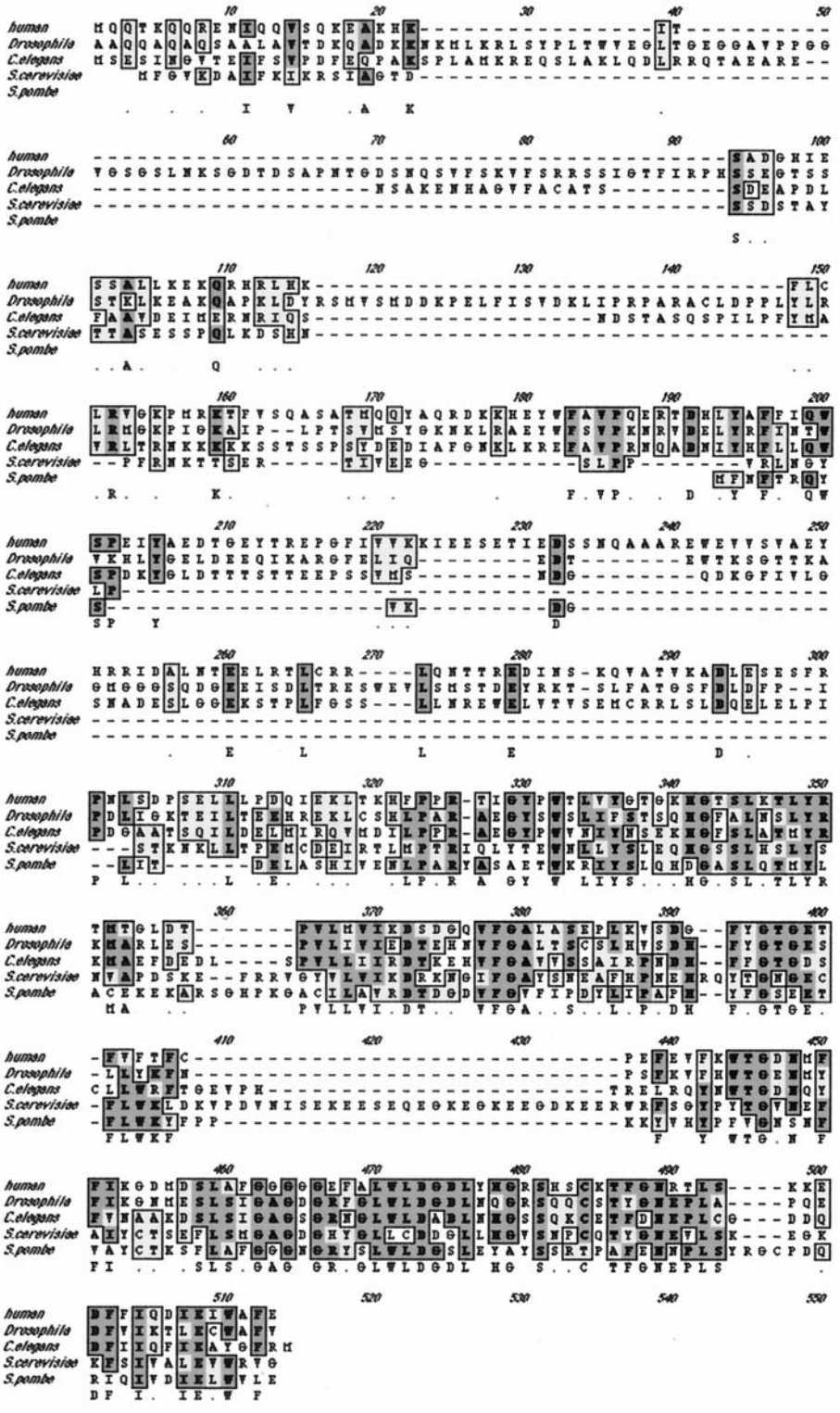

Fig. 2. Alignment of *OXR1* homologues. Numbering corresponds to the human *OXR1* protein sequence. It is compared with its homologues from *C. elegans* (AAD31551), beginning with residue 425; *Drosophila melanogaster* L82C (AAD28510), beginning with residue 421; and the entire sequences of *S. cerevisiae* YPL196w (CAA97909) and *S. pombe* (CAB16289) alleles. Highly conserved, identical residues are indicated in bold type with a dark background; conserved residues and their conservative substitutions are in plain type with a light gray background. The consensus sequence is indicated on the bottom line; regions containing conservative substitutions are indicated by dots.

Analysis of the h*OXR1* DNA Sequence. DNA sequence analysis shows that the h*OXR1*-expressing plasmid pMV520 carries a cDNA insert of approximately 1.7 kbp encoding a previously unidentified human gene that matches several human and mouse expressed sequence tag database sequences and shares homology with genes found in *S. cerevisiae, Schizosaccharomyces*

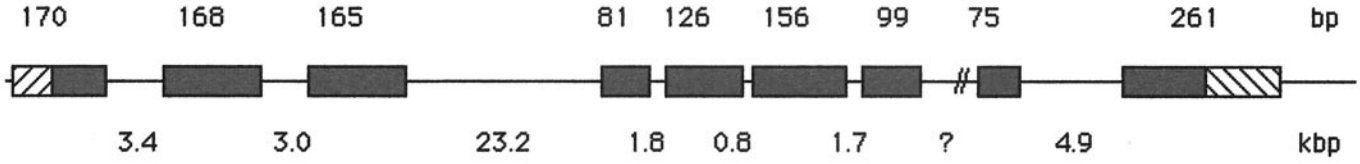

Fig. 3. Genomic structure of *OXR1*. The *OXR1*-containing region of chromosome 8q23 is shown (not to scale). The sizes (in bp) of exons (black boxes) are shown above the line; the sizes of introns (in kbp) are shown below the line. The region between exons 7 and 8, indicated by the question mark, contains a sequencing gap of unknown size. Untranslated regions that are also present in the cDNA clone are shown as striped boxes.

pombe, Caenorhabditis elegans, and *Drosophila*. Homologues are not found in *E. coli* or other bacterial species. Thus h*OXR1* appears to be a member of a conserved family of genes present in a wide variety of eukaryotic species. Fig. 2 compares the predicted h*OXR1* protein sequence with the corresponding regions of its homologues and shows a consensus sequence for the *OXR1* family of genes. The highest degree of homology is in the carboxyl-terminal half of the protein, and several consensus motifs are identifiable in this region (Fig. 2). Two additional regions conserved primarily among the higher eukaryotes are found in the region corresponding to h*OXR1* amino acid residues 100–200 (Fig. 2). The conserved motifs of *OXR1* do not correspond to motifs of known function; thus their functions cannot currently be predicted. The h*OXR1*-related genes are the *S. cerevisiae* OXR1 homologue (sc*OXR1*), which is 27% identical and 43% similar to the h*OXR1* gene. It is known only as the ORF YPL196w, and no phenotype has been ascribed to this gene. Similarly, the *S. pombe* and *C. elegans OXR1* homologues have been identified by genomic sequencing efforts and are known only as open reading frames. Only the Drosophila gene has been characterized to date (see *Drosophila* below).

Genomic h*OXR1* Structure and Locus. The current release of the human genome sequence indicates that the h*OXR1* gene is located on chromosome 8 (q23). Its genomic structure is shown in Fig. 3. It comprises nine exons. The first exon includes 74 bp of upstream untranslated sequence present in the cDNA, and the last exon includes 156 bp of downstream untranslated DNA sequence. The full length of the genomic h*OXR1* DNA cannot be predicted from the existing data because of a sequencing gap of unknown length between exons 7 and 8. A second homologous sequence is present on chromosome 15 (q21). This region of chromosome 15 corresponds to the region of h*OXR1* shown in Fig. 2 beginning with amino acid 204. It is likely to be a pseudogene, based on its lack of introns and the presence of a frameshift mutation early in the *OXR1* coding sequence that disrupts the ORF, leaving only a small portion of the *OXR1* gene as a potential ORF.

Yeast *OXR1* Mutants Are Sensitive to Hydrogen Peroxide Treatments. To determine whether the *OXR1* gene affects oxidative damage resistance in a eukaryotic organism, we constructed a *S. cerevisiae* strain deleted for the sc*OXR1* (YPL196w) ORF. sc*OXR1* was cloned along with approximately 300 bp of both upstream and downstream flanking sequences, and deletion was constructed by the use of PCR methods to replace all but six codons of the *OXR1* coding sequence with a cassette that expresses the *URA3*$^+$ gene. The yeast strain R117 (22) was transformed with the linear DNA fragment carrying the *URA3*$^+$ gene surrounded by the sc*OXR1* sequences, and *URA3*$^+$ recombinants were selected. *URA3*$^+$ diploid recombinants were then sporulated and tetrads were analyzed. *URA3*$^+$ haploid segregants were viable, indicating that sc*OXR1* is not an essential gene (data not shown). Fig. 4*A* shows the expected sc*OXR1*$^+$ and scΔ*oxr1*::URA3$^+$ gene structures, and Fig. 4*B* shows that sc*OXR1* restriction fragments of the appropriate sizes are present in the *OXR1*$^+$ ura3$^-$ strain and the scΔ*oxr1*::*URA3*$^+$ mutant haploid strains.

To determine whether mutation of *oxr1* adversely affects oxidation sensitivity in yeast, cells were grown to mid-log phase and treated with up to 200 mM H_2O_2, then plated on YEPD agar to determine viable cell numbers. Fig. 5 compares the hydrogen peroxide sensitivity of wild-type and *oxr1* mutant haploid yeast strains and shows that mutation of *oxr1* results in increased sensitivity to hydrogen peroxide, thus demonstrating that the wild-type sc*OXR1* provides protection against the deleterious effects of oxidation. Similar results were obtained with an independent Δ*oxr1*::*URA3* isolate, and introduction of the wild-type sc*OXR1* gene cloned, along with its upstream DNA sequence, onto the yeast vector pRS315 (26) restores wild-type resistance to the *oxr1* deletion mutant, confirming that the sensitivity is due to the loss of *OXR1* function (data not shown). The level of peroxide sensitivity resulting from the *oxr1* mutation is greater than that resulting from mutations inactivating yeast oxidative repair genes such as *ogg1*, *ntg1*, *ntg2*, *apn1*, *rad1*, *rev3*, or *rad52* (27, 28). Individually, these mutations have no adverse effect on peroxide resistance, and even the combination of *ntg1 ntg2 apn1* and *rev3* mutations has no detectable effect on peroxide sensitivity (28). A level of peroxide sensitization similar to that conferred by the scΔ*oxr1*::*URA3*$^+$ mutation requires either the combination of *ntg1 ntg2 apn1* with *rad52* or *rad1*, or the combination of *rev3*

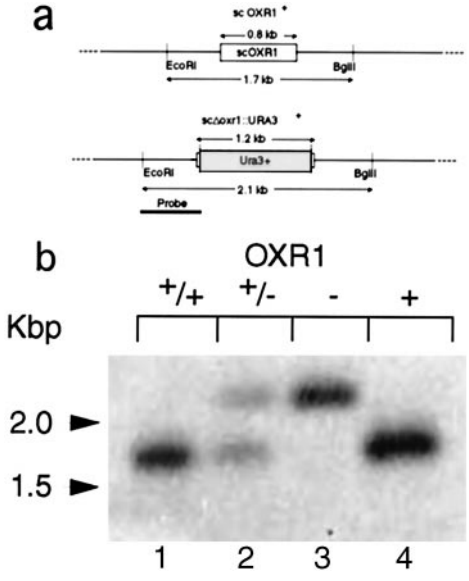

Fig. 4. Analysis of a yeast scΔ*oxr1*::URA3 mutant. (*a*) Sizes of the wild-type *OXR1*- and scΔ*oxr1*::*URA3*$^+$-containing DNA restriction fragments. Also shown is the probe used in *b*. (*b*) Hybridization analysis of scΔ*oxr1*::*URA3*$^+$ and *OXR1* wild-type strains. Lane 1, Wild-type diploid parent strain, R117; lane 2, *OXR1*/Δ*oxr1*::*URA3*$^+$ diploid strain, N1; lane 3, Δscoxr1::URA3$^+$ haploid segregant strain, N1–9; lane 4, *OXR1*$^+$ *ura3*$^-$ haploid segregant N1–4.

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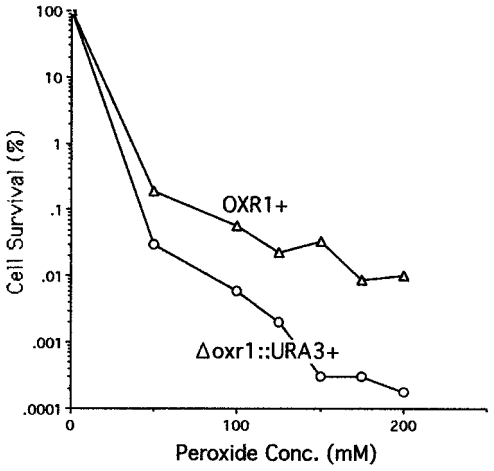

Fig. 5. Hydrogen peroxide resistance of wild-type (○) and sc*Δoxr1::URA3* (△) haploid strains of yeast. The titers of surviving cells were determined by plating on YEPD agar plates that were incubated for 2–3 days at 30°C. Representative data from at least four experiments are shown, all showing similar levels of difference between the wild type and mutant strains. Similar results were also seen with an independent *oxr1* deletion mutant strain.

with *rad52*. These observations indicate that the *OXR1* gene plays an important role in oxidative protection in yeast and, presumably, other eukaryotes that have *OXR1* homologues.

Discussion

Interspecies Complementation of Antimutator Activity. The use of spontaneous oxidation as the DNA damaging treatment provides a high degree of sensitivity compared with methods used by others (18, 29), because mutagenic oxidative damage is constantly occurring, thereby allowing mutations to accumulate in these sensitive strains of *E. coli*. Expression of cDNAs that result in either a small reduction in the production of DNA damage or a small increase in DNA repair activity reduces the number of spontaneous mutations. Genes counteracting the low constant rate of oxidative damage are likely to be important for protection against the low, spontaneous level of oxidative damage normally produced within cells. The use of this approach resulted in the discovery of the human *OXR1* gene as an antimutator mutation that reduced oxidative mutagenesis in an *E. coli* mutator strain.

***OXR1* Homologues.** *OXR1* is conserved among eukaryotes, and homologues have been identified in a number of different species. Comparison of the various known forms of *OXR1* indicates that the most highly conserved region of the gene is its the carboxyl-terminal half. However, because the gene has been identified primarily in sequencing projects rather than genetic studies of DNA repair, little is known about its physiological function.

The *S. cerevisiae OXR1* gene is composed primarily of the highly conserved carboxyl-terminal domain of the human *OXR1* gene (Fig. 2). The presence of *OXR1* in *S. cerevisiae* allowed the use of yeast genetics to construct an *OXR1* deletion mutant and to analyze its function in oxidative protection. This mutant was found to be sensitized to treatments with exogenous hydrogen peroxide, thus demonstrating that *OXR1* is required for normal levels of resistance to oxidative damage and that this function is contained within the most conserved region of the *OXR1* family.

Genetic Analysis of the *Drosophila OXR1* Homologue. Genetic studies of the *Drosophila OXR1* homologue (30) implicate it in other cellular processes in addition to oxidation protection. The Drosophila homologue is encoded by the *L82* gene, which produces seven different known isoforms, *L82A* through *L82G* (30). The h*OXR1* homology region of *L82* is contained within its carboxyl-terminal exon, and all known isoforms contain this exon. The largest isoform of *L82* encodes a protein of 1270 amino acids, whereas the smallest encodes only the 192-amino acid protein that makes up just the *OXR1* homology region. Mutants of *L82* are defective in eclosion and, therefore, fail to release adults from pupae (30). This developmental defect can be complemented by expression of the largest isoform, *L82A* (30). Because other isoforms have not been tested, it is uncertain whether the developmental deficiency of the *L82* mutant strain results from loss of the *OXR1* region alone, or if the other upstream domains of *L82A* are important for this process. This stage of *Drosophila* development is associated with dramatic increases in catalase and superoxide dismutase expression (31, 32), suggesting that oxidative stress may increase at this stage of development and that *Drosophila* induces protective mechanisms to counteract this stress. These observations raise the possibility that one function of *L82* gene expression during eclosion may be to contribute to a general increase in protection against oxidative damage to DNA.

The identification of the *OXR1* family is a step toward identifying all of the genes that contribute to protection against ROS in humans. The functional genomic approach we have taken has the potential to support the identification of genes that complement *OXR1* family members, interact with them, or provide alternative pathways for response to ROS. The elucidation of these pathways should be of importance in understanding human disease processes.

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