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Prevention of Oxidative Damage by Yeast and Human OXR1: A Dissertation

Nathan Andrew Elliott
University of Massachusetts Medical School

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PREVENTION OF OXIDATIVE DAMAGE BY YEAST AND HUMAN OXR1

A Dissertation Presented By

Nathan Andrew Elliott

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PREVENTION OF OXIDATIVE DAMAGE BY YEAST AND HUMAN OXR1

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Nathan Andrew Elliott

Approved as to style and content by:

Richard Baker, Ph.D. Chair of Committee

Anthony Poteete, Ph.D. Member of Committee

Martin Marinus, Ph.D. Member of Committee

Timothy Kowalik, Ph.D. Member of Committee

Leona Samson, Ph.D. Member of Committee

Michael Volkert, Ph.D. Thesis Advisor

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences
Portions of this dissertation appear in the following publications:


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CHAPTER I

INTRODUCTION

Oxidative damage

With the advent of an oxygen-rich atmosphere on Earth came a unique challenge to existing life forms: to adapt to the new lifestyle of aerobic metabolism. To meet this challenge, organisms had to overcome a major obstacle in the utilization of oxygen to produce energy—namely, the production of highly reactive oxygen molecules from the incomplete reduction of $O_2$ during respiration. These reactive oxygen species (ROS) have the potential to attack virtually all cellular molecules (DNA, proteins, lipids), and unless countered effectively will surely result in DNA damage, mutagenesis or cell death.

The mechanisms by which cells prevent and or repair damage by ROS are complex and in many cases not fully understood. A better understanding of these processes will be crucial to combating diseases associated with oxidative damage.

There are numerous species of reactive oxygen molecules, the most common and widely studied being the superoxide radical ($\cdot$O$_2$), the hydroxyl radical (•OH) and the peroxides. Hydrogen peroxide ($H_2O_2$) itself is relatively inert, but can be broken down readily by the Fenton reaction to form the hydroxyl radical:
Nucleic acids bind iron well, and as a result production of hydroxyl radicals through Fenton chemistry is a significant source of oxidative DNA lesions (112).

Reactive oxygen can be produced from both intracellular and extracellular sources. Cells are continually barraged by ROS from the environment. Radiation (X-ray and Gamma ray) damages cells primarily by producing reactive oxygen through the electrolysis of water (14). Other chemicals present in the environment, the herbicide paraquat, or menadione (vitamin K3) for example, are redox-cycling compounds that produce superoxide radicals (22, 26). However, the biggest threat of oxidative damage to the cell comes not from the environment but from the cell’s very own energy production machinery. At the inner membrane of the mitochondria, the electron transport chain (ETC) culminates in the reduction of molecular oxygen to water, and the energy derived from this process is coupled to production of ATP. However, it is believed that electron leakage from both complex I and complex III of the ETC may lead to the incomplete reduction of O₂ and the production of reactive oxygen intermediates (mainly superoxide radicals)(9, 134). Superoxide is rapidly converted to H₂O₂, which is a much more stable molecule and is believed to diffuse passively throughout the cell. It is estimated that as much as 1% of all O₂ consumed during respiration is converted to ROS (21). Due to this intimate interaction between energy production and reactive oxygen production, the mitochondria require highly efficient means of controlling ROS activity. As will be
discussed later, the mitochondria are believed to be important sites of ROS-related processes such as apoptosis and senescence.

**Cellular defenses to oxidative damage**

Reactive oxygen will attack macromolecules indiscriminately, targeting DNA, proteins and the cell membrane. Dozens of oxidative DNA lesions have been described (27), but probably the most common and well studied is 8-oxoGuanine (8-oxoG). The importance of this lesion is illustrated by the numerous repair mechanisms employed by cells to avoid 8-oxoG incorporation or persistence in DNA. All organisms studied to date express 8-oxoG DNA glycosylases that remove the oxidized base from DNA. In *E. coli*, this is the MutM protein, and in yeast and humans the OGG1 protein. Interestingly, *E. coli* MutM shares no sequence homology with the eukaryotic OGG1 enzymes, but is both structurally and functionally homologous, suggesting these enzymes evolved independently to counteract the troublesome 8-oxoG. Some plants, for example the well studied *Arabidopsis thalania*, have acquired both a prokaryotic mutM homolog as well as the eukaryotic OGG1 homolog (47). And what is the reason for such pressure to counteract 8-oxoG? Unlike many types of DNA damage that produce bulky or helix distorting lesions (e.g. UV-induced crosslinks or alkylation adducts) and thus trigger replication arrest (110, 133), 8-oxoG is a relatively small chemical addition and does not itself alter the conformation of duplex DNA sufficiently to block DNA synthesis (72). However, the 8-oxoG lesion leads to mispairing with adenine at a frequency of
approximately 50%, causing a high rate of G-C to T-A transversions following DNA replication through the lesion (16).

A second line of defense for the cell is directed against this 8-oxoG-A mispairing. *E. coli* and mammals both express DNA glycosylases (MutY and MYH, respectively) that excise adenine when incorrectly paired with 8-oxoG (5, 88). The yeast *S. cerevisiae* does not express a MutY homolog, however in this organism 8-oxoG:A mispairs are a substrate for the mismatch repair proteins MSH2 and MSH6 (99), again highlighting the varied ways in which cells solve the oxidative DNA damage problem.

Finally, a third mechanism exists to counteract 8-oxoG in both *E. coli* and mammals- the MutT homologs. These are 8-oxo-dGTPases that remove 8-oxo-dGTP from the nucleotide pool by hydrolyzing it to 8-oxo-dGMP, thus preventing its incorporation during DNA synthesis (83).

Of course, cells have also devised many ways of preventing oxidative damage from occurring in the first place. The most ubiquitous anti-oxidant function within the cell is the tripeptide gamma-glutamyl-cystyl-glycine, or glutathione (GSH). Two reduced glutathione molecules (GSH) can be oxidized to GSSG, thus serving as a sink for reactive oxygen within the cell. A complex reduction system exists in both prokaryotes and eukaryotes to convert GSSG back to reduced glutathione. The enzyme glutathione reductase reduces GSSG using electrons donated from NADH or NADPH. The breakdown of peroxides into water and oxygen can be accomplished through the activity
of peroxidases, which use the reducing equivalents derived from the thiol containing molecules glutathione and thioredoxin (see figure 1):

![Diagram of redox cycling of glutathione peroxidase and thioredoxin peroxidase systems.]

Figure 1-1. Redox cycling of glutathione peroxidase and thioredoxin peroxidase systems. GPx, glutathione peroxidase. GR, glutathione reductase. G6PDH, glucose-6-phosphate dehydrogenase. TPx, thioredoxin peroxidase. TR, thioredoxin reductase. ROOH, peroxides (taken from Inuoe et.al. (65)).
Cells also express several enzymatic anti-oxidant functions. These include superoxide dismutase, catalase and the peroxidase family of proteins. Superoxide dismutase catalyzes the conversion of superoxide anion (\(O_2^{•−}\)) to \(O_2\) and \(H_2O_2\). Catalase can then break down hydrogen peroxide into oxygen and water:

\[2H_2O_2 \rightarrow 2H_2O + O_2\]

The peroxidase families of enzymes (GPx and TPx) are characterized by a C-X-X-C motif at the active site of the enzyme (45), and are highly conserved in both prokaryotes and eukaryotes. In *E. coli*, the enzyme responsible for scavenging endogenously produced H\(_2\)O\(_2\) during aerobic growth is Ahp, alkyl hydroperoxide reductase. This is an NAD(P)H-dependent peroxidase which is required to detoxify low levels of hydrogen peroxide produced during respiration, whereas higher levels of H\(_2\)O\(_2\) (produced in stationary phase, for instance) are more efficiently scavenged by the catalases *katE* and *katG* (121).

*S. cerevisiae* expresses two catalase genes, *CTT1*, encoding the cytosolic catalase T, and *CTA1*, encoding the peroxisomal catalase A (24, 58). Interestingly, neither single mutants of these genes nor the double *Δcta1 ctt1* mutant are sensitive to hydrogen peroxide in the exponential growth phase. However, stationary phase cells of the *cta1 ctt1* double mutant have a roughly 100 fold sensitivity to hydrogen peroxide killing
relative to wild type cells when tested with a hydrogen peroxide survival curve. Furthermore, the localization of the enzyme does not seem to be critical to peroxide resistance, as either CTA or CTT are capable of compensating for the absence of the other (66). This indicates that, while important to protect cells during stationary phase oxidative stress, catalase plays only a minor role during active growth in yeast.

*S. cerevisiae* also expresses two forms of superoxide dismutase: the cytoplasmic SOD1 and the mitochondrial SOD2 (30, 138). Cells lacking Sod2 are sensitive to superoxide generating agents (e.g. paraquat) and hyperoxia (138). In these mutants, oxygen sensitivity can be suppressed by mutations that inhibit respiration (54), indicating that SOD2 serves to protect cells from endogenous ROS produced from the mitochondrial electron transport chain.

Yeast also express a number of enzymes in the peroxidase family, including at least three glutathione peroxidases (GPx) and five thioredoxin peroxidases (TPx) (65). These enzymes have been shown to be important in detoxification of hydrogen peroxide. However, they appear to have largely redundant functions, as single mutants in either family display little sensitivity to oxidative stress (65, 96). The double mutant of the thioredoxin peroxidases Δtsa1 tsa2 is only mildly sensitive to hydrogen peroxide during the exponential growth phase (146). The glutathione peroxidase GPX3 appears to be the most critical for protection from oxidative stress. Δgpx3 cells are sensitive to hydrogen peroxide, while Δgpx1gpx2 cells are not (65).
It is important to note that the enzymatic functions described above are very well conserved in both prokaryotes and eukaryotes and they contain distinct functional domains or motifs characteristic of their protein family. It is likely that any newly discovered human proteins falling into any of these protein families would contain these conserved domains and or motifs characteristic of its function.

Oxidation sensitivity of repair mutants and antioxidant mutants

Human genes identified based on their ability to suppress oxidative damage are likely to fall into one of two categories: those that repair oxidative DNA damage or those that prevent the damage from occurring initially (antioxidant function). In the yeast *S. cerevisiae*, the phenotypes of mutants of each class of gene may help to shed light on the function of any newly discovered gene believed to be involved in these processes.

There are several DNA repair genes involved in the repair of oxidative DNA damage in yeast. These include base excision repair genes *OGG1* (8-oxoG glycosylase), *NTG1* and *NTG2* (endonuclease III homologs), *APN1* (abasic site endonuclease). The nucleotide excision repair (NER) pathway is also believed to be involved in the repair of some oxidative DNA lesions (63). During NER repair of these lesions, RAD1 is required to carry out an endonucleolitic cleavage of the DNA backbone 5’ to the oxidized lesion that initiates the repair process (55). Also, oxidative DNA damage can be bypassed during replication by specialized DNA polymerases that insert nucleotides opposite oxidized template nucleotides. One member of this family of bypass polymerases is
REV3 (Pol zeta) (97). Finally, recombinational repair is involved in the repair of double strand breaks, which frequently occur as intermediates in the repair of oxidative lesions on opposite strands of DNA, and is carried out by the RAD52 homologues (40).

In yeast, these genes appear to have overlapping functions to repair oxidative DNA damage. Single mutations in ogg1, ntg1 or ntg2, radl, rev3 and rad52 have little or no sensitivity to killing by hydrogen peroxide (127). In fact, several mutations have to be combined to produce a significant level of peroxide sensitivity. For instance, a quadruple mutant of ntg1 ntg2 apn1 rad52 results in approximately the same sensitization to hydrogen peroxide as the oxr1 mutation (140) as measured by H₂O₂ survival curves. It is also interesting to note that these mutant strains have nearly identical sensitivities to both hydrogen peroxide and the superoxide generating drug menadione (127). This suggests that these families of DNA repair genes function to repair a variety of oxidative lesions caused by multiple oxidizing agents.

In addition to the DNA repair genes, oxidative damage can be prevented by the action of numerous antioxidant functions within the cell. Several of these genes, and the phenotypes of their mutants, have been described above in the section on “Cellular defenses to oxidative damage”. Here I will point out some interesting contrasts between mutants of DNA repair genes vs. those of antioxidant genes. First, whereas DNA repair pathways are often capable of compensating for defects in others, antioxidant genes are more likely to display oxidation sensitivity when single genes are mutated. A good example is the sod2 mutant of yeast, which is sensitive to superoxide damage [133],
while a similar sensitivity to superoxide requires several mutations in DNA repair genes (ntg1ntg2apn1rad52 or rad52rev3) (127). A second difference often observed when comparing the phenotypes of repair mutants and antioxidant mutants is their specificities for one type of oxidizing agent. For example, superoxide dismutase mutants are sensitive to superoxide radical generating compounds, but not to hydrogen peroxide. Likewise, catalase mutants may be sensitive to hydrogen peroxide, but not to menadione or paraquat. This is in contrast to the DNA repair mutants described above, which display roughly the same sensitivities to both hydrogen peroxide and menadione. Thus these different phenotypes may offer clues to the function of newly identified mutants in S. cerevisiae that are believed to be involved in suppression of oxidative damage.

Mitochondria and oxidative damage

Within the inner membrane of the mitochondria, oxidative respiration is coupled to energy production through the activities of the electron transport chain and F₀F₁ ATPase. As O₂ is consumed, superoxide radicals are produced that have potentially deleterious effects on the mitochondria and the cell as a whole.

Estimates vary as to the amount of ROS produced during respiration, although it is generally accepted that between 0.1 – 5% of the molecular oxygen consumed during respiration is converted to ROS (21). These molecules can then react with mitochondrial DNA, proteins and lipids to cause potentially lethal damage to the mitochondrial machinery. Owing to the close association of the mitochondrial genome with the inner
membrane, mitochondrial DNA (mtDNA) is particularly sensitive to oxidative damage. Not surprisingly, it has been shown that mitochondrial DNA incurs a higher rate of oxidative DNA damage than nuclear DNA (116). The amount of damage to mtDNA, as well as proteins, is further enhanced by mutations in respiration function that increase mitochondrial ROS production (143). It has been hypothesized that the accumulation of such mutations is the cause of age-related degenerative disorders (77, 104). For these reasons, mitochondria have evolved several defense systems to counteract the production of ROS (see intro to Chapter IV).

Recent research has also focused on the regulatory properties of ROS within the mitochondria. It has been proposed that ROS plays a critical role in signaling to transcription factors in the nucleus (74), regulating apoptosis (98) and contributing to the aging process (89).

Several nuclear transcription factors are regulated by oxidative stress. Both NF-κB and AP-1 are activators of many stress-response genes and can be induced by H₂O₂ (91). Intracellular ROS generated in the mitochondria has the potential to signal these redox-regulated transcription factors and activate stress-response pathways to alter the redox state of the cell.

Much research recently has focused on the role played by mitochondria in the regulation of apoptosis. In the mitochondria-dependent, or intrinsic, pathway of
programmed cell death, cell stress or damage signals converge on the mitochondria to trigger the apoptotic cascade (150). The first step in this process is an alteration of the mitochondrial membrane permeability and the release of mitochondrial proteins into the cytosol (120). One such protein is cytochrome c, a critical component of the respiratory complex IV within the inner membrane (84). Release of cytochrome c may be associated with an increase in ROS production, as the electron transport chain is rendered less efficient (115).

It is also clear that ROS plays a role in the triggering of the mitochondrial apoptosis pathway. Mutations in mitochondrial anti-oxidant functions (thioredoxin, MnSOD) have been shown to increase ROS production and trigger apoptosis (100, 153). Also, overexpression of antioxidant proteins such as SOD can lower ROS levels and suppress apoptosis (85).

ROS generation in mitochondria has also been implicated as a cause of cellular senescence and whole organism aging (73). Since the 1950’s, evidence has pointed to a correlation between oxidative damage and aging, leading Harmon to propose the free radical theory of aging (57). Later it was demonstrated that proteins of Drosophila accumulate oxidative damage (43), and that aged mice show more elevated levels of oxidative protein damage than young mice (46). Oxidative damage has also been shown to accumulate in the mitochondria of aged organisms (protein and DNA), and to affect the respiratory chain and mitochondria function (56, 94).
More recently, direct evidence has pointed to a role for ROS in regulating lifespan of cells and organisms. Senescence of cells in culture appears to be regulated by oxidative stress. Inhibition of SOD1 expression by RNAi in human fibroblasts induces senescence (12). In addition, murine embryonic fibroblasts (MEFs) undergo senescence when cultured in 20% O₂, and this transition can be bypassed by lowering the O₂ content of the culture to 3% (17). These MEFs also accumulate more mutations when senescing in high oxygen conditions than when cultured in low oxygen conditions (17). Through the use of transgenic and knock-out technology, redox regulation has been identified as a critical component in determining lifespan. In *Drosophila*, overexpression of SOD has been shown to significantly increase lifespan. Also, treatment of flies with compounds that mimic superoxide dismutase and catalase extends lifespan as well (89). In mice, mutation of the p66Shc gene increases lifespan by roughly 30% (93). p66Shc is an adaptor protein involved in regulation of receptor tyrosine kinase signaling (108), and appears to play a role in oxidative stress response and apoptosis. p66 knockout mice survive up to 40% longer than wild type mice following exposure to paraquat (93). Also, MEFs from p66-/- mice fail to undergo oxidative stress-induced apoptosis at the same levels as wild type cells (93). Recently p66 protein has been shown to partially localize to the mitochondria where it interacts with mitochondrial HSP70 to regulate apoptosis in response to oxidative stress (103). These recent genetic experiments point to a crucial role for oxidative stress response genes in the regulation of cellular senescence and whole organism aging.
Screening human cDNA libraries for genes involved in oxidative DNA damage prevention or repair

The expression of human genes in heterologous systems has proven to be an effective way to identify and characterize genes involved in DNA damage and repair. As pointed out by Memisoglu and Samson, this is probably due in large part to the fundamental conservation of genetic material in all organisms: double stranded DNA. As a consequence, proteins that recognize a specific type of lesion to the DNA double helix should be able to recognize that damage in the genome of any organism, be it bacteriophage or human (90). By the same reasoning, one might expect heterologous protein expression to also be an effective way to identify those proteins involved in oxidative damage prevention. Since all aerobically respiring organisms are subject to damage by the same types of molecules (ROS), and the detoxification of these molecules follow the same chemical principles, then proteins involved in oxidation prevention should function similarly from one organism to the next (provided they can work independently of other species-specific proteins).

An early and impressive example of interspecies complementation came nearly 30 years ago from the introduction of bacteriophage T4 endonuclease V protein into human cells deficient in nucleotide excision repair (NER) of UV-induced DNA damage (129). The phage protein was able to restore the ability of the human cells to repair UV damage. Later, similar experiments allowed for the cloning of human NER genes through expression in NER-deficient rodent cells (137).
**E. coli** mutator strains have been used previously to study heterologously expressed proteins from both yeast and humans. For instance, **E. coli** strains deficient in the *ada* and *ogt* alkylation repair genes exhibit a mutator phenotype, and have to been used to study the function of yeast and human methyl transferases (MTase), a class of alkylation DNA damage repair protein (130, 149). However, these studies only involved the expression of specific Mtase clones in **E. coli** to study the functional relationships of proteins from different organisms. A similar approach has also been employed to study base excision repair proteins in **E. coli**. A strain mutated in the *alkA* and *tag* 3-methyladenine DNA glycosylase genes was used to find yeast and human cDNAs capable of conferring MMS resistance on this strain (10, 20). A limitation of these approaches is that they only involve screening for genes that protect the bacteria from lethal doses of an exogenously added DNA damage agent.

Based on the proven usefulness of heterologous protein expression in **E. coli**, our lab has developed a screening approach to find human genes capable of preventing or repairing oxidative DNA damage. Our approach builds on previous work (examples of which are outlined above), but is unique in other respects. First, we are looking for genes involved in preventing the most ubiquitous form of damage to most organisms, oxidative damage. Second, our approach is highly sensitive in that it utilizes **E. coli** strains with very high mutation rates, such that human genes with even subtle functions can be identified using this method. Third, this screening method measures spontaneous DNA damage and repair, without the need for exogenously added damaging agents. This also allows for the identification of genes whose function is important to limit oxidative
damage, but not critical for surviving lethal doses of damage. Lastly, our approach does not limit the search to genes of a particular class of repair protein, or to genes related by sequence homology or functional homology. In theory, any gene that functions to suppress oxidative DNA damage, whether it be detoxification of ROS or repair of DNA, can be identified by this screening method.
CHAPTER II

FUNCTIONAL GENOMICS REVEALS A NEW FAMILY OF EUKARYOTIC
OXIDATION PREVENTION GENES

Abstract

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 *E. coli* a large number of genes are involved in 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 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.
Introduction

Reactive oxygen species (ROS) are formed as by-products of normal metabolism of aerobic organisms and react with DNA to produce damage (114). Cells protect themselves from ROS by detoxification mechanisms and by mechanisms that repair the damage ROS produce (13, 31, 32, 37, 61). In humans oxidative damage results in mutagenesis, triggers apoptosis, and has been implicated as a contributing cause to a number of human diseases including cancer and neurodegenerative diseases. Oxidative damage has also been implicated as a contributing factor to the aging process (31, 79, 86). 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 (51) and mutations in the superoxide dismutase gene, which affects the cell’s ability to detoxify reactive oxygen species predisposes patients to amyotrophic lateral sclerosis (102). Protective mechanisms also interfere in cancer therapies, preventing or repairing oxidative DNA damage produced by radiation treatments and other therapies (113). 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 (13, 31, 32, 37, 61). 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 (11, 67, 118, 141, 152). Most of these mutant strains also exhibit a spontaneous mutator phenotype that results largely, or exclusively, from their inability to repair spontaneous oxidative damage (11, 67, 118, 141). 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::Tn10 nth-1::kan,ble* derivative of MV1161(33). 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 (109) 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 (NIEHS, North Carolina)(139).

**Culture media.** LB ampicillin (LB-amp) plates were standard LB medium (33) containing 100 μg/ml ampicillin. ESEM plates are standard SEM plates in which the salts solution has been replaced by E salts (33, 145). The low level of arginine supplied
is sufficient to allow a background growth of arginine requiring cells to reach a growth ceiling of approximately $5 \times 10^9$ cells per plate. Once the arginine is exhausted, only Arg$^+$ revertants will continue to grow to form colonies (1). Standard YEPD plates and broth were used for routine growth of yeast. Minimal drop-out medium lacking uracil was used to select for Ura$^+$ recombinants (1). 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 IPTG inducible synthetic promoter that functions in *E. coli* (139). Transformants were selected on LB-amp plates. Transformants were then picked, inoculated into 96 well microtiter trays containing 250 μl LB amp and grown overnight for subsequent testing. Trays were spotted onto two ESEM-amp plates using a multi-prong device; one plate contained IPTG (2mM) for induction of cDNA transcription. Spontaneous mutation frequencies 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 (Figure 2-1) and clones showing a clear decrease in mutagenesis were selected, their plasmids 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. In order to eliminate clones that either interfered with the Arg reversion assay, or had non specific effects on mutagenesis, cDNA clones were transformed either into an ung or 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 *S. cerevisiae OXR1*.** The *Saccharomyces cerevisiae*, OXR1 gene (scOXR1) was cloned via a PCR approach. Primers 1 and 2 (see below) were used to amplify the scOXR1 coding sequence and 300 bp of flanking DNA on each side from the wild type yeast R117 (119). These primers also included new restriction sites that allowed cloning of the amplified sequence into the EcoRI and BamHI sites of the vector pTrc99A (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 scOXR1 and introduced restriction sites compatible with the SacI XmaI 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 3 and last 3 codons of scOXR1 and are flanked on each side by 300 bp of scOXR1 flanking DNA. This fragment (shown in Results, Fig. 2-4) was then purified as a single PvulII fragment. Competent *ura3\(^-\)* mutant yeast cells (strain R117) were then transformed by Li acetate transformation with the *URA3\(^+\)*-carrying PvulII 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 N2-3 (*URA3\(^+\)*) and N2-9 (*ura3\(^-\)*) were selected for further study. Genetic structures of the mutants were confirmed by Southern blotting. Primers used were:
Primer 1: ATCATCGAATTCTATGACCGACTCGTAAT,
Primer 2: ATCATCGGATCCTTTTTTTTCACATTGGGAG 3',
Primer 4: ATCATCGAGCTCTCCAAACATTGTCGCTCC,
Primer 5: ATCATCCCCGAGGATAGTGTATTCATCTA,
Primer 6: ATCATCTGAGTTTTTTTCACATTGGGAG.

**Southern hybridizations.** Yeast genomic DNA was prepared as described by Adam et al. (1). Standard hybridization methods were used to measure the size of the OXR1, or *URA3* replacement allele carrying DNA fragments (119). The 300 bp *EcoRI*- *SacI* fragment from pMV603, corresponding to the upstream OXR1 flanking DNA (shown in Results, Fig. 2-4) was used as a probe.

**Yeast strains.** Yeast strains used in this study are derivatives of R117 (8), a strain congenic to 381G (59). Strains were grown in standard YEPD medium at 30°. Additional strains constructed in this study were derivatives of R117 and carry the following additional genetic markers: N2, MATa/MATα Δoxr1::URA3/OXR1; N2-3, MATa Δoxr1::URA3; and N2-9, MATa OXR1.

**Peroxide sensitivity testing.** Peroxide sensitivity tests were performed as described by Ramotar et al. (114) Briefly, overnight cultures of yeast strains were diluted to an OD<sub>600</sub> of approximately 0.3 in standard YEPD medium and grown with aeration to an OD<sub>600</sub> of approximately 0.8 to 1. Cells were harvested, washed once in sterile water and resuspended in phosphate buffered saline, pH 7.4. Samples were treated with H<sub>2</sub>O<sub>2</sub> at the indicated concentrations for 1 hour at 30°C with aeration. After treatment, cells were diluted in PBS and titered on YEPD plates. Experiments were repeated at least three times and representative data are shown.
DNA sequencing. DNA sequencing was performed either by the MIT Center for Cancer Research, the University of Massachusetts Medical Center, and Iowa State University DNA sequencing facilities. DNA and predicted protein sequences were analyzed using Blast sequence searches (2).

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 nth double mutant strain as the mutagenesis indicator strain. This strain is highly sensitive to peroxide treatments and both mutations contribute to this phenotype (67, 141, 152). Figure 2-1 compares spontaneous Arg" mutagenesis in wild type and mutH nth double mutant strain, and shows that IPTG induction of the vector has no effect. However when the human OXR1 (hOXR1) gene is induced by IPTG it causes about a five-fold reduction in spontaneous Arg" mutagenesis in the mutH nth strain (Fig. 2-1 F) without a detectable effect on
growth (data not shown). Thus, hOXR1 functions as an antimutator in this E. coli genetic background.

Figure 2-1. Effect of hOXR1 expression on Arg$^+$ mutagenesis in E. coli. A,B, MV3626 (wild type/pTrc99A (Pharacia)); C, D, MV4174 (mutH nth/pTrc99A); E, F, MV4300 (mutH nth/pMV420 (hOXR1)). No IPTG induction A, C, E. IPTG (1 mM) induction, B, D, F.
Analysis of the hOXR1 DNA sequence. DNA sequence analysis shows that the hOXR1 expressing plasmid, pMV520, carries a cDNA insert of approximately 1.7 kbp encoding a previously unidentified human gene that matches several human and mouse EST database sequences and shares homology with genes found in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, and *Drosophila*. Homologues are not found in *E. coli*, or other bacterial species. Thus hOXR1 appears to be a member of a conserved family of genes present in a wide variety of eukaryotic species. Figure 2-2 compares the predicted hOXR1 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. 2-2). Two additional regions conserved primarily among the higher eukaryotes are found in the region corresponding to hOXR1 amino acid residues 100 through 200 (Fig. 2-2). The conserved motifs of OXR1 do not correspond to motifs of known function, thus their functions can not presently be predicted. The hOXR1-related genes are the *S. cerevisiae* OXR1 homologue (scOXR1) which is 27% identical and 43% similar to the hOXR1 gene. It is known only as the open reading frame 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).
Fig. 2
Figure 2-2. 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 and regions containing conservative substitutions are indicated by dots.

**Genomic hOXR1 structure and locus.** The current release of the human genome sequence indicates that the hOXR1 gene is located on chromosome 8 (q23). Its genomic structure is shown in Figure 3. It is comprised of 9 exons. The first exon includes 74 bp of upstream untranslated sequence present in the cDNA, and the last exon includes 156 bp downstream untranslated DNA sequence. The full length of the genomic hOXR1 DNA can not 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 hOXR1 shown in Fig 2-2 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 open reading frame, leaving only a small portion of the OXR1 gene as a potential open reading frame.
Figure 2-3. Genomic structure of OXR1. The OXR1 containing region of chromosome 8q23 is shown (not to scale). Size of exons (black boxes), in bp, is shown above the line, size of introns, in kbp, is 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.
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 scOXR1 (YPL196w) open reading frame. scOXR1 was cloned along with approximately 300 base pairs 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 (8) was transformed with the linear DNA fragment carrying the URA3+ gene surrounded by the scOXR1 sequences and URA3+ recombinants were selected. URA3+ diploid recombinants were then sporulated and tetrads analyzed. URA3+ haploid segregants were viable, indicating that scOXR1 is not an essential gene (data not shown). Fig. 2-4A shows the expected scOXR1+ and scΔoxr1::URA3 gene structures and Fig. 2-4B shows that scOXR1 restriction fragments of the appropriate sizes are present in the OXR1 ura3 strain and the scΔoxr1::URA3 mutant haploid strains.
Figure 2-4 (A). Analysis of a yeast scΔoxr1::URA3 mutant. A. Shows the sizes of the wild type OXR1 and scΔoxr1::URA3+ containing DNA restriction fragments. Also shown is the probe used in B. (B). Hybridzation analysis of scΔoxr1::URA3+ and OXR1 wild type strains. Lane 1, Wild type diploid parent strain, R117; lane 2, OXR1/Δoxr1::URA3+ diploid strain, N2; lane 3, Δscoxr1::URA3+ haploid segregant strain, N2-3; lane 4; OXR1+ ura3- haploid segregant N2-9.
To determine if mutation of *oxr1* adversely affects oxidation sensitivity in yeast, cells were grown to mid-log phase and treated with up to 200 mM H$_2$O$_2$, then plated on YEPD agar to determine viable cell numbers. Figure 2-5 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 scOXRI provides protection against the deleterious effects of oxidation.

![Figure 2-5](image)

**Figure 2-5.** Hydrogen peroxide resistance of wild type (O) and scΔoxr1::URA3 (Δ) 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 are shown from at least four experiments, all showing similar levels of difference between the wild type and mutant strain. Similar results were also seen with an independent *oxr1* deletion mutant strain.
Similar results were obtained with an independent Δoxr1::URA3 isolate, and introduction of the wild type scOXR1 gene cloned, along with its upstream DNA sequence, onto the yeast vector pRS315 (124) restores wild type resistance to the oxrl 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 oxrl mutation is greater than that resulting from mutations inactivating yeast oxidative repair genes such as ogg1, ntg1, ntg2, apn1, rad1, rev3, or rad52 (127). 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 (127). A level of peroxide sensitization similar to that conferred by the scΔoxr1::URA3+ mutation requires either the combination of ntg1 ntg2 apn1 with rad52 or rad1, or the combination of rev3 with rad52. These observations indicate that the OXR1 gene plays an important role in oxidative protection in yeast and, presumably, other eukaryotes which have OXR1 homologues.

Discussion

Interspecies complementation of antimitator activity. The use of spontaneous oxidation as the DNA damaging treatment provides a high degree of sensitivity compared to methods used by others (90, 109), 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, since 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 comprised primarily of the highly conserved carboxyl terminal domain of the human OXR1 gene (Fig. 2-2). 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 (125) 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 (125). The hOXR1 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 comprises just
the OXR1 homology region. Mutants of L82 are defective in eclosion and, therefore, fail to release adults from pupae (125). This developmental defect can be complemented by expression of the largest isoform, L82A (125). Since other isoforms have not been tested, it is uncertain if 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 (87, 101), 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 first step towards identifying all of the genes which contribute to protection against ROS in humans. The functional genomic approach we have taken has the potential to support the identification of genes which 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.
CHAPTER III

UNPUBLISHED OBSERVATIONS ON THE HUMAN AND YEAST OXR1 GENES

Abstract

The human OXR1 gene is the first of a family of eukaryotic OXR1 homologues to be cloned and characterized. Initial studies have shown the OXR1 genes of humans and S. cerevisiae to be involved in oxidative damage prevention and stress response (41, 140). hOXR1 is capable of suppressing oxidative DNA damage induced mutagenesis in E. coli (140). In yeast, an oxr1 deletion mutant is approximately 10 fold more sensitive to hydrogen peroxide induced cell death than wild type cells (140). In the present studies, the human and yeast OXR1 genes are further characterized with respect to their function is suppressing oxidative damage. A hOXR1 cDNA sequence sufficient for suppressing mutagenesis in E. coli is defined. The yeast oxr1 mutant is shown to suffer a higher level of hydrogen peroxide induced DNA damage than a wild type strain. This damage is likely specific for hydrogen peroxide, as the oxr1 mutant is not sensitive to the superoxide radical generating agent mendione.
Introduction

Recently a novel system for screening human cDNA has been devised that allows for the identification of genes that function to prevent or repair oxidative DNA damage in *E. coli* (137). Like the Arg reversion assay described in Chapter II, this system relies on the mutator phenotype of *E. coli* strains deficient in oxidative DNA damage repair functions. Human cDNAs are expressed in these repair deficient strains to determine if the human gene is capable of suppressing the mutator phenotype.

The *E. coli* strains used to assay human gene function contain a mutant *lacZ* allele, rendering the strain phenotypically lac-. This mutant *lacZ* allele has a single base substitution within a codon that codes for the critical active site residue of the β-galactosidase enzyme (34). Only a G:C to T:A transversion mutation can revert this allele to wild type *lacZ*, thus making the strain phenotypically lac+. The sequence of the wild type *lacZ* gene encoding the critical residues of β-galactosidase are shown below, along with the sequence of the mutant allele used in the screen for human gene function:

\[
\begin{align*}
\text{GLY} & \quad \text{ASN} & \quad \text{GLU} & \quad \text{SER} & \quad \text{GLY} \\
\text{GGG} & \quad \text{AAT} & \quad \text{GAG} & \quad \text{AGT} & \quad \text{GGC} \\
\text{GGG} & \quad \text{AAT} & \quad \text{GCG} & \quad \text{AGT} & \quad \text{GGC}
\end{align*}
\]

wild type β-gal amino acid sequence

wild type *lacZ* DNA sequence

*lacZ* – allele DNA sequence

Since the lesion 8-oxoGuanine has an approximately 50% chance of mispairing with adenine (123), DNA replication of this lesion can result in the mutation G:C to T:A at the active site codon of *lacZ* in this strain (assuming the lesion escapes normal base
excision repair pathways). Thus, reversion of $\text{lacZ}^{-}$ to $\text{lacZ}^{+}$ provides a useful read-out for endogenous oxidative damage in this $E. coli$ strain during normal aerobic growth.

The mutagenic potential of endogenous oxidative damage is illustrated by $E. coli$ strains deficient in oxidative damage repair pathways, such as $\text{mutM}$, $\text{mutY}$, $\text{nth}$, and $\text{mutH}$. As described previously, MutM and MutY are DNA glycosylases that excise 8-oxoG and adenine mispaired with 8-oxoG, respectively. Nth (endonuclease III) is a DNA glycosylase that repairs the thymine glycol lesions, while the mismatch repair protein MutH has been shown to be involved in the repair of oxidative DNA damage as well (148). Mutation of these genes greatly increases the frequency of spontaneous mutagenesis in $E. coli$ (see figure 2-1). These mutator strains are useful tools for assaying the ability of human genes to prevent or repair oxidative DNA damage when overexpressed in $E. coli$.

The mutator $E. coli$ system has been used successfully to identify the human OXR1 gene, as described previously (Chapter II and (140)). The hOXR1 gene represents a family of eukaryotic genes, well conserved evolutionarily from yeasts to humans. In the yeast $S. cerevisiae$, deletion of the OXR1 gene results in sensitivity to lethal doses of hydrogen peroxide (140).

In this study I use the mutator $E. coli$ system to further characterize the hOXR1 gene, identifying a domain sufficient for its oxidative damage prevention function. In addition, the yeast homolog of hOXR1 is further characterized with respect to its
sensitivity to DNA damaging agents, and its role in preventing DNA damage induced by hydrogen peroxide.

Materials and Methods

Strains and growth media. *E. coli* strains MV4504 (WT) is congenic to strain CC104 containing the lacZ reversion allele (34). Strain MV4708 is the *mutM mutY* derivative of MV4504 (142), and MV4631 is the *mutH nthH* derivative of MV4504. For complementation analysis, strains were transformed with plasmids expressing hOXR1 or empty vector alone, and plated on selective indicator plates (92)(1x E salts, 0.2% D-glucose, 1mM MgSO₄, 5μg/ml thiamine hydrochloride, 0.5mM IPTG (isopropyl-β-D-galactopyranoside), 40μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.5mg/ml P-gal (phenyl-β-D-galactopyranoside), 50μg/ml carbenicillin, 15%agar) and grown at 37℃ for 4-5 days to allow papilae formation. *S. cerevisiae* strains N1-4 and N1-9 have been described previously (140), and were propagated on standard YEPD media at 30℃ for all experiments.

Yeast Survival Analysis. Hydrogen peroxide and menadione survival analysis was conducted as described previously (140). Cells were grown to OD600=0.8, harvested and washed twice in PBS. Cells were resuspended in PBS, and aliquots were treated with the indicated doses of hydrogen peroxide (Sigma) and menadione (Sigma) for 1 hour. For hydrogen peroxide experiments, treatments were terminated with addition
of 38.5 μg/ml catalase (Sigma) after 1 hour. For menadione experiments, after 1 hour treatment cells were immediately diluted in PBS and plated on YEPD plates. Colonies were counted after 3 days growth at 30°C.

Quantitative PCR analysis of DNA damage. QPCR of DNA damage in yeast was performed by Ben Van Houten et. al. as described previously (68). Wild type (N1-4) and Δoxr1 mutant (N1-9) cells were grown to OD600=0.8, harvested, washed in PBS and treated with 50mM hydrogen peroxide for 30 minutes. Alternatively, cells were resuspended in PBS and left untreated as controls. Following treatment, cells were immediately washed in PBS, and the cell pellet was frozen in dry ice and sent to B. VanHouten for QPCR analysis.

DCFDA fluorescence assay. Yeast strains were grown to saturation over night. The following day, strains were diluted 1:10 in fresh media (5ml per sample) and grown at 30°C to log phase (OD600=0.5). Cells were washed once in PBS, and resuspended in 400ul PBS and transferred to 0.5ml Eppindorf tubes. DCFDA was added to experimental samples at the desired concentrations (0 to 20μM). Cells were incubated with probe at 30°C for 10 minutes, followed by the addition of 400ul of PBS to each sample to make the total volume 800ul. Samples were incubated for an additional 50 minutes at 30°C. 100ul aliquots were removed at placed in microtiter plates and read with a fluorescence microplate reader (Molecular Devices). Excitation wavelength was 490nm, emission wavelength was 520nm.
Results

The original hOXR1 cDNA isolated by Volkert et al. (140), pMV520, was transformed into the lacZ reporter strain MV4708 to determine its ability to suppress oxidative mutagenesis in a mutM mutY repair deficient background. This strain contains a lacZ allele mutated at the active site codon and requires a G-C to T-A transversion to revert to lacZ+ (34). This strain has been used successfully to screen human cDNAs for genes that function to suppress oxidative DNA damage-mediated mutagenesis (142). Figure 3-2 shows the results of hOXR1 expression in strain MV4708. hOXR1 expression markedly reduces the number of lac+ papillae appearing during growth of the lac- colony (left panel), indicating that hOXR1 functions to suppress oxidative damage induced mutagenesis in E. coli.

The hOXR1 cDNA was also introduced into an E. coli strain mutated for the genes mutH and nth. MutH is a mismatch repair endonuclease which has been shown to be involved in repair of oxidative DNA damage (148). Nth is the DNA glycosylase endonuclease three, which removes thymine glycol lesions from DNA, a significant contributor to oxidative DNA damage. Figure 3-3 demonstrates that hOXR1 functions to suppress oxidative damage induced mutagenesis in the mutH nth strain of E. coli, when compared to the strain containing the vector alone. Also, the suppression in this strain is comparable to that seen in the mutM mutY E. coli mutator strain (Figure 3-2), indicating that hOXR1 can function equally well to counter oxidative DNA damage in multiple DNA repair deficient E. coli host strains.
In addition to the hOXR1 cDNA described in Chapter II, pMV520, a second hOXR1 cDNA was isolated in a screen for human genes that suppress oxidative mutagenesis in the mutM mutY mutator E. coli system (142). This cDNA, pMV669, is identical to the hOXR1 gene in pMV520, but also contains additional upstream sequence 5’ to the start codon of pMV520. This additional 5’ sequence corresponds to human chromosome 8 and is also homologous to sequences present in the Drosophila L82 gene, indicating that it most likely represents hOXR1 exonic sequence. However, it is unclear if this upstream sequence is contributing to the anti-oxidative damage activity of hOXR1 in the E. coli mutator assay. Since it is the C-terminal domain of OXR1 that is most highly conserved between organisms, and this is the only domain present in scOXR1, it is likely that it is sufficient to suppress E. coli mutagenesis in the absence of any additional upstream sequence. To test this hypothesis, the additional upstream sequence in pMV669 was removed, leaving only the C-terminal 296 amino acids (position 358 to 654, see figure 3-1), giving clone pMV634. This construct was again introduced into E. coli strain MV4708 to test for anti-mutator activity. The results in Figure 3-4 demonstrate that this domain alone is sufficient to suppress mutagenesis in the mutM mutY strain background. I will refer to this 296 amino acid domain, capable of suppressing oxidative DNA damage in E. coli, as the OXR1 homology domain (shaded regions in figure 3-1). Referring to figure 2-2, the OXR1 homology domain corresponds to the consensus sequence from amino acid position 169 to 513 in the multiple sequence alignment.
Figure 3-1. Protein domains of hOXR1 clones. White boxes represent upstream OXR1 amino acid sequences present in hOXR1 from clone pMV669. pMV633 was derived by truncation of pMV669 to express only the C-terminal 296 amino acids. This 296 amino acid domain defines the OXR1 homology domain (grey boxes).
Figure 3-2. *E. coli* strain MV4708 was transformed with pMV520 (phOXR1) and transformants were diluted and plated on papillation assay plates as described in materials and methods.
mutH nth/strain MV4631 was transformed with the human OXR1 expressing plasmid pMV520 (pOXR1). Transformants were diluted and plated on selective indicator plates as described in materials and methods.
Figure 3-4. Plasmid pMV634 was introduced into the mutM mutY mutator strain MV4708, and transformants were diluted and plated on selective indicator plates as described in materials and methods. Left, MV4708 transformed with the vector, pTrc99. Right, MV4708 transformed with pMV634 expressing hOXR1ΔN.
Experiments with the *S.cerevisiae oxi1* mutant

The yeast *S. cerevisiae* expresses a homologue of hOXR1, scOXR1, which allows for the study of OXR1 function in a genetically tractable model system. The sequence homology between hOXR1 and scOXR1, as well as the construction of the scoxr1Δ mutant, are described in detail in Chapter II. This mutant yeast strain has been used to study the role OXR1 plays in oxidative damage protection in a eukaryotic model system.

The first evidence that OXR1 functions to protect cells from oxidative damage came from the hydrogen peroxide survival curves of wild type and oxi1Δ yeast strains (see figure 2-5). To determine if this oxidation sensitivity also corresponded to an increase in DNA damage, we conducted a quantitative PCR analysis of these yeast strains following oxidative damage in collaboration with Ben Van Houten. This experimental approach is described in the materials and methods, however the rationale for the experiment is quite simple. If oxidative damage results in an increase in DNA polymerase-stalling lesions (those lesions that prevent DNA polymerase from replicating a template molecule containing a lesion) then these lesions can be measured as a quantitative decrease in PCR product when comparing reactions of damaged DNA template molecules to undamaged DNA template molecules. This method would therefore measure an increase in DNA lesions in a mutant strain that suffers more oxidative DNA damage compared to a wild-type strain.
The experimental procedure for estimating DNA lesions by quantitative PCR is diagrammed in figure 3-5:

Figure 3-5. Methodology for detection of DNA damage by QPCR. DNA is isolated from cells exposed to damaging agents, quantified, and subjected to quantitative PCR. PCR products are then statistically analyzed using the Poisson expression to estimate the number of lesions per 10 kb of template molecule (taken from Ayala-Torres et. al. (7)).
After DNA is isolated from the experimental samples, it is quantified and used as template in a PCR reaction. Radiolabeled nucleotides are used in the PCR reaction so that amplification products can be quantified using phosphorimaging and band density analysis software (ImageQuant, Molecular Dynamics, Sunnyvale CA). Lesion frequencies are then calculated using the Poisson equation, \( f(x) = e^{-\lambda} \frac{\lambda^x}{x!} \), which assumes that lesions are randomly distributed along the template molecule.

Amplification is directly proportional to the fraction of undamaged DNA templates \((x=0)\). Average lesion frequency per template molecule is then calculated as \(- \ln A_D / A_o\), where \(A_D\) represents the amount of amplification of the damaged template and \(A_o\) is the amplification product from undamaged DNA (7).

The data in figure 3-6 show that the \(oxr1Δ\) strain contains approximately 2-fold more oxidative DNA lesions in nuclear DNA than wild type. In addition, the \(oxr1\) mutant shows an increase in mitochondrial DNA damage as well (bottom), although to a lesser extent than the nuclear genome. This data is consistent with previous studies measuring DNA damage by QPCR. The Van Houten lab has measured DNA damage at the human \(β\)-globin gene locus using this technique and produced lesion frequencies in the range of 0.4 to 1.2 lesions per 10 kb (7). In this range of lesion frequency, which is comparable to the data shown in figure 3-6, the error range was approximately 10% at the 1 lesion/10kb frequency and rises to about 20% at lower lesion frequencies (0.2 lesions/10kb). Given the small error ranges common in this QPCR assay, it is likely that the difference observed between the \(oxr1\) mutant and wild type is significant.
The yeast $\textit{oxr}1\Delta$ mutant has been shown to be sensitive to killing by hydrogen peroxide (140). To determine if this oxidation sensitivity was limited to hydrogen peroxide, or extended to other sources of ROS, the $\textit{oxr}1\Delta$ mutant was treated with menadione, a source of superoxide radicals (22). The menadione survival curves were repeated three times, and figure 3-7 shows two representative data plots of these experiments. The results indicate that in these experiments, the $\textit{oxr}1$ mutant is no more sensitive to superoxide killing than a wild type strain. Although this data suggests that $\textit{OXR1}$ does not function to protect cells from superoxide damage, these results are by no means conclusive. Given the variation in survival seen between experiments, it would be necessary to repeat these survival curves several more times to attain a better understanding of the sensitivity of these strains to menadione. Also, it would be of interest to compare wild type, $\textit{oxr}1$, and a $\textit{sod}$ mutant strain in the same experiment to determine how the sensitivity of the $\textit{oxr}1$ mutant compares to that of a mutant known to be sensitive to superoxide radical damage.

The survival data presented in Figs 3-7 and 2-5 suggest that, in yeast, $\textit{OXR1}$ functions to protect cells from the lethal effects of hydrogen peroxide-induced damage. However, the mechanism of this protective effect is unclear. If $\textit{OXR1}$ provides an antioxidant activity to the cell (as opposed to damage repair activity), then loss of $\textit{OXR1}$ function should lead to increased levels of ROS within the cell. To test this hypothesis, I employed an assay to measure levels of reactive oxygen molecules in wild type versus $\textit{oxr}1\Delta$ mutant yeast strains. The assay relies on a molecular probe, $2'-7'$-.
dichlorofluorescein diacetate (DCFDA), that undergoes modification by cellular esterases and intracellular oxidation to yield the fluorescent product dichlorofluorescein (DCF) (60). This probe has been used in several cell systems to measure intracellular ROS levels (38, 136, 151). Figure 3-8 represents data from preliminary experiments using the DCFDA probe in undamaged, and hydrogen peroxide treated yeast cells. The data in the left hand panel of figure 3-8 indicates that fluorescence intensity is proportional to the concentration of DCFDA probe loaded into the cells. In addition, the higher fluorescence measured in the oxr1 mutant indicates that this strain may contain higher levels of intracellular ROS than wild type cells. I therefore wanted to continue using this assay to measure ROS in yeast cells, the data in the right hand panel of figure 3-8 represents a second experiment using this technique. In this experiment, cells were loaded with one concentration of DCFDA (20μM), either in unstressed cells (middle) or in cells pre-treated with H₂O₂ (right). The oxr1Δ mutant cells appear to have an elevated basal level of ROS compared to wild type cells (middle). The level of ROS detected increases upon exposure of cells to H₂O₂ as expected, however the difference between wild type and mutant becomes smaller (right). Based on these initial results, it is tempting to speculate that OXR1 may function to suppress the production or persistence of ROS molecules with the cell. However, it should be stressed that these are preliminary experiments only, and they should be followed up carefully before any conclusions can be made regarding the role of OXR1 in detoxification of intracellular reactive oxygen species. Also, other techniques for assessing intracellular oxidation should be used to corroborate this data. I have attempted to use other assays for this purpose. First, measurement of the ratio of reduced and oxidized glutathione (GSH/GSSG) in cell extracts using an in vitro assay,
and secondly measurement of oxidized proteins (protein carbonyls) by western blot using indirect chemiluminescent detection of the oxidized protein. In both of these systems I was unable to obtain consistent, reproducible data due to continued technical difficulties. For this reason I continued with the DCFDA oxidation assay, and believe that it has produced the most reproducible data and will offer the best chances to accurately measure intracellular oxidation in yeast and mammalian cells.

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Sample</th>
<th>Ad/Ao</th>
<th>lesions/10kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.78</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>oxr1</td>
<td>0.52</td>
<td>0.7</td>
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<tr>
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Figure 3-6. Quantitative PCR analysis of DNA damage in wild type and oxr1Δ yeast strains for both nuclear (top) and mitochondrial (bottom) DNA. For each strain, the ratio of PCR product obtained from damaged DNA template (A_d) to that of undamaged DNA template (A_o) is given. This ratio is used to calculate the frequency of DNA lesions per 10kb of template DNA (7).
Figure 3-7. Menadione survival curves of wild type and *oxr1* mutant strains. Cells were treated with the indicated concentrations of menadione in PBS buffer for 1 hour at 30°C. Cells were diluted and plated on YPD plates, and colonies counted after three days of growth at 30°C. Two representative survival curves are shown. Despite the internal consistency and reproducibility of survival differences between *oxr1* and wild type, these are preliminary experiments due to the wide variation in lethality seen from one experiment to the next.
Figure 3-8. DCFDA oxidation assays. **Left panel.** Wild type and oxr1 mutant cells were loaded with the indicated concentrations of DCFDA in PBS for 1 hour. Fluorescence was measured at ex 490nm and em 520nm. **Right panel.** Wild type (hatched bars) or oxr1Δ (closed bars) yeast cells were loaded with 20uM DCFDA in PBS for 1 hour. They were then either left untreated, or exposed to 50mM H₂O₂ for 15 min. Cells were washed once in PBS, and fluorescence was measured at ex490 and em520nm. All fluorescence values given are arbitrary fluorescence units, and expressed as fluorescence per A600 units of yeast cells used in each measurement. The two experiments shown should be considered preliminary data, and due to wide variation in fluorescence emission and differences between oxr1 and wild type strains, should be repeated.
Discussion

Mutagenic *E. coli* strains have proven to be a useful tool in studying the ability of human genes to repair or protect against oxidative DNA damage (140, 142). Through the use of a new *E. coli* lacZ reversion system (described in this work and (142)), new clues have emerged as to the function of the human OXR1 gene in protecting cells from oxidative damage.

The major source of oxidative mutagenesis in *E. coli* is the 8-oxoGuanine lesion. This lesion is produced under normal, aerobic growth conditions, although the DNA repair systems of the cell are robust enough to keep the mutagenic effect of this lesion relatively low (observe the low number of lac+ papillae in wild type strain of *E. coli*, Figure 3-2). However, the frequency of production of 8-oxoG can be unmasked by mutation of DNA repair functions that normally keep this damage in check. In the *mutM mutY* strain, deficient oxidative damage DNA glycosylases, the number of lac+ revertant papillae are drastically increased, indicating a high level of spontaneous 8-oxoG production in aerobically growing cells (Figure 3-2). Expression of hOXR1 is capable of suppressing this high level of mutagenesis in the *mutM mutY* strain, suggesting hOXR1 functions to either repair 8-oxoG or protect DNA from oxidation indirectly (Figure 3-2). This latter explanation is supported by the results shown in figure 3-3. The *mutH nth E. coli* strain also displays a high frequency of oxidative mutagenesis. In this case, mutagenesis is due to defective repair of a different spectrum of DNA damage than in the *mutM mutY* strain. Nth (Endonuclease III) is a DNA glycosylase that removes thymine glycol, another
common lesion produced by oxidative damage. The chemical structures of thymine glycols and 8-oxoG are very distinct, and as a result the DNA glycosylases responsible for their removal are highly specific (Nth has little activity on 8-oxoG; MutM has little activity on thymine glycol (32)). MutH is a nicking endonuclease and part of the mismatch repair system. Strains deficient in mutH exhibit a G:C to T:A mutator phenotype, and evidence suggests that this mutagenesis is a result of oxidative DNA damage (148). Given the diverse substrates for DNA repair in the mutator strains presented in figures 3-2 and 3-3, it would be unlikely that hOXR1 provides a repair function capable of acting on all of them. I believe it is more likely that OXR1 prevents reactive oxygen species from generating DNA lesions, and hence an anti-mutator effect can be seen in these various mutator E. coli strains.

Consistent with this hypothesis are the results presented in Figure 3-5. Quantitative PCR of template DNA from peroxide damage and undamaged yeast cells indicates that oxr1Δ mutant cells contain a higher amount of hydrogen peroxide induced lesions than wild type cells. In nuclear DNA, oxr1Δ mutant shows an approximately 2-fold increase in the number of lesions per template molecule in the PCR reaction (top table). A similar increase is also seen for mitochondrial DNA templates, although the increase is lower in magnitude (approximately 50%, lower table). This data indicates that loss of oxr1 function tends to increase oxidative damage throughout the entire cell (at least in the nuclear and mitochondrial compartments). This result is most consistent with the idea that OXR1 functions to detoxify reactive oxygen, and in its absence ROS levels
increase resulting in greater DNA damage in both the nuclear and mitochondrial genomes.

Additional support of the hypothesis that OXR1 functions to prevent hydrogen peroxide induced damage comes from experiments described in Figures 3-6 and 3-7. First, data in figure 3-7 indicates that the oxr1Δ mutant strain is not sensitive to superoxide induced damage, suggesting that there may be specificity of the Oxr1 protein for preventing hydrogen peroxide induced damage. Secondly, figure 3-8 shows the results of a preliminary experiment in which intracellular ROS are assayed using the oxidation dependent fluorescent probe DCFDA. Increased fluorescence is observed in oxr1Δ cells compared to wild type cells, even in the absence of exogenous oxidative stress. After addition of hydrogen peroxide, DCF fluorescence increases in both mutant and wild type strains as more of the probe is activated to fluoresce, while the oxr1Δ mutant still shows an increased level compared to wild type. This preliminary data suggests that oxr1Δ mutant cells have an increased basal level of ROS compared to wild type cells, consistent with notion that Oxr1 protein serves to detoxify ROS, or prevent its production, rather than repair oxidative damage.

Finally, information regarding the function of Oxr1 can be gained from determining the possible substrates for Oxr1 activity. Previously I have observed that the yeast oxr1Δ mutant is not sensitive to either UV irradiation or the alkylating agent MMS (data not shown). These results suggest that the action Oxr1 protein is somehow specific for reactive oxygen molecules rather than simply providing a “molecular shield” to cellular molecules like DNA and proteins. If this were the case, one would expect Oxr1
to provide the cell protection from virtually any type of insult, regardless of its chemical nature. As mentioned in the Introduction, it is well known that cells possess several antioxidant defence systems that are specific for certain molecules. For instance, catalase and peroxidases act on peroxides, while superoxide dismutase acts on superoxide radicals. I therefore asked if the activity of Oxr1 was specific for hydrogen peroxide induced damage or superoxide induced damage. Figure 3-7 shows the results of a menadione survival experiment for both wild type and oxr1Δ mutant yeast strains. Menadione is a well-characterized drug that produces superoxide damage primarily (22). No difference in survival is observed between wild type and oxr1Δ mutant cells after menadione treatment, while at a dose of hydrogen peroxide that causes a similar level of lethality in wild type cells, oxr1Δ cells show a nearly ten fold increase in sensitivity (see figure 2-5). These results suggest that Oxr1 protein provides protection from oxidative damage associated with hydrogen peroxide, and acts on such molecules specifically rather than simply insulating DNA (or other cellular components) from their damaging effects.
CHAPTER IV
STRESS INDUCTION AND MITOCHONDRIAL LOCALIZATION OF OXR1 PROTEINS IN YEAST AND HUMANS

Abstract

Reactive oxygen species (ROS) are critical molecules produced as a consequence of aerobic respiration. It is essential for cells to control the production and activity of such molecules in order to protect the genome and regulate cellular processes such as stress response and apoptosis. Mitochondria are the major source of ROS within the cell, and as a result numerous proteins have evolved to prevent or repair oxidative damage in this organelle. The recently discovered OXR1 gene family represents a set of conserved eukaryotic genes. Previous studies of the yeast OXR1 gene indicate that it functions to protect cells from oxidative damage. In this report we show that human and yeast OXR1 genes are induced by heat and oxidative stress, their proteins localize to the mitochondria, and function to protect against oxidative damage. We also demonstrate that mitochondrial localization is required for OXR1 protein to prevent oxidative damage.
**Introduction**

It is critical for the aerobically respiring cell to defend against oxidative damage to cellular macromolecules in order to carry out metabolic activities and faithfully maintain the genome. Recently, much research has focused on the importance of reactive oxygen species (ROS) in various cellular processes including DNA damage and repair (27), redox regulation of protein activity (50, 74), signal transduction (18, 29), ageing (89) and apoptosis (44, 69, 98).

There are numerous sources of ROS. Chemical agents such as menadione and paraquat produce mainly superoxide radicals (26), while hydrogen peroxide can be converted to the hydroxyl radical (•OH) by the Fenton reaction (71). Ionizing radiation such as X rays and Gamma rays damage cells primarily through reactive oxygen intermediates formed by the electrolysis of water (14). However, the major source of reactive oxygen species within the cell is the mitochondria (78). Electron transport activities occurring at the inner membrane of mitochondria have been shown to produce ROS at significant levels; as much as 1-5% of the O₂ consumed by respiring cells is converted to ROS (21). As a result, cells express numerous antioxidant defenses that protect mitochondria, including MnSOD, thioredoxins, glutathione, as well as DNA repair enzymes (3, 82, 128, 135). Recent investigations have also highlighted a role for antioxidant activity in the regulation of the mitochondrial apoptosis pathway. Inhibition of the electron transport chain, resulting in increased ROS production, has been shown to result in increased apoptosis (75, 131). Conversely, mutations in mitochondrial
antioxidant functions (Trx-2, MnSOD) have also been demonstrated to increase apoptosis through the mitochondria-dependent pathway (100, 153). The important relationship between oxidative damage prevention and disease is illustrated by mice deficient in the mitochondrial Apoptosis Inducing Factor (AIF), which display increased oxidative stress and cell death in neuronal cells (70). It is becoming increasingly apparent that identification and characterization of ROS regulating proteins in the mitochondria will be crucial in understanding how cells avoid oxidative injury and control apoptosis.

Recently we have identified a novel human gene, OXR1, based on its ability to suppress oxidative DNA damage in *Escherichia coli* (140). OXR1 is an evolutionarily conserved gene, as homologues are present in many eukaryotic organisms from yeast to man. To date there is little known about its function. Deletion of the OXR1 gene in *Saccharomyces cerevisiae* (scOXR1) results in sensitivity to hydrogen peroxide damage (140). This suggests the OXR1 gene product may play a particularly important and unique role, as many other mutations in individual genes that prevent or repair oxidative damage do not result in oxidation sensitivity phenotypes (6, 53). *Drosophila melanogaster* expresses seven isoforms of OXR1 (L82A-G), and a mutant deleted for the entire locus is lethal as a result of a defect in eclosion (hatching from the pupae case) (125). The mouse homologue of OXR1, C7, was identified in a screen for genes induced upon cell attachment to extracellular matrix (42). The eukaryotic OXR1 genes encode proteins of various sizes, although they all contain a conserved, ~300 amino acid C-terminal domain. As this domain corresponds to the entire *S. cerevisiae* Oxr1 protein, it
likely represents a unique functional domain and possesses the proposed oxidation protection function of the yeast and human OXR1 proteins.

In this report we further characterize the expression of the yeast and human OXR1 genes, as well as the cellular localization of their respective proteins. We provide evidence that the yeast and human OXR1 genes exhibit a stress response in both yeast and human cells. We also present the first evidence that the human OXR1 protein provides protection from oxidative damage in a eukaryotic cell.

Materials and Methods

Yeast strains, plasmids and media. The S. cerevisiae strain N1-4 (140) was used for wild type controls. Strains N74 and N76 (containing plasmids pMV656 and pMV657 respectively) were derived from the oxr1Δ::URA3 strain N1-9 (see Table 4-1). The mitochondrial targeting sequence (MTS) of SOD2 was fused to hOXR1 by PCR using the primer: 5'
aaggatcatggcggaaacagcagctgctattaaccttaaccgaaaggggtggtttgtcattgcttcacaagggaaatattcacc 3'

The product was inserted into the vector pMV611 to generate pMV656. pMV657 was constructed in the same way, except the upstream hOXR1 primer lacked the SOD2 MTS sequence. Strains N34 and N39 were derived from strain N1-4 by integration into the OXR1 locus of a PCR product containing the C-terminal GFP or HA tag fused to the 3' end of the OXR1 open reading frame and flanked by 40 bp of chromosomal sequence.
upstream and downstream of the $OXR1$ open reading frame, following published procedures (81). Correct integration into the chromosome was confirmed by PCR analysis using an internal primer to $OXR1$ and an external primer to the kanamycin resistance selectable marker. Also, strains were tested phenotypically for wild-type sensitivity to hydrogen peroxide to confirm $OXR1$ function. Plasmid template for the HA tag was gift of M. Longtine (Oklahoma State Univ., Stillwater). Yeast peptone dextrose (YPD) media and synthetic minimal dextrose media (SD) were prepared as described by Adams, et al. (1).

**Cell lines and culture.** HeLa cells were cultured in Dulbecco's modified Eagle medium (GIBCO BRL) supplemented with 10% fetal bovine serum (Sigma), 1 mg/ml L-glutamine (Gibco) and penicillin/streptomycin (Gibco) at 37°C in a 5% CO$_2$ incubator.

Immunofluorescence microscopy. Yeast immunofluorescence was carried out as described (1) using mouse anti-HA monoclonal antibody 16B12 (Covance) at 1:1000 dilution and secondary anti-mouse AlexaFluor 568 antibody (Molecular Probes) used at 1:500 dilution. For HeLa cell immunofluorescence, cells grown on 12-mm poly-D-lysine coated glass coverslips (Becton Dickinson) were washed 5x in phosphate buffered saline (PBS), fixed in 2.5% paraformaldehyde, washed 1x in PBS, and permeabilized with 0.1% Triton X-100. Cells were incubated with a 1:200 dilution of either rabbit anti-C7C (0.78 mg/ml stock), or anti-C7M antibodies (0.25 mg/ml stock), gifts from E. Engvall (Burnham Institute, LaJolla, CA). Antibodies were produced and affinity purified using the C7C or C7M domain peptides as described by Fischer et al. (42). Cells were then washed 3X in PBS and incubated with anti-rabbit AlexaFlour 488 secondary antibody (Molecular Probes) at 1:200 dilution, followed by visualization by fluorescence
microscopy. For mitochondrial labeling, cells were incubated with 100nM Mitotracker Red (Molecular Probes) for 15 minutes prior to fixation.

**Protein extracts and Immunoblotting.** Yeast and HeLa cells were pelleted, washed 1X in phosphate buffered saline, and resuspended in SDS protein loading buffer and immediately boiled for 10 minutes. Proteins were then separated by SDS PAGE and immunoblotted using mouse anti-HA monoclonal antibody 16B12 (Covance) and anti-mouse HRP-conjugated secondary antibody (Amersham Life Science). Where shown, mouse anti-alpha tubulin antibody (Lab Vision) was used to probe blots as a loading control. Bands were quantified with a Bio-Rad scanning densitometer and Bio-Rad Multi Analyst software (Hercules, CA).

**RNA isolation and Northern blotting.** Total yeast RNA was extracted using the acid phenol method as described by Sambrook et al. (119). Approximately 20 μg of total RNA per sample was separated on a 1% formaldehyde gel and transferred to a nylon membrane (Hybond) by capillary transfer. PCR products of the scOXR1 open reading frame or SCR1 open reading frame were used as templates in random primed labeling reactions to generate a 32P-labeled probes. Hybridization was carried out at 42°C overnight. Band intensities were scanned on a Personal Densitometer SI (Molecular Dynamics) and quantified using Molecular Analyst software (Bio-Rad). Fold-induction values are given relative to the 0 minute control, and normalized to the SCR1 loading control.
Results

Mitochondrial localization of scOxr1p. Sequence analysis of the yeast \textit{OXR1} open reading frame revealed a putative mitochondrial localization signal at the N-terminus of the protein (PSORTII, http://psort.nibb.ac.jp). We therefore sought to confirm this prediction with the use of a fluorescently tagged scOxr1 protein. The GFP open reading frame was fused in-frame with the 3' end of the \textit{OXR1} gene, and this fusion construct was integrated by homologous recombination, replacing the endogenous \textit{OXR1} gene on chromosome XVI. This allows expression of an \textit{OXR1}-GFP fusion protein from the endogenous \textit{OXR1} promoter in the normal \textit{OXR1} chromosomal context. Figure 4-1 shows the localization of \textit{OXR1}-GFP to discrete cytoplasmic compartments which correspond with the extra-nuclear DAPI stained regions (compare panels A and B), indicating that \textit{OXR1}-GFP and mitochondrial DNA reside in the same compartment. To further support this, yeast cells were stained with the mitochondrial specific probe Mitotracker (Molecular Probes). As seen in Figure 4-1C, the regions stained with Mitotracker clearly overlap with those identified by \textit{OXR1}-GFP fluorescence. Very little if any protein is associated with the nucleus (large DAPI stained region), indicating that scOxr1p resides almost exclusively in the mitochondria.

We also confirmed the localization of Oxr1p with the use of an epitope-tagged Oxr1 protein and immunofluorescence techniques. A C-terminal HA tagged form of Oxr1p was generated and expressed from the endogenous \textit{OXR1} locus, as was done for \textit{OXR1}-GFP. Immunofluorescence was then performed to determine the localization of the HA tagged protein. Figure 4-1, panels D and E show the co-localization of
mitochondrial DNA and the Oxr1-HA protein. These results are in agreement with the OXR1-GFP studies, and strongly suggest that scOxr1p is associated with the yeast mitochondria.

Table 1. Yeast strains and plasmids used in this study.

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<td>(140)</td>
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<td>N1-9</td>
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<td>(140)</td>
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<td>N76</td>
<td>[N1-9]/phOXR1-myc</td>
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Figure 4-1. Localization of scOxr1p in yeast. Fluorescence microscopy was carried out on strain N34, expressing a C-terminal GFP tagged Oxr1p. Cells were pre-loaded with 100μM MitoTracker Green probe for 30 minutes prior to collection and mounting in DAPI containing mounting buffer. (A) DAPI stained cells. (B) MitoTracker. (C) Oxr1p-GFP. Immunofluorescence was conducted on strain N-39 expressing Oxr1p-HA. (D) DAPI. (E) anti-HA immunostaining.
Figure 4-2. The N terminus of \(\text{yOxr1p}\) is required for mitochondrial localization. Full length (FL) \(\text{yOxr1p}\) fused at the C-terminus with GFP was expressed in wild type yeast cells and localized by fluorescence microscopy (top right). Mitochondrial localization was compared to DAPI stained compartments (top left). The same construct lacking the first 50 N-terminal amino acids was expressed and localized as above (bottom right), and compared to DAPI stained cells (bottom left).
To confirm that the N-terminus of yOxr1p contained a mitochondrial targeting sequence as predicted *in silico*, a C-terminally GFP tagged Oxr1p was generated lacking the first 50 amino-terminal amino acids (Δ50 Oxr1-GFP). This construct was expressed in wild type strain N1-4 and protein localization was determined by fluorescence microscopy. The data in figure 4-2 demonstrates that the Δ50 Oxr1-GFP protein does not localize to the mitochondria, rather it displays a more diffuse cytoplasmic pattern. As expected, the full length Oxr1-GFP protein localizes properly to the DAPI stained, mitochondrial compartment (compare top left panel to top right panel in figure 4-2). This data confirms that the N-terminus of yOxr1p contains a targeting signal that is required for the protein to be sorted into the mitochondrial compartment in yeast cells.

**Expression of yeast OXR1 is induced by heat and oxidative stress.** Global transcription profiling experiments in yeast have demonstrated that *OXR1* is one of a subset of genes induced by stress conditions, particularly those conditions associated with an increase in oxidative stress (19, 49). To confirm that *OXR1* expression is stress-inducible, we subjected yeast cells to both heat and oxidative stress conditions, and the levels of *OXRI* transcripts were monitored by Northern blotting. Figure 4-3A shows the results of a northern blot of total yeast RNA from untreated cells, and from those subjected to a 37°C heat stress. The levels of *OXR1* transcript increase within the first 15 minutes of the treatment, and return to unstressed levels by 45 minutes. This data closely resembles that obtained with a probe to the classical yeast heat shock gene *HSP12* (data not shown) (111). The heat stress inducible expression of *OXR1* is also apparent at the translational level. As seen in figure 4-3B, heat stress causes the accumulation of Oxr1-
HA protein as determined by immunofluorescence. These results suggest that expression of OXR1 is regulated at least in part by growth temperature. We also asked if OXR1 expression could be induced by oxidative stress. Figure 4-3A shows the effects of a 0.5 mM H₂O₂ exposure on the levels of OXR1 transcript as monitored by Northern blot. As with heat stress, there was a rapid increase in OXR1 transcript level during the first 15 minutes of treatment, although transcript levels remained elevated throughout the time course of the experiment. Together with the heat stress data and previously published micro-array data, these results strongly suggest that OXR1 is a stress induced gene in *Saccharomyces cerevisiae*.

![Figure 4-3A](image)

Figure 4-3 continued on next page
Figure 4-3. Heat and Oxidative stress induction of scOXRI. (A) Yeast cells were grown at 22° C to log phase, and shifted to 37° C for the given time points (left). Alternatively, cells were grown at 30° C and treated with 0.5 mM H₂O₂ for the indicated times (right). For each time point cells were harvested, and total RNA was analyzed by Northern blot. Fold induction values are given for each time point relative to the 0 minute control, and are normalized to SCR1 loading control. (B) Yeast cells were grown at 22° C to log phase and incubation was either continued at 22° C (upper panels) or shifted to 37° C and continued for 1 hour (lower panels). Cells were then fixed and stained with anti-HA antibody to visualize Oxr1p-HA. Panels A and C, DAPI staining. Panels B and D, anti-HA staining.
The human OXR1 protein is localized to mitochondria in human cells.

Previous work with the mouse homolog of hOXR1, C7, has generated antibodies to two domains of the C7 protein. An antibody to domain II of C7 was used to demonstrate nucleolar localization of C7 in several rodent cell lines (42). A second antibody, called C7C, was also generated to the C-terminal domain (domain III) of C7. This domain is highly homologous to the corresponding region of the human OXR1 protein (~90% identity), and is also homologous to scOxr1p. (We reasoned that the mouse antibody C7C would provide a useful tool for examining the localization of the hOXR1 protein in the human HeLa cell line, since it recognizes human OXR1 expressed in bacteria (data not shown).) We conducted immunofluorescence experiments on HeLa cells using the C7C antibody, and as shown in Figure 3, the human OXR1 protein is localized to a specific cytoplasmic compartment, enriched around the nuclear periphery and also found in long, tubular shaped projections extending from the perinuclear region to the tips of the adherent cell. The observed staining is strikingly similar to that of the mitochondria in HeLa cells (25). We therefore stained the cells with Mitotracker prior to immunofluorescence in order to establish co-localization. Figure 4-4 clearly shows that Mitotracker stains the same cellular compartment as the OXR1 antibody, indicating that in HeLa cells hOXR1 is associated with the mitochondria. As is the case in yeast, little if any OXR1 protein is detected in the nucleus. Similar localization results were obtained in experiments with two additional mammalian cell lines, human Hep2 cells and monkey COS cells (data not shown).
Figure 4-4. Localization of human OXR1 protein in HeLa cells. HeLa cells were pre-loaded with 100 μM MitoTracker Red probe, fixed and immunostained with rabbit anti-C7C antibody and anti-rabbit AlexFluor 488 secondary antibody. (A) anti-C7C staining. (B) MitoTracker. (C) Merged images of panels A and B.
**Human OXR1 protein is induced by heat and oxidative stress in HeLa cells.** Given the similarities between yeast and human OXR1 in terms of protein homology and localization, we asked if human OXR1 expression was also induced by stress conditions in human cells. First, we subjected HeLa cells to a 1mM dose of H$_2$O$_2$ for 15 minutes and allowed 1 hour to recover in fresh growth medium. Human OXR1 protein was then visualized by immunofluorescence, as shown in Figure 4-5A. The staining of OXR1 protein is more intense in peroxide treated cells compared to untreated cells, and the protein accumulation appears to be localized primarily to the mitochondria with some diffuse staining in the cytoplasm (compare OXR1 staining with MitoTracker staining). There is no visible difference in intensity of MitoTracker staining between peroxide treated and untreated cells, indicating that the differences seen in OXR1 staining is due to protein levels and not mitochondria content. To support the immunofluorescence results, we also monitored hOXR1 protein levels during stress by Western blot (Figure 4-5B). In unstressed HeLa cells, the C7C antibody recognizes an 37.5 kD protein, which is consistent with the predicted size (41.6 kD) of the human OXR1 gene product originally isolated (140). Following 1mM H$_2$O$_2$ treatment, OXR1 protein accumulates within the first hour after treatment, and by 4 hours the level of protein reaches a maximum and begins to decrease by 5 hours. We also observed the accumulation of a higher molecular weight protein recognized by the C7C antibody at 58.8 kD, which presumably reflects a larger splice variant that includes additional upstream exons detected by DNA sequence analysis of chromosome 8. We also asked if heat stress induces expression of human OXR1 protein, as it does for scOXR1p. Figure 4-5C shows a Western blot of HeLa cells extracts following a 30' heat stress at 42$^\circ$C. Human OXR1 protein accumulates in a
manner very similar to that of peroxide treated cells, with a maximum level occurring by 2.5 hours and reduction apparent by the 4.5 hour mark. The immunofluorescence results of peroxide treated HeLa cells, together with the Western blots of peroxide and heat treated cells, indicates that human OXR1 protein expression is induced by heat and oxidative stress in human cells.

Figure 4-5. (A) H₂O₂ induced accumulation of hOXR1 protein in HeLa cells. Cells were loaded with 100 μM Mitotracker Red for 15 minutes, then treated with 1mM H₂O₂ for 15 minutes (top panels) or left untreated (bottom panels) and recovered in fresh media for 1 hour prior to fixation and staining with anti-C7C antibody.
Figure 4-5. (B) HeLa cells were treated with 1mM H$_2$O$_2$ for 15 minutes and recovered in fresh media for the indicated time points. Cells were harvested and crude protein extracts separated by SDS PAGE and immunoblotted with anti-C7C antibody. Samples were also stained with Coomassie as a loading control. (C) Cells were heat stressed at 42°C for 30 minutes and recovered at 37°C for the indicated time points. Western blots were conducted as in panel (B). Blots were probed with anti-α-tubulin as a loading control. Numbers under bands represent fold-induction values relative to the untreated sample and are normalized to loading controls (coomassie or α-tubulin).
Multiple transcripts of hOXR1 are expressed in a tissue specific manner. To further characterize hOXR1 expression, we determined the abundance of hOXR1 message in multiple human tissues by Northern blot. A blot of polyA⁺ RNA from several human tissues was hybridized with a hOXR1 cDNA probe corresponding to the entire C-terminal OXR1 homology domain, and the results shown in Figure 4-6. Two transcripts, 2.9 and 4.9 kb, are observed in nearly all tissues with the exception of brain, which expresses a unique 5.1 kb transcript in addition to the 2.9 kb transcript. Also, the relative abundances of each transcript differ in the tissues examined, with the 4.9 kb transcript being most abundant in placenta, lung, liver, kidney and pancreas. In heart and skeletal muscle, the smaller transcript appears to be equally as abundant as the 4.9 kb transcript. From this data we conclude that hOXR1 is expressed as two transcripts whose relative abundances differ among various human tissues.
Figure 4-6. Multiple tissue Northern blot of hOXR1. A blot of polyA⁺ RNA from several human tissues (Clonetech) was probed with ³²P-labelled hOXR1 cDNA.
Mitochondria-targeted hOXR1 can complement the peroxide sensitivity of a yeast oxr1 mutant strain. Previous work has shown that the yeast oxr1Δ mutant is approximately ten-fold more sensitive to hydrogen peroxide lethality than a wild type strain (140). In order to determine if the human OXR1 protein was capable of complementing the peroxide sensitivity of the scoxr1Δ mutant, we expressed mitochondria-targeted and untargeted hOXR1 proteins in the scoxr1Δ mutant background and tested resistance to hydrogen peroxide. To target hOXR1 protein to the yeast mitochondria, the MTS from yeast Sod2p was fused to the N-terminus of the hOXR1 protein. This targeting signal has been used previously to direct ectopically expressed proteins into the yeast mitochondria (39). The protein was tagged at its C-terminus with the 13-Myc epitope tag for detection by immunofluorescence and Western blotting. The untargeted hOXR-myc protein lacked the Sod2p MTS. Figure 4-7A shows hydrogen peroxide survival curves of wild type, oxr1Δ, and oxr1Δ strains expressing either the mitochondria-targeted hOXR1 (mt-hOXR1-myc) or untargeted hOXR1 (hOXR1-myc) gene from the constitutive GPD promoter. The mt-hOXR1-myc expressing strain shows wild type resistance to hydrogen peroxide, while the untargeted hOXR1-myc expressing strain is as sensitive as the oxr1Δ mutant strain. Even though the untargeted hOXR1-myc protein is more highly expressed than the mt-hOXR1-myc protein (Figure 6B), it does not confer peroxide resistance to the strain in which it is expressed, indicating that mitochondrial localization is required for protection from oxidation by OXR1 protein. We also confirmed the mitochondrial localization of the mt-hOXR1-myc protein by immunofluorescence as shown in figure 4-7C. The anti-myc antibody staining co-localizes with the Mitotracker probe (compare panels B and C). In contrast, the
untargeted hOXR1-myc protein displays a more diffuse staining pattern, with a significant amount of signal present in the nucleus (panels D and E) and little co-localization with MitoTracker (panel F). These results indicate that hOXR1 can functionally complement the hydrogen peroxide sensitivity of an scoxr1Δ mutant strain and that mitochondrial localization is a requirement for function.

Figure 4-7. Mitochondria targeted hOXR1 protein complements the peroxide sensitivity of an oxr1Δ::URA3 mutant S. cerevisiae strain. (A) Yeast strains were grown to OD₆₀₀ 0.6, treated with the indicated doses of H₂O₂ and dilutions were plated on SD –leu plates. Colonies were counted after 3 days of growth at 30°C. Strains bearing plasmids expressing either the mitochondria-targeted (pmt-hOXR1-myc) or untargeted (phOXR1-myc) forms of human OXR1 are compared with wild type (WT) and vector control (oxr1Δ::URA3) strains. Results are data representative of three independent experiments.
(B) Protein extracts from *oxr1Δ::URA3* strains expressing myc-tagged human OXR1 protein fused to the mitochondrial targeting sequence (mt-hOXR1-myc) or untargeted (hOXRD1-myc) were immunoblotted with anti-myc 9E10 monoclonal antibody. (C) Yeast cells were loaded with 100μM MitoTracker Green probe and immunostained for hOXR1-myc proteins using anti-myc 9E10 antibody and AlexaFluor 568 secondary antibody. Cells expressing targeted and untargeted human OXR1 proteins are labeled as in panel B.
Discussion

The recently discovered human OXR1 gene represents a family of well conserved, eukaryotic genes whose function is proposed to include resistance to oxidative damage (140). In this report we demonstrate that the regulation of gene expression, protein localization and function of OXR1 are also conserved from yeast to humans.

Numerous proteins are localized to the mitochondria to counteract the deleterious effects of ROS, including glutathione peroxidase, thioredoxin, superoxide dismutase as well as multiple DNA repair enzymes (3, 82, 135) (128). Despite the seeming overabundance of these oxidative damage resistance functions, the yeast oxrΔ mutant remains sensitive to oxidative damage, indicating an important role for this gene in protecting cells from oxidative damage (140). Consistent with this idea are the results of several micro array experiments addressing stress-induced gene expression in yeast. The scOXR1 gene has been shown to be induced during conditions of heat stress, stationary phase, and diauxic shift (19, 49). Interestingly, these same conditions are reported to result in significant increases in reactive oxygen species within the cell (36, 52, 80). Our findings corroborate the micro array data and expand these observations by showing that scOXR1 is part of a stress-response pathway turned on during conditions of ROS production, and provide further evidence that scOxr1p serves to protect yeast cells from oxidative damage. We also demonstrate that the scOXR1 protein can be functionally replaced by its human orthologue containing the OXR1 homology domain.
The mouse homologue of hOXR1, C7, was isolated by others in a screen for genes up regulated upon cell attachment to extracellular matrix. Using the C7M antibody generated to domain II of C7, this protein was shown to localize to the nucleolus in several rodent cell lines (42). We have used the C7C antibody produced from this study and shown it to recognize specifically mitochondrial protein in HeLa cells (Figure 4-4), as well as in Hep2 and COS cells. This is consistent with the western blot data showing that the C7C antibody recognizes only one major protein in untreated HeLa cell extracts (Figure 4-5B, untreated control lane). A second species is detectable after induction by oxidative or heat stress, and also appears to be largely mitochondrial. No nucleolar staining is detectable even after stress induction. Our results indicate that in the cell lines tested hOXR1 is associated with the mammalian mitochondria. That we observed no nucleolar staining with the C7C antibody suggests either the nucleolar isoform of OXR1 lacks the amino acid sequence recognized by C7C, or that the antibody cannot access such sequences. Recent studies failed to detect nucleolar staining in human cells (Eva Engvall, personal communication) and are similar to our results using the C7C or C7M antibodies (Figures 4-4 and 4-5 and data not shown). This suggests that the nucleolar staining in rodent cells is due to a species difference, or is a species related artifact.

Mitochondrial localization of the OXR1 homology domain is consistent with the finding that OXR1 must be targeted to this cellular compartment for the antioxidant function of this domain in yeast.

As is the case with scOXR1, the human OXR1 gene is induced by stress conditions in human cells. The first evidence of stress-induced expression of the human
OXR1 gene came from immunofluorescence experiments in HeLa cells after hydrogen peroxide treatment (Figure 4-5A). During a one hour recovery from oxidative stress, hOXR1 protein visibly accumulates in the mitochondria. We also see more intense signal in the cytoplasm, which may be due to leakage of hOXR1 protein from the mitochondria, incomplete importation of all protein into the mitochondria, or to the expression of a distinct, cytoplasmic isoform of hOXR1. This latter possibility is consistent with the western blot results showing the appearance of multiple OXR1 bands following peroxide treatment (Figure 4-5B). This western blot data also confirms the oxidative stress-induced accumulation of the 37.5 kD hOXR1 protein. As in yeast, heat stress has been shown to lead to increased ROS and induction of antioxidant functions in mammalian cells (126). We have shown that heat stress induces expression of human OXR1 protein in a manner very similar to oxidative stress (Figure 4-5C). Although the induction of mitochondrial heat shock proteins by heat and oxidative stress is well known (48) there is little evidence of mitochondrial proteins outside of this well conserved protein family induced by both heat and oxidative stress. Also, the most well characterized mitochondrial heat shock proteins are chaperonins (105), and it is unclear what, if any, antioxidant activity they possess. Human OXR1 may therefore represent one of a small set of proteins that are responsive to multiple stress conditions and provide protection against reactive oxygen species in human mitochondria. In this respect it is interesting to note that hOXR1 mRNA appears to be abundant in tissues with relatively high respiration capacity (heart, skeletal muscle, brain; Figure 4-6), where it would be advantageous to counteract mitochondrial ROS production.
It has been hypothesized that reactive oxygen species play a role in mediating cell death in mammalian cells, particularly through the mitochondrial apoptosis pathway (44, 69, 98). Conditions that increase the amount of mitochondrial ROS production (for example, inhibition of the electron transport chain) lead to increased apoptosis. Conversely, depletion of mitochondrial antioxidant functions has also been shown to increase cell death by apoptosis (70). Regulation of the mitochondrial redox state has been shown to be important for resistance to oxidative stress in *S. cerevisiae*, as well as in mammals. Deletion of the mitochondrial thioredoxin reductase *TRR2* in yeast causes increased sensitivity to hydrogen peroxide (106), while homozygous mutation of mitochondrial thioredoxin (Trx-2) in mice results in elevated apoptosis and embryonic lethality (100). We have found that targeting human OXR1 to the yeast mitochondria is necessary for complementing the hydrogen peroxide sensitivity of an *oxr1Δ* mutant (Figure 4-7A). The human OXR1 protein containing an N-terminal MTS is targeted to the yeast mitochondria (Figure 4-7C) and exhibits wild-type resistance to peroxide, particularly at the highest doses tested. A strain expressing an identical copy of hOXR1 lacking the MTS is as sensitive to peroxide as the *oxr1Δ* mutant. These data suggest that the peroxide induced lethality seen in yeast is mediated by a mitochondrial process, and mitochondrial localization of OXR1 function (either yeast or human) is required for wild-type resistance to peroxide damage. Furthermore, these results support the claim that human and yeast OXR1 proteins are functionally homologous. Our findings suggest that both human and yeast OXR1 may be part of a mitochondrial stress response. Since human OXR1 is capable of providing yeast cells protection from oxidative damage when localized to the mitochondria, it is likely that it plays a similar role in oxidative stress.
resistance in human cells as well. It will be interesting to ask if human OXR1 is involved in regulation of ROS production or detoxification and protection from oxidation-mediated apoptosis in human cells.
Chapter V

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Isolation of the human OXR1 gene

Heterologous protein expression and genetic complementation in *E. coli* has proven to be a useful method to identify and characterize human genes. This work describes the identification of a human gene, OXR1, based on its ability to suppress mutagenesis in DNA-repair defective strains of *E. coli*.

The screening approach used in these studies is unique in that it allows for the identification of genes that function to limit oxidative DNA damage either through direct repair of lesions or indirectly through inhibiting the activity of ROS. The system does not require that genes be necessary for survival of the cell, either during normal aerobic growth or during treatment with exogenous oxidizing agents. Also, the system does not rely on sequence or structural homologies between genes, which allows for the identification of entirely novel genes whose function and sequence may not be previously described. Finally, the visual-based system of identifying genes whose activity can decrease mutagenesis (*i.e.*, decreasing the number of colonies on a selection plate or decreasing the number of blue papillae on an indicator plate) makes this a very versatile system. Genes with moderate activities can be identified as easily as those with very high activities. Although untested, it is possible that this system could also be useful in identifying mutator alleles, those that increase, rather than decrease, the level of
mutagenesis in *E. coli*. This would greatly expand the classes of genes that could be identified by this screening method, and allow for the characterization of mutants in known DNA damage repair or prevention genes.

Because of the advantages outlined above, it is unlikely that the human OXR1 gene would be identified by previous screening methods (described in Chapter I). However, because of the strength of its anti-oxidative damage properties, it appears to be an important gene in the cell’s defence against oxidative stress. It is interesting that hOXR1 was identified on two independent occasions during the screening process. First, pMV520 was isolated in the Arg+ screening system described in Chapter II. Additionally, Jen-Yeu Wang isolated the pMV669 clone of hOXR1 in the lacZ reversion system (142). While this may be an artifact due to over-amplification of the cDNA library used in these screens, it could also point to the importance of this particular message in the tissue from which the library was derived (human testis).

**The OXR1 Gene Family**

One important factor in the decision to pursue hOXR1 for further study was the presence of several will conserved eukaryotic OXR1 homologues in the database of sequenced genomes. In particular, the presence of a yeast homologue allowed for the study of an OXR1 family member in a genetically tractable organism. I therefore began characterizing the *S. cerevisiae OXR1* gene.
Prior to this work, scOXR1 was only identified as an open reading frame in the yeast genome (YPL196w). I was the first to clone this open reading frame and create the deletion mutant oxr1Δ, which I then demonstrated was a non-essential gene and that the mutant was sensitive to hydrogen peroxide. This data has been used to update the gene information for YPL196w, including the new designation OXR1, on the Stanford Saccharomyces Genome Database website: [http://www.yeastgenome.org/](http://www.yeastgenome.org/). This database also contains information gained from transcription profile experiments that may shed light on yeast OXR1 function. The yeast gene is induced under conditions that enhance oxidative stress: heat stress, stationary phase, and diauxic shift. I then confirmed by Northern blot that scOXR1 transcription is upregulated by both heat stress and hydrogen peroxide treatments. Furthermore, scOXR1 protein accumulates following heat stress. These are the first results to indicate that a eukaryotic OXR1 family member is required for oxidative damage avoidance and is expressed in response to oxidative stress.

The *Drosophila* OXR1 homologue, designated L82, is expressed as seven different isoforms (L82A-G). This locus was identified based on its regulation by the steroid hormone ecdysone, a hormone controlling metamorphosis in *Drosophila* (4) (125). Deletion of the entire L82 locus results in lethality during eclosion, since the newly formed adult fly is unable to extricate itself from the pupae case. Interestingly, if the adult fly is manually removed from the case, it survives and remains active for several weeks (125). In addition, L82 mutants exhibit a developmental delay, taking longer to progress from embryo through metamorphosis to adulthood, of between 1 to 15 days (125). The length of the developmental delay depends on the severity of the L82
mutation. The seven isoforms of L82 form an allelic series, with the length of developmental delay being proportional to the number of L82 isoforms disrupted in the mutant fly (125). The eclosion-lethal phenotype of L82 mutants is particularly interesting when viewed in light of other studies of oxidative stress during *Drosophila* development. It has been hypothesized that flies undergo a crisis of oxidative stress during late metamorphosis and eclosion (62). In support of this, mutants with decreased expression of catalase or superoxide dismutase, in combination with thioredoxin deficiencies, leads to lethality during eclosion (95). Also, this stage of fly development is associated with marked transcriptional induction of both catalase and superoxide dismutase genes (87, 101). Also noteworthy is the observation that transcriptional induction of catalase, like L82, is regulated by the steroid hormone ecdysone. Thus, antioxidant functions including catalase and OXR1 may be part of a transcriptional program induced by the metamorphic hormone ecdysone. These studies suggest that metamorphosis and eclosion are developmental stages associated with high levels of oxidative stress, and the fly’s survival depends on appropriate expression of antioxidant functions, including OXR1.

The mouse OXR1 homolog, C7, was cloned by Engvall and colleagues as a gene induced upon cell attachment to extracellular matrix (42). Like the human gene, mouse C7 is expressed as multiple isoforms, all of which contain the highly conserved, C-terminal OXR1 homology domain (42). Although nothing is known about the function of the mouse OXR1 homolog, its regulation by matrix binding is quite interesting. It is known that cellular response to ionizing radiation, a source of ROS, is dependent on cell-
cell and cell-ECM contacts (35). Lung cancer cells are more resistant to ionizing radiation when in contact with fibronectin, and this resistance appears to be due to the regulation of the damage response and cell cycle genes Chk1, Chk2, and Cdk1 (28).

Also, ECM attachment regulates apoptosis as well. In a phenomenon known as "anoikis", cell detachment from ECM induces cytochrome c release from mitochondria and apoptosis (117). Consistent with this process would be a model in which OXR1 is down regulated following detachment from extracellular matrix, leading to an increase in reactive oxygen generation in the mitochondria, which triggers apoptosis via the mitochondrial pathway.

In humans there appears to be two widely expressed OXR family genes. I have discussed OXR1, found on chromosome 8, in detail. However, computer based searching of the human genome has revealed a second OXR1 paralogue. I will refer to this gene as OXR2, although it has also been cloned and characterized by Miles Brown and coworkers as ERAP140 (122). The proteins encoded by these genes are 51% identical and 65% similar to one another. As with the other eukaryotic OXR1 homologues, the OXR2 protein is most similar in its C-terminal domain. Figure 5-2 shows the similarities between OXR1 and OXR2 both in terms of intron/exon structure (A) and protein sequence (B) (Durand, manuscript in preparation). The predicted intron/exon structure of both OXR1 and OXR2 show extensive similarities with regard to number and spacing of exons, as well as the boundaries between introns and exons. This suggests that OXR1 and OXR2 have evolved from a common ancestral gene, most likely through a duplication event.
Figure 5-1. (A) Organization of OXR1 and ERAP140 (OXR2) genes. Exon-intron structures both genes are shown. OXR1 is located on Chromosome 8q22, ERAP140 is located on Chromosome 6q22.3. The dark, solid boxes represent exons, or portions of exons shared between all forms of OXR1 and ERAP140. Exons shown in light gray are those regions present only in the longest forms of OXR1 and ERAP140. Areas in white are unique to ERAP140. The striped exon is unique to a predicted, alternatively spliced form of OXR1 which is similar to that present in the mouse OXR1 homologue, C7B (42). The length of the lines connecting exons is an indication of the relative size of the intron.
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<th>Yeast</th>
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<td>TASESSPQLKDHSN-P-FRNKTSERTIVEE---GSLPPVRLNGYLPSTKN</td>
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**Figure 5-1 (B) Comparison of the predicted protein sequences of the OXR1 homology domains of full length OXR1 beginning with aa 624 (C-terminal 215aa); OXR2/ERAP140 beginning with amino acid residue 620 (C-terminal 216aa); and yeast Oxr1p beginning with aa 28 (C-terminal 245 aa).**
Both full length OXR2 and a truncated allele only consisting of the OXR1 homology domain are capable of suppressing mutagenesis in the *E. coli lacZ* mutator system (figure 5-2). This suggests that the OXR2 protein contains the C-terminal OXR1 homology domain that functions to prevent oxidative damage in a manner very similar to OXR1 protein. The Brown lab has cloned OXR2 as an estrogen receptor interacting protein of 140 kD (ERAP140), which functions as an activator of estrogen receptor mediated transcription. As expected, the protein contains a nuclear localization signal, and is targeted to the nucleus. However, unlike OXR1, it does not appear to be induced by oxidative stress (Brown, personal communication). The protein contains approximately 600 amino acids N-terminal to the 296 amino acid OXR1 homology domain, a region that is homologous to Drosophila L82A and mouse C7, but is not present in the human OXR1 protein described in Chapter II of this work (clone pMV520). This N-terminal region does contain homology with the longest isoform of OXR1 (figure 5-1A, light blue shaded boxes), although there are three unique exons present in OXR2 that are not found in the corresponding OXR1 gene (figure 5-1A, white boxes). In addition, there are two exons of OXR2 that do not contain significant homology with OXR1: exons 8 and 11. It is within the coding region of exon 8 that the estrogen receptor binding activity of OXR2 protein has been mapped (122). Furthermore, receptor binding does not require the OXR1 homology domain. It appears then that OXR2 is a dual-function protein, containing estrogen receptor binding and coactivation activity in the N-terminus, and oxidative damage protection activities in the C-terminus. It remains to be determined if the OXR1 homology domain is required *in vivo* to modulate receptor binding and or coactivation functions. An interesting possibility is that the estrogen
receptor/transcription activator complex is controlled in part by oxidation state, and thus OXR2 may function in redox regulation of estrogen signaling in the nucleus.

There is precedence for the idea of damage-induced regulation of estrogen signaling in the nucleus. Several DNA repair proteins have been shown to interact with the estrogen receptor to modulate transcription, including 3-methyladenine DNA glycosylase (76), T:G mismatch thymine DNA glycosylase (23), and the human DNA repair and TFIIH regulator MMS19 (147). In addition, the human DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) illustrates an interesting mechanism of estrogen receptor regulation. This enzyme directly reverses DNA alkylation damage by transferring the alkyl group of O6-alkyl guanine to its own active site cysteine, repairing the DNA damage while at the same time inactivating the MGMT enzyme (107). This inactive, alkylated form of the enzyme, R-MGMT, undergoes a conformational change that allows it to interact with the estrogen receptor and negatively regulate transcriptional activity (132). This provides an elegant mechanism by which DNA damage sensing is coupled to regulation of cell growth control in estrogen responsive cells. An interesting model is then suggested in which the OXR1 homology domain of OXR2 functions to sense oxidative damage and regulate estrogen receptor signaling in a manner analogous to MGMT.

It is also possible that OXR2 contains the nuclear counterpart to OXR1, and the function of the C-terminal domain (the OXR1 homology domain) of OXR2 is unrelated to estrogen receptor activation. In this case, the N-terminal region of OXR2 that contains
the nuclear localization signal may serve as a shuttle to get the OXR1 homology domain into the nucleus.

Figure 5-2. Anti-mutator activity of OXR2/ERAP140 cDNA clones. Left, strain MV4708 expressing vector alone. Middle, MV4708 expressing the OXR1 homology domain region of OXR2/ERAP140, amino acids 657-942. Right, MV4708 expressing full length OXR2/ERAP140. Strains were plated on minimal indicator plates as described in Materials and Methods, and photographed after 5 days. (from Durand et.al. manuscript in preparation).
Multiple isoforms of human OXR1

Analysis of the genomic sequence of chromosome 8, in conjunction with database analysis of human cDNA clones, has suggested that OXR1 is expressed as multiple isoforms arising from mRNA splicing (see figure 5-2). These predictions have been partially confirmed by Northern blot analysis of human tissues (figure 4-6), which demonstrate that at least three different transcripts of OXR1 are expressed in human tissues.

Whole brain tissue RNA contains a unique 5.1 kb transcript in addition to the 2.9 kb transcript found in other tissues, demonstrating the presence of a splice variant of OXR1 mRNA. Consistent with this Northern blot data, several cDNA clones have been produced from human brain tissue that contain an addition exon approximately 21 kb upstream of exon 2. I will refer to this transcript as OXR1A (figure 5-2, second line from top). Human cDNA clones also predict the presence of another splice variant, OXR1C, containing exon 10 that corresponds to the pMV520 OXR1 clone described in detail in these studies. Finally, a small splice variant is also predicted containing exon 11 (OXR1D) that corresponds to the mouse C7C protein. This unique exon 11 is also predicted to contain a mitochondrial targeting signal similar to both mouse C7C and yeast OXR1 (described in Chapter IV).

As shown in figure 5-2, four splice variants of OXR1 are predicted. This is in agreement with Northern blot data of mouse embryonic mRNA, which also indicates the
presence of four different C7 transcripts (42). The sizes of the two most abundant mouse transcripts, 4.8 kb and 2.8 kb, also correspond closely to those of the two primary human transcripts (figure 4-6). In addition, 3.7 kb and 1.8 kb transcripts are detected in mouse embryonic samples, although there do not appear to be corresponding transcripts of this size in adult human tissues. In the mouse studies by Fischer et al., it was found that C7 is most highly expressed during development, from embryonic day 7 to 17 (42). Much lower abundance of transcripts were observed from adult mouse tissues. The fact that the multiple tissue Northern blot in figure 4-6 only shows three different transcripts is most likely due to either low abundance of one transcript in these tissues, or possibly a cell type or developmental stage-specific expression of an OXR1 isoform that cannot be detected in the adult tissue samples on this blot.
Figure 5-3. Genomic map of exon and intron regions from chromosome 8q22.3 and OXR1 splice variants. Splice variants are predicted from known cDNA sequences and database analysis of chromosome 8. Dark boxes represent exons unique to only one OXR1 isoform.
Function of OXR1 proteins

Much progress has been made in characterizing both yeast and human OXR1 with respect to protection from oxidative stress, gene expression, and protein localization. However, one question still remains: What is the molecular function of OXR1 proteins? The work I have presented here suggests that OXR1 proteins function in the mitochondria of eukaryotic cells as an antioxidant protein active against ROS derived from hydrogen peroxide.

First, evidence in *E. coli* indicates that OXR1 is capable of suppressing endogenous oxidative damage in bacterial cells. Initially, OXR1 was identified based on its ability to suppress oxidative mutagenesis in a *mutH nth* mutant *E. coli* strain ((140) and Chapter I). Subsequently I determined that OXR1 was also capable of suppressing mutagenesis in a *mutM mutY* mutant *E. coli* strain (Chapter II). These results indicated the either OXR1 was complementing the DNA repair defects in these strains, or it was serving to lower the amount of oxidative DNA damage occurring within these strains. However, the known functions and specificities of these DNA repair genes make the former possibility unlikely. The *mutH nth* strain contains defects in two distinct DNA repair pathways. MutH is a mismatch repair endonuclease that recognizes and incises hemi-methylated GATC sequences during the mismatch repair process (144). Nth on the other hand, is a base excision repair DNA glycosylase which recognizes and repairs the oxidative lesion thymine glycol (15). Neither protein is capable of providing the specific function of the other. Furthermore, MutH is a prokaryotic-specific function that does not
exist in eukaryotes. Similarly, MutM and MutY both provide specific base excision repair functions. MutM excises 8-oxoG lesions, while MutY excises adenine mispaired with 8-oxoG (5). Also, neither of these proteins show any redundancy of function. Therefore, it is unlikely that OXR1 can suppress mutagenesis in strains defective in these repair processes by directly complementing the DNA repair defects (i.e., acting as a DNA repair protein), since that would require that OXR1 function to recognize and repair several distinct forms of oxidative DNA lesions. It is more likely that OXR1 functions to suppress the amount of ROS damage to DNA occurring in all bacterial cells in which it is expressed.

This idea is supported by the quantitative PCR experiments done in collaboration with B. VanHouten (figure 3-5). This data indicates that in oxr1Δ mutant yeast cells there is a higher degree (approximately 2-3 fold) of oxidative DNA lesions in nuclear DNA induced by hydrogen peroxide than in wild type cells. This is also true for mitochondrial DNA, although the increase in lesion frequency is slightly less for mitochondrial DNA. It should be noted that these oxidative DNA lesion frequencies are likely underestimates, since they are only measurements of DNA lesions which block DNA synthesis in the PCR reaction. The ubiquitous lesion 8-oxoG, for example, does not block DNA polymerase in vitro, and would therefore not be accounted for in these experiments. Since yOxr1p has been shown to localize almost exclusively to the mitochondrial compartment, with little or no protein detectable in the nucleus, it is doubtful that these results could be due to a lack of DNA repair activity. More likely,
Oxr1 protein serves to lessen the amount of reactive oxygen in the cell, thereby protecting both mitochondrial and nuclear compartments from oxidative DNA damage.

That OXR1 proteins might possess an anti-oxidant function is also suggested by analysis of the Δoxr1 mutant phenotypes in yeast. Initially, the oxr1Δ mutant was found to be hypersensitive to hydrogen peroxide damage (figure 2-5). Subsequently, I tested this mutant for sensitivity to other damaging agents. I found that the mutant was no more sensitive to UV light or the methylating agent MMS than wild type. Interestingly, I also found that the mutant was not sensitive to menadione. This is a chemical known to produce damage mainly through production of superoxide radicals (22). Until this point, it remained a possibility that OXR1 protein functioned simply to protect DNA (and other cellular components) by coating them and acting as a shield to damage. This however appears unlikely since the oxr1 mutant appears only to be sensitive to hydrogen peroxide damage. If OXR1 could provide some type of general shielding defense, then one would expect it to also confer protection to many different types of damage, including UV light, MMS and superoxide radicals. In addition, the results shown in figure 3-7 indicate that OXR1 can limit the steady-state levels of ROS within the cell, as oxr1 mutant cells incubated with the oxidation sensitive probe display a higher level of fluorescence than wild type cells. It now appears likely that OXR1 functions to protect cells by suppressing damage caused by hydrogen peroxide derived ROS (hydroxyl radicals, for example).

The results presented in figure 4-6 are consistent with the idea that both yeast and human OXR1 function to prevent damage caused by hydrogen peroxide-derived ROS.
More importantly, these results suggest that such an activity is specifically required at the mitochondria. When properly localized to the mitochondria, the human OXR1 protein confers wild-type resistance to the oxr1 mutant (figure 4-7C). However, hOXR1 protein lacking a mitochondrial targeting sequence is unable to protect the oxr1 mutant from cell killing by hydrogen peroxide, even when expressed at much higher levels than the mitochondria-targeted protein (figure 4-7B). This raises the question: Why does OXR1 only function at the mitochondria to prevent oxidative killing in the oxr1 mutant strain? It seems reasonable that if OXR1 is active against peroxide-derived ROS, it should be able to confer resistance to hydrogen peroxide when expressed in the cytoplasm as well. One possible explanation is that the lethality seen in the oxr1 mutant is due primarily to ROS generation in the mitochondria, and in the absence of OXR1 these molecules can cause lethal damage to mitochondrial components and also escape to the nucleus and other cellular compartments. In this case, even if OXR1 is present in high levels in the cytoplasm, it will not provide any protection because it is unable to access the primary sites of ROS production and damage. An alternative explanation is that OXR1 does require the activity of a protein partner, or protein modification, that only occurs in the mitochondria. While this seems unlikely, given that OXR1 can function by itself in the E. coli system, it cannot be ruled out since in a eukaryotic context there may be significant differences in how the production, and detoxification, of ROS is compartmentalized. For instance, suppose a multi-step pathway requiring other host cell factors produces the substrate for OXR1. In bacteria, these initial steps may take place in the cytoplasm or at the inner membrane but they are not compartmentalized. However, in yeast and humans these steps may take place in the mitochondria and would therefore
require that OXR1 be properly localized for it to function in the pathway. In other words, the activity of OXR1 in bacteria may be representative of its role in the most "prokaryotic" of eukaryotic compartments, the mitochondria.

Model of OXR1 function

Based on the results presented here, I propose the following model for the function of OXR1 proteins in protecting cells from oxidative damage:

When expressed in E. coli, OXR1 proteins are capable of detoxifying reactive oxygen species produced during normal aerobic growth through activities in the C-terminal OXR homology domain of the protein. Thus, OXR family proteins are capable of suppressing oxidative DNA damage-induced mutagenesis in E. coli.

In the eukaryotic cell, OXR1 proteins protect against oxidative damage by suppressing the production or reactivity of ROS molecules derived from hydrogen peroxide. The expression of OXR1 proteins is regulated by stress conditions, including heat stress and oxidative stress. The anti-oxidant activity of OXR1 is carried out in the mitochondria, where proteins must be properly localized in order to function. Figure 5-4 represents the OXR1 model for both E. coli and eukaryotic cells:
Figure 5-4. Model for OXR1 activity in human, yeast and E. coli cells.
Future Directions

Future research on this project will be geared toward better understanding the molecular function of OXR1 proteins in yeast and mammalian cells.

I. How does OXR1 protect cells from oxidative damage? There is now evidence that OXR1 functions to lower the steady-state levels of ROS within yeast cells. These results will be confirmed with assays designed to detect intracellular ROS production (e.g., fluorescent DCFDA assays) in wild type, oxr1 mutant, and oxr1 mutant strains expressing recombinant OXR1 proteins in order to determine if OXR1 can reduce the production or activity of ROS in yeast. These experiments can also be extended to human cells, where it has yet to be determined if OXR1 acts in a manner analogous to the yeast system. siRNA knock-down experiments will be conducted to determine if OXR1 deficiency sensitizes human cells to oxidative stress. Also, overexpression of the protein will be used to determine if OXR1 can enhance survival of human cells following oxidative stress.

II. Biochemical analysis of OXR1 protein function. Yeast and human OXR1 proteins will be purified and assayed for activity against various species of ROS in vitro. In addition, the purified proteins will be analyzed for post-translational modifications and cofactor binding that may provide clues as to OXR1’s function. For instance, flavoproteins involved in redox
regulation can be identified spectroscopically based on binding to FAD and NADPH.

**III. Mouse knockout.** I have begun working in collaboration with David Housman at MIT on construction of an OXR1 knockout mouse strain. The phenotype of the resulting mice should provide clues as to the role OXR1 plays in the mammalian system. Crossing these mutants to other knockout strains (tumor suppressor mutants, oxidation sensitive mutants) may uncover a function for OXR1 that is not apparent based on the phenotype of the OXR1 knockout itself.

These experiments, in addition to the data already presented here, will help to better define the function of this newly identified and important gene family.
References


44. **Fleury, C., B. Mignotte, and J. L. Vayssiere.** 2002. Mitochondrial reactive oxygen species in cell death signaling. Biochimie **84:**131-41.


