Identification of Factors Interacting with hMSH2 and hMLH1 in the Fetal Liver and Investigations of how Mitochondrial Dysfunction Creates a Mutator Phenotype

Anne Karin Rasmussen

Ph.D. Thesis

December 2001
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Preface

This Ph.D. thesis is based on work carried out at Department of Life Sciences and Chemistry, Roskilde University, Denmark in Dr. Lene Juel Rasmussen's laboratory and at Johns Hopkins Oncology Center, Baltimore, MD, USA in Dr. Keshav K. Singh's laboratory from 1998 to 2001.

On the beginning, my Ph.D. research focus area was primarily the DNA mismatch repair pathway. However, after a stay at Johns Hopkins the scope of my thesis ended up being more comprehensive. In the Ph.D. thesis I am also addressing the following issues: molecular mechanisms associated with DNA damage and DNA repair, and mutagenesis in mitochondria.
Acknowledgements

First, I wish to express my gratitude to Dr. Lene Juel Rasmussen for critical reading of my manuscripts and thesis and particularly for being an extraordinarily positive, dedicated and stimulating supervisor.

Secondly, I wish to give my thanks to Dr. Keshav Singh for letting me work in his laboratory at Johns Hopkins and for widening my intellectual horizon in the field of mutagenesis in mitochondria. Furthermore, I want to thank him for the critical reading of my manuscripts and for being an inspiring supervisor.

Thirdly, I want to express my thanks to Lene Markussen and Gerda Olesen for excellent technical assistance.

In addition, I want to give my thanks to all my colleagues and friends at Department of Life Sciences and Chemistry at Roskilde University and at Johns Hopkins Oncology Center. A special thanks to Rob Delsite and Barbara Sigala for technical support and to Anne Lützen and Jonas Andreasen for critical reading of my thesis and for creating a cheerful atmosphere.

Finally, I want to express my gratitude to my dearest family and boyfriend for all the love and support they gave me while completing my Ph.D.

Anne Karin Rasmussen
Copenhagen 2001
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Publications/Manuscripts

The present thesis is based on one original publication (I), one submitted manuscript (III), and two manuscripts in preparation (II, IV). The manuscripts referred to in the thesis are identified by the following roman numerals:


Structure

The thesis is divided into two sections:

1. Section one is a general introduction where important results from my research are included. The section is divided into three parts: The first part is an introduction to the publication (I). The second part is an introduction to manuscript II. The third part is an introduction to manuscripts III and IV.
2. Section two consists of one published paper and three manuscripts.
### Abbreviations

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<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>AD</td>
<td>Activation Domain</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine Nucleotide Translocator</td>
</tr>
<tr>
<td>AP</td>
<td>Apurinic/apyrimidinic</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BD</td>
<td>Base Excision DNA Repair</td>
</tr>
<tr>
<td>BER</td>
<td>Binding Domain</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>Complex I</td>
<td>NADH dehydrogenase or NADH:ubiquinone oxidoreductase</td>
</tr>
<tr>
<td>Complex II</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>Complex III</td>
<td>Ubiquinol:cytochrome c oxidoreductase</td>
</tr>
<tr>
<td>Complex IV</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>Complex V</td>
<td>ATP synthase</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q, also called ubiquinone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>FEN1</td>
<td>Flap endonuclease-1, <em>S. cerevisiae</em> rad27 homolog</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GGR</td>
<td>Global genome repair</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>hAPE1</td>
<td>Human AP endonuclease</td>
</tr>
<tr>
<td>hEXO1</td>
<td>Human exonuclease 1</td>
</tr>
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<td>hMLH1-hMLH3</td>
<td><em>E. coli</em> MutL homolog complex</td>
</tr>
<tr>
<td>hMLH1-hPMS1</td>
<td><em>E. coli</em> MutL homolog complex</td>
</tr>
<tr>
<td>hMLH1-hPMS2</td>
<td><em>E. coli</em> MutL homolog complex</td>
</tr>
<tr>
<td>hMSH2-hMSH3</td>
<td><em>E. coli</em> MutS homolog complex, recognizes insertion/deletions</td>
</tr>
<tr>
<td>hMSH2-hMSH6</td>
<td><em>E. coli</em> MutS homolog complex, recognizes base:base mispairs and insertion/deletions</td>
</tr>
<tr>
<td>hMYH</td>
<td>Glycosylase, Human MutY Homolog</td>
</tr>
<tr>
<td>HNPPC</td>
<td>Hereditary Non-Polyposis Colon Cancer</td>
</tr>
<tr>
<td>hNTH1</td>
<td>Glycosylase, Human Nth homolog</td>
</tr>
<tr>
<td>hOGG1</td>
<td>8-OxoGuanine DNA Glycosylase</td>
</tr>
<tr>
<td>HO*</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>Msh1</td>
<td>Mitochondrial homolog of the <em>E. coli</em> MutS</td>
</tr>
<tr>
<td>MNU</td>
<td>Methylnitrosourea</td>
</tr>
<tr>
<td>MPG</td>
<td>N-methylpurine-DNA glycosylase</td>
</tr>
<tr>
<td>Msh1</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>nDNA</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complexes</td>
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<tr>
<td>O2-</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>O6-MeG</td>
<td>O6-methylguanine</td>
</tr>
<tr>
<td>O6-MeT</td>
<td>O6-methylthymine</td>
</tr>
<tr>
<td>O2</td>
<td>7,8-dihydro-8-oxoguanine</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>POL</td>
<td>Polymerase γ</td>
</tr>
<tr>
<td>Pol ε</td>
<td>DNA polymerases (Pol II)</td>
</tr>
<tr>
<td>Pol δ</td>
<td>DNA polymerases (Pol III)</td>
</tr>
<tr>
<td>rho−</td>
<td>Mutations in the mitochondrial genome</td>
</tr>
<tr>
<td>rho0</td>
<td>Lack of mitochondrial genome</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>SOD1</td>
<td>Nuclear superoxide dismutase</td>
</tr>
<tr>
<td>SOD2</td>
<td>Mitochondrial superoxide dismutase</td>
</tr>
<tr>
<td>SSB</td>
<td>Single-strand binding protein</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TCR</td>
<td>Transcription coupled repair</td>
</tr>
<tr>
<td>UNG</td>
<td>Uracil-DNA Glycosylase</td>
</tr>
<tr>
<td>XPG</td>
<td><em>S. cerevisiae</em> Rad27 homolog</td>
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</tbody>
</table>
Summary

Increased spontaneous mutation frequency is associated with increased cancer risk. However, the relative contribution of spontaneous endogenous mutagenesis to carcinogenesis is not known today. Defects in the postreplication DNA mismatch repair (MMR) pathway are recognized to increase spontaneous mutations. Mutations in MMR genes cause hereditary non-polyposis colon cancer.

In an effort to identify unidentified genes involved in MMR and tissue-specific MMR-associated factors, we employed the yeast two-hybrid system, using the human hMSH2 as bait and a human fetal liver cDNA library as prey. We demonstrated that hMSH2 interacts with a human 5’→3’ exonuclease 1 (hEXO1).

Data presented in this thesis also support the conclusion that mitochondrial dysfunction leads to spontaneous nuclear DNA damage. We employed the yeast Saccharomyces cerevisiae as a model system to investigate a potential link between mitochondrial activity and genomic instability. Mitochondrial dysfunction and genetic instability are characteristic features of cancer cells. Furthermore, mitochondrial dysfunction is a key feature of aging due to accumulation of mutations in mtDNA. Our studies in a yeast model system suggest that mitochondria contain some intrinsic properties that control the generation of the mutator phenotype associated with cancer cells. We hypothesize that cancer cells by losing their mitochondrial function create a mutator phenotype.

Given the importance of maintaining the integrity of the mitochondrial genome we have found that it might be valuable to further investigate the molecular processes and components responsible for mtDNA repair. It has recently been recognised that base excision DNA repair (BER) is operating in the mitochondria, however, knowledge about other repair pathways is still very limited. We decided to investigate O6-methylguanine-DNA methyltransferase (MGMT) because of the fact that its sub-cellular localization has not been determined. We determined that it was localized to nucleus but not to mitochondria in HeLa and breast epithelial cells.
Sammendrag (Summary in Danish)

En øget frekvens af spontane mutationer er forbunden med øget risiko for cancer. Hvor stor en del af cancinenogenese som skyldes spontan endogen mutagenese, er endnu ikke kendt. Fejl i det postreplikative DNA mismatch repair (MMR) system har imidlertid vist sig at forøge den spontane mutationsfrekvens, da mutationer i MMR-generne kan forårsage hereditær non-polypos colon cancer.

For at finde uidentificerede gener, som er involveret i MMR, samt vævsspecifikke MMR-associerede faktorer, gjorde vi brug af gær two-hybrid systemet. Vi brugte hMSH2 som “bait” og et humant føtalt lever cDNA bibliotek som “prey”. Vi viste, at hMSH2 interagerer med en human 5’ → 3’ exonuclease 1 (hEXO1).


Givet vigtigheden af at opretholde/reparere det mitokondrielle genom for at forhindre mitokondriel dysfunktion, fandt vi det værdifuldt yderligere at undersøge de molekylære processer og komponenter ansvarlige for mtDNA reparation. Det er i dag anerkendet at base excision DNA repair (BER) virker i mitokondrierne. Viden om andre reparationssystemer i mitokondrierne er dog stadig meget begrænset. Vi undersøgte O6-methylguanine- DNA methyltransferase (MGMT) subcellulære lokalisation, og fandt at MGMT i bryst epithelialceller er lokaliseret til kernen, men ikke til mitokondrierne.
1. Analysis of Human DNA Mismatch Repair

1.1 Introduction

The postreplication DNA mismatch repair (MMR) pathway is responsible for the maintenance of DNA fidelity upon replication (Buer Meyer et al., 1999; Kolodner & Marsischky, 1999; Harfe & Jinks-Robertson, 2000; Jiricny et al., 2000). MMR captures errors in the newly synthesized DNA strand that are missed by the polymerase proofreading and lowers the mutation frequency by a factor of 100-1000-fold as compared to MMR deficient cells (Bhattacharyya et al., 1994; Eshleman et al., 1995). In humans, accumulation of mutations is a critical step in carcinogenesis. Loss of a single allele of one of the mismatch repair proteins causes Hereditary Non-Polyposis Colon Cancer (HNPCC), a form of cancer that accounts for 1-5% of all cases of colon cancer (Lynch & de la Chapelle, 1999; Peltomäki, 2001). HNPCC is caused by inherited mutations in MMR genes. Most HNPCC families have germline mutations in the hMSH2 (2p22-p21) and hMLH1 (3p21) genes. Mutations in the other known MMR genes hMSH6 (2p16), hMSH3 (5q11-q12), hPMSH2 (7p22), hPMSH1 (2q31), and hMLH3 (14q24) are either rare or non-existent in HNPC families (Peltomäki & Vasen, 1997; Kolodner & Marsischky, 1999; Wood et al., 2001).

Some HNPCC families fail to display mutations in known MMR genes. Therefore, we argue that these HNPCC families must harbor mutations in yet unidentified genes that are involved in MMR. In an attempt to identify such genes, we employed the yeast two-hybrid system, using the human MMR proteins hMSH2 (Rasmussen et al., 2000) or hMLH1 as bait and a fetal liver matchmaker cDNA library as prey.

1.2 DNA Mismatch Repair

**MMR in Escherichia coli**

Mechanisms and functions of mismatch correction are best understood in Escherichia coli, therefore a short introduction to MMR in E. coli is given. MMR is directed by the state of adenine methylation of GATC sequences. Since DNA adenine methylation (Dam) occurs after replication, an unmethylated newly synthesized strand is temporarily paired with a fully methylated parental strand, which provides a strand discrimination signal for MMR in
Analysis of human DNA Mismatch Repair

*E. coli* (Lahue & Modrich, 1989). Initiation of MMR occurs via mismatch recognition and binding of a MutS homodimer followed by binding of a MutL homodimer. In *E. coli* MutL serves to couple mismatch recognition with downstream MMR events. Interactions between MutL, MutS, and ATP are believed to result in translocation of the MutS-MutL complex away from the mispair, leading to the activation of the MutH endonuclease (Allen *et al*., 1997; Hall & Matson, 1999). After activation, MutH introduces a nick in the nascent strand of the nearest hemi-methylated GATC sequences. MutL helps to load DNA helicase II (UvrD) at the nicked GATC site and UvrD unwinds DNA from the nick toward and past the mismatch (Hall *et al*., 1998). Removal of the error-containing DNA strand is facilitated by one of four single-stranded, DNA-specific exonucleases (RecJ, ExoI, ExoVII, ExoX) depending on the polarity of the reaction (Viswanathan & Lovett, 1998; Burdett *et al*., 2001). The resulting single-stranded gap, is stabilized by single-strand binding protein (SSB) and filled by DNA polymerase III holoenzyme. The remaining nick is closed by DNA ligase and Dam methyltransferase finishes the MMR pathway in *E. coli* by methylating the newly synthesized strand (Rasmussen *et al*., 1998).

**Mismatch recognition - in eukaryotes**

In eukaryotes, mispaired bases in DNA are recognized by the heterodimeric complexes, MSH2-MSH6 and MSH2-MSH3 (*MutS* homologs) (Acharya *et al*., 1996; Guerrette *et al*., 1998, Genschel *et al*., 1998). Analysis of mismatch binding specificities of the human hMSH2-hMSH6 and hMSH2-hMSH3 complexes showed that they were overlapping but not identical. The hMSH2-hMSH6 complex recognizes base:base mispairs and insertion/deletion mispairs of up to 8 unpaired bases. In comparison, hMSH2-hMSH3 has a high affinity for insertion/deletions of 2-8 unpaired bases, weak affinity for single-nucleotid insertion/deletion mispairs, and do not bind base:base mispairs (Drummond *et al*., 1997; Genschel *et al*., 1998). The predominant DNA-binding protein in the hMSH2-hMSH6 complex appears to be hMSH6 when binding to a mismatched oligonucleotide (Matton *et al*., 2000). Hence, the hMSH2-hMSH6 complex appears to provide the predominant mismatch-binding activity in human cells (Genschel *et al*., 1998; Marra *et al*., 1998). The hMSH3 protein is believed to compete with hMSH6 for the available hMSH2, as the interacting regions of hMSH2 with hMSH3 and hMSH6 are identical (Guerrette *et al*., 1998). This prediction is supported by two independent findings: extracts prepared from HCT15 cells, which lack hMSH6, contain approximately three-fold higher levels of hMSH2-hMSH3 complex compared to MMR proficient cells (Genschel *et al*., 1998); and
cells overexpressing hMSH3 preferentially form hMSH2-hMSH3 complexes (Marra et al., 1998). Thus, hMSH3 interacts with all hMSH2 to form the hMSH2-hMSH3 complex, making cells functionally deficient in hMSH2-hMSH6 complex. Consequently, these cells lack base:base mispair correction (Drummond et al., 1997; Marra et al., 1998).

### Table 1.1 • DNA mismatch repair proteins
(Kolodner & Marsischky, 1999; Burdett et al., 2001; Wood et al., 2001)

<table>
<thead>
<tr>
<th></th>
<th><strong>E. coli</strong></th>
<th><strong>S. cerevisia</strong></th>
<th><strong>H. sapiens</strong></th>
<th><strong>Function</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MutS</em></td>
<td>Msh2-Msh6</td>
<td>hMsh2-hMsh6</td>
<td></td>
<td>Recognizes single-nucleotide and insertion/deletion mispairs.</td>
</tr>
<tr>
<td></td>
<td>Msh2-Msh3</td>
<td>hMsh2-hMsh3</td>
<td></td>
<td>Recognizes insertion/deletion mispairs.</td>
</tr>
<tr>
<td><em>MutL</em></td>
<td>Mlh1-Pms1</td>
<td>hMLH1-hPMS2</td>
<td></td>
<td>Couples mismatch recognition with downstream MMR events in <em>E. coli</em>. The function in eukaryotes is unclear.</td>
</tr>
<tr>
<td></td>
<td>Mlh1-Mlh2</td>
<td>hMLH1-hPMS1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mlh1-Mlh3</td>
<td>hMLH1-hMLH3</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>MutH</em></td>
<td>not identified</td>
<td>not identified</td>
<td></td>
<td>Endonuclease; nicks hemimethylated GATC sequences.</td>
</tr>
<tr>
<td><em>UvrD</em></td>
<td>not identified</td>
<td>not identified</td>
<td></td>
<td>Helicase; facilitates DNA unwinding</td>
</tr>
<tr>
<td><em>RecJ &amp; ExoVII</em></td>
<td>not identified</td>
<td>not identified</td>
<td></td>
<td>5’→3’ single-stranded DNA exonucleases</td>
</tr>
<tr>
<td><em>ExoI &amp; ExoX</em></td>
<td>not identified</td>
<td>not identified</td>
<td></td>
<td>3’→5’ single-stranded DNA exonucleases</td>
</tr>
<tr>
<td><em>not identified</em></td>
<td>Exo1</td>
<td>hEXO1</td>
<td></td>
<td>5’→3’ DNA exonucleases that have a preference for degrading double-stranded DNA</td>
</tr>
</tbody>
</table>

**The step after mismatch recognition - in eukaryotes**

Except for the initial mismatch recognition step, relatively little is known about the MMR mechanism in humans. However, human MutL homologs have been identified (table 1.1 & figure 1.1). The human MutL complexes consist of three different heterodimers: hMLH1-hPMS2, hMLH1-hPMS1 and hMLH1-hMLH3.

A glutathione-S-transferase (GST) fusion protein assay has shown that the interactions between hMLH1 and hPMS2 (Guerrette et al., 1999), hMLH1 and hPMS1 (Kondo et al., 2001), and hMLH1 and hMSH3 (Lipkin et al., 2000) all are mediated through the same C-terminal region of hMLH1 (Kondo et al., 2001). This fact could imply that hPMS2, hPMS1 and hMSH3 are competing for hMLH1.
To date, the exact biochemical roles of hMLH1-hPMS1 and hMLH1-hMSH3 complexes have not been determined. Only hMLH1-hPMS2 has been shown to be involved in MMR (Li & Modrich, 1995; Nicolaides et al., 1995), although, hMLH1 seems to have greater affinity for hPMS1 than for hPMS2, when measured in the yeast two-hybrid assay (Räschele et al., 1999) and hPMS1 has been shown to be mutated in one HNPCC family (Nicolaides et al., 1994).

Overproduction of an hMLH3 N-terminal deletion protein is associated with a microsatellite instability phenotype (Lipkin et al., 2000). Furthermore, results obtained in *S. cerevisiae* imply that the Mlh1-Mlh3 heterodimer repairs insertion/deletion mispairs, most likely in cooperation with the Msh2-Msh3 heterodimer (Flores-Rozas & Kolodner, 1998). These results suggest that the hMLH1-hMLH3 complex can substitute for the hMLH1-hPMS2 complex in the repair of insertion/deletion loops recognized by the hMSH2-hMSH3 complex (figure 1.1).

**Figure 1.1: Protein complexes involved in MMR and their diverse functions.** Base:base mispairs are only recognized by hMSH2-hMSH6/hMLH1-hPMS2 complexes. Whereas insertion/deletion loops are recognized by hMSH2-hMSH6/hMLH1-hPMS2 and hMSH2-hMSH3/hMLH1-hPMS2. Studies in yeast suggest that hMSH2-hMSH3/ hMLH1-hMLH3 recognize insertion/deletion loops but this has not yet been proven in human, therefore the question mark.

As in *E. coli*, the human MutL homologs have been shown to interact with the mismatch recognition complex; hMSH2, hMSH6, hMLH1, and either hPMS2 or hPMS1 (but not both together) have been co-precipitated from HeLa nuclear extracts in the absence of added ATP.
Analysis of human DNA Mismatch Repair

(Matton et al., 2000). The exact function of the MLH heterodimers as well as the signal for strand discrimination is not clearly defined in eukaryotes. However, it is known that strand-specific mismatch repair in human cells can be directed by a single strand nick in the DNA helix (Holmes et al., 1990; Thomas et al., 1991).

It has been demonstrated that PCNA (Proliferating Cell Nuclear Antigen)\(^1\) is required for initiation of repair as well as for resynthesis in MMR (Umar et al. 1996; Gu et al., 1998). Studies (two-hybrid screens) in yeast have shown that Mlh1 interacts with the replication accessory factor PCNA, suggesting that Mlh1 heterodimers serve as an interface between Msh heterodimers (mismatch recognition) and DNA replication components (Umar et al., 1996). The involvement of PCNA in MMR initiation has been supported by observations, which demonstrated that hMSH2, hMLH1, hPMS2, and PCNA can be co-immunoprecipitated from HeLa nuclear extracts in the presence of double-stranded circular DNA, Mg\(^{2+}\) and ATP. Furthermore, PCNA could not be precipitated from either hMSH2-defective LoVo or hMLH1-defective H6 cells or if ATP was eliminated from the immunoprecipitation step suggesting that formation of the complex requires functional hMSH2 and hMLH1 proteins (Gu et al., 1998). Other research has suggested that PCNA plays an important role in steps proceeding mismatch recognition. Bowers et al. (2001) demonstrated that following mismatch recognition, PCNA could disrupt ternary complexes in yeast composed of Msh2-Msh6, Mlh1-Pms1 and linear mismatched substrate (Bowers et al., 2001).

Genetic analyses in yeast also indicate that mutations in PCNA can create mutator phenotypes (increase frameshifts in simple sequence repeats) consistent with disrupted MMR, possibly as the result of defects in strand discrimination (Johnson et al., 1996; Kokoska et al., 1999; Chen et al., 1999). Recent work in yeast has suggested that PCNA-Msh2-Msh6 interactions play a key role in facilitating specific binding of Msh2-Msh6 to mispairs and/or that the specific activity of Msh2-Msh6 is increased by the interaction with PCNA (Flores-Rozas et al., 2000). The interaction between PCNA and Msh2-Msh6 seems to be mediated by a specific PCNA-binding site present in Msh6 (Flores-Rozas et al., 2000).

Therefore, PCNA seems to be implicated in steps in all phases of mismatch recognition. PCNA interacts with a number of DNA factors, including DNA polymerases (Pol \(\delta\), Pol \(\epsilon\)),

\(^1\) Proliferating cell nuclear antigen (PCNA) is a replication accessory factor encoded by the essential gene POL30 in Saccharomyces cerevisiae. It is a homotrimeric ring-shaped protein that serves as an accessory factor for DNA polymerase \(\delta\) (Pol \(\delta\)) and DNA polymerase \(\epsilon\) (Pol \(\epsilon\)). DNA-bound PCNA forms a sliding clamp that tethers Pol \(\delta\) and Pol \(\epsilon\) to template DNA and thus promotes processive DNA synthesis (Chen et al., 1999).
DNA endonucleases (FEN1, XPG), DNA ligases (Ligase 1), and methyltransferases (DNA-(cytosine-5) methyltransferase), indicating that it may have multiple roles in DNA repair (Gary et al., 1999). However, it is still not clear how PCNA is involved in the MMR pathway.

**Excision**

One of the interacting proteins we found in the two-hybrid screening with hMSH2 as a bait was the human exonuclease 1 (hEXO1) (Rasmussen et al., 2000). Therefore, the excision step in MMR will be described in greater detail in the next section.

**1.3 The Human Exonuclease 1**

The human exonuclease 1 (hEXO1) belongs to a family of nucleases with structure-specific nuclease activity that is conserved from phage to human (Lieber, 1997; Ceska & Sayers, 1998). The strong sequence homologies are limited to two discrete regions, designated to the N (N-terminal) and I (internal) regions that comprise the catalytic domain responsible for exo- and endonuclease activities. Based on sequence comparisons, positioning of the N-terminal and internal regions, and their biochemical and biological functions, this nuclease family can be divided into three subfamilies (Lee & Wilson III, 1999).

One subfamily includes human XPG (xeroderma pigmentosum group G) and its *S. cerevisiae* (Rad2) and *S. pombe* (Rad13) homologs. Another consists of FEN1 (flap endonuclease-1), *S. cerevisiae* (Rad27) and *S. pombe* (Rad2) homologs. The third subfamily includes human exonuclease 1 (hEXO1), *S. cerevisiae* (Exo1) and *S. pombe* (Exo1).

**The first subfamily – XPG**

The XPG proteins possess both endonuclease and 5’→ 3’ exonuclease activities and are known to operate in Nucleotide Excision Repair (NER) (Habraken et al., 1994). Two NER subpathways exist; repair of damage that blocks elongating RNA polymerases, transcription coupled repair (TCR), and repair of lesions over the entire genome, referred to as global genome repair (GGR). In both the TCR and GGR pathways the XPG and ERCC1-XPF proteins are responsible for cleaving 3’ and 5’ respectively of the damaged strand which occurs after the damage is detected and a region around the damage site has been opened. Cleavages with XPG and ERCC1-XPF result in the removal of 24-32 nucleotides containing the lesion. The resulting gap is filled in by the combined action of DNA polymerase δ or ε,
PCNA, single-strand binding protein (RPA) and ligase. In mammalian cells, at least 25 polypeptides are required for the NER process (de Laat et al., 1999; Hoeijmakers, 2001). Mutations in the XPG gene have been found to lead to the human disorder xeroderma pigmentosum, characterized by a hypersensitivity to sunlight and an increased likelihood of developing skin cancer (van Steeg & Kraemer, 1999).

The second subfamily – FEN1

The FEN1-like proteins exhibit a 5'→3' flap-specific exo/endonuclease that plays an important role in multiple DNA metabolic processes. The flap-specific endonuclease activity is required for branched DNA structures produced by DNA polymerase strand displacement during lagging strand DNA synthesis, or as intermediates during DNA recombination (Harrington & Lieber, 1994a, 1994b). The 5'nuclease function of FEN1 is responsible for the excision of Okazaki fragments, (FEN1 excises the final 5'-terminal ribonucleotide at the RNA-DNA junction) (Bambara et al., 1997) and for long-patch base excision repair (Kim et al., 1998).

In S. cerevisiae, a deletion of the FEN1 homolog Rad27 results in sensitivity to the alkylating agent methylmethane sulfonate, modest sensitivity to ultraviolet light, increased spontaneous chromosome instability, and temperature sensitivity (Johnson et al., 1995; Reagan et al., 1995; Vallen & Cross, 1995). These phenotypes are consistent with participation of FEN1 in both DNA replication and repair.

The third subfamily – EXO1

The human exonuclease 1 (hEXO1) gene consists of 14 exons, and is transcribed to yield a 3-kb mRNA. The hEXO1 gene is located on chromosome 1 (1q42-43) (Schmutte et al., 1998; Tishkoff et al., 1998; Wilson III et al., 1998). There exist two forms of exonuclease 1, hEXO1a/HEX1 and hEXO1b (Schmutte et al., 1998; Tishkoff et al., 1998; Wilson III et al., 1998). The hEXO1a/HEX1 protein is 803 amino acids long, whereas the hEXO1b protein is 846 amino acids. This difference in length arises from alternatively spliced RNA transcripts involving only C-terminal content outside of the nuclease domain (Schmutte et al., 1998; Tishkoff et al., 1998).

The ratio between hEXO1a/HEX1 and hEXO1b ESTs, found during database searches, was 1:6, which suggests that hEXO1b is the more abundant species (Tishkoff et al., 1998). The DNA sequences of the hEXO1 two-hybrid clones we isolated showed homology to the C-terminal region of hEXO1b (Rasmussen et al., 2000).
It should be noted that hEXO1 will be used as nomenclature for human exonuclease 1 when hEXO1a/HEX1 and hEXO1b are not compared.

**Interactions with hEXO1**

The *S. cerevisiae* and human exonucleases 1 (Exo1 & hEXO1) interact with mismatch repair protein Msh2/hMSH2 as demonstrated by the two-hybrid system and immunocoprecipitation, suggesting that hEXO1 may play a role in MMR (Tishkoff *et al.*, 1997; Schmutte *et al.*, 1998; Tishkoff *et al.*, 1998; Rasmussen *et al.*, 2000). The interaction is mediated through C-terminal domains for both the *S. cerevisiae* and human exonucleases 1 (Tishkoff *et al.*, 1997; Rasmussen *et al.*, 2000). We have shown that hMSH2 interacts with both forms of human exonuclease 1, suggesting that the interacting domain is located between exons 8 and 13 (~amino acids 384-870) (Rasmussen *et al.*, 2000). Recently, Schmutte *et al.* (2001) suggested that the carboxy-terminal amino acids 603-846 in hEXO1 are the specific interacting region with hMSH2 (Schmutte *et al.*, 2001).

Like hMSH2, the hMLH1 protein interacts with hEXO1 through the C-terminal domain of the hEXO1, as demonstrated by the two-hybrid system and via *in vitro* pull-down assay (Jäger *et al.*, 2001; Schmutte *et al.*, 2001). Two other MMR proteins, hMSH6 and hPMS2, have been shown not to interact with hEXO1 in the two-hybrid system (Rasmussen *et al.*, 2000; Jäger *et al.*, 2001). However, the N-terminal (amino acids 129-390) of hEXO1 seems to interact with the N-terminal of hMSH3 (Schmutte *et al.*, 2001). This result indicates that hEXO1 and hMSH3 proteins interact with same region of hMSH2. The interacting region of hMSH2 with hEXO1 are amino acids 261-671 of hMSH2 (261-669aa are essential for interaction and 261-600aa stabilize the interaction) and the interaction region between hMSH2 and hMSH3 are amino acids 378-625 of hMSH2. (Schmutte *et al.*, 2001).

**Expression/localization of hEXO1**

We have shown that hMSH2 and hEXO1 are co-expressed at high levels in fetal liver, adult testis and thymus. hEXO1 transcripts are expressed in the fetal tissue of liver, spleen and kidney but not in adult liver, spleen, and kidney tissue, suggesting a role for hEXO1 in development of these tissues. Northern Blot analysis revealed that hEXO1 is highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas but not in the corresponding non-neoplastic tissue (Rasmussen *et al.*, 2000).
Sub-cellular localization of hEXO1 was restricted to the nucleus of murine NIH3T3 cells transfected with YFP-hEXO1b plasmids (Jäger et al., 2001), indicating that hEXO1 has no function in the mitochondria.

**Mutator gene**

Disruption of EXO1 increased the mutation rate in *S. cerevisiae* cells, indicating a role for EXO1 in DNA repair (Tishkoff et al., 1997; Tran et al., 1999). However, *S. cerevisiae exo1* mutants show a moderately lower mutator phenotype than that caused by mutations in MSH2 (Tishkoff et al., 1997; Tran et al., 1999). This suggests that there are additional exonucleases involved in MMR in *S. cerevisiae*.

FEN1 may be another exonuclease involved in MMR. It has been observed that *rad27* and *exo1* mutations are lethal in combination with one another, unlike the corresponding single mutants (Tishkoff et al., 1997; Gary et al., 1999). Overexpressions of Exo1 or hEXO1 proteins suppress both the temperature sensitive and the spontaneous mutator phenotype of *rad27* mutants. (Tishkoff et al., 1997; Qiu et al., 1999).

hEXO1 remains unverified as a colon cancer predisposition gene. However, Wu et al. (2001) have detected germline hEXO1 variants in HNPCC families. All hEXO1 variants were identified in families in which no germline hMSH2, hMLH1, and hMSH6 mutations had been found. One hEXO1 variant found in a family with HNPCC resulted in a truncated protein that was 106 amino acids shorter than the wild-type gene product detected (Wu et al., 2001). Because hEXO1 interacts with hMSH2 through its C-terminal, the shorter gene product could have functional consequences for MMR.

**DNA exonuclease activity**

All of the Exo1 homologous (*S. cerevisiae, S. pombe*, and human) posses a 5’→3’ double-stranded DNA exonuclease activity (Szankasi & Smith, 1995; Tishkoff et al., 1997; Qiu et al., 1999). The hEXO1 and Exo1 proteins act both on single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) substrate but have preference for dsDNA. The human hEXO1 does not discriminate between RNA and DNA substrates *in vitro* (Qiu et al., 1999). This suggests that the lethality of the *exo1*rad27 double mutants are caused by inability to remove primer RNAs during lagging strand DNA synthesis. As mentioned earlier, the function of removing RNA primers of the lagging DNA strand is known to be performed by the FEN1/Rad27 nuclease in eukaryotes (Bambara et al., 1997). However, the efficiency of
hEXO1 to remove RNAs indicates that it may also be involved in removal of RNA primers during lagging strand DNA synthesis.

A detailed analysis of the nuclease domain (HEX1-N2) of hEXO1 showed that HEX1-N2 has 5’ flap and pseudo flap-like structure-specific endonuclease activities analogous to FEN1 (Lee & Wilson, 1999). However, neither HEX1-N2 nor FEN1 were active on 3’ flap structures (Lee & Wilson, 1999). An analysis revealed that HEX1-N2 degrades blunt duplex substrate at a rate roughly 7-fold faster than ssDNA, but the analysis did not show specific endonuclease activity at 10-base pair bubble-like structures, G:T mismatches, or U:G mismatches (Lee & Wilson, 1999).

Both hEXO1 and FEN1 appear to be directed by their C-terminal domains. As mentioned earlier hEXO1 associates with hMSH2 and hMLH1 through a C-terminal interaction, a physical association that may direct hEXO1 to sites of mispaired nucleotides. The C-terminus of FEN1 has been identified as the PCNA binding sequence (Gary et al., 1997; Warbrick et al., 1997). PCNA stimulates FEN1 nuclease activity and PCNA stabilizes FEN1 on a DNA substrate (Tom et al., 2000). These properties suggest that FEN1 and PCNA interact during the course of DNA replication, DNA repair, or both.

Gomes & Burgers (2000) have found that protein-protein contacts between FEN1 and PCNA differ depending on whether the proteins are in complex with DNA or not (Gomes & Burgers, 2000). In the absence of DNA, FEN1 interacts with PCNA mainly through the InterDomain Connector Loop (IDCL). However, when PCNA encircles the DNA, the C-terminal domain of PCNA rather than its IDCL is important for binding FEN1 (Gomes & Burgers, 2000). This interesting bimodal interaction between PCNA and FEN1 may represent a model for several other proteins with the PCNA-binding motif (QX[ILM]XXF[Y]X). The PCNA-binding motif has been identified in a large number of proteins involved in DNA metabolic processes, such as DNA methylation (MCMT, cytosine-5-methyltransferase), NER (XPG endonuclease), base excision repair (hMYH glycosylase), MMR (hMSH3 and hMSH6) and cell cycle control (p21) (Warbrick, 1998; Tsurimoto, 1999; Boldogh et al., 2001; Kleczkowska et al., 2001).

**3’ → 5’ exonuclease**

Strand-specific mismatch repair in HeLa cell extracts can be directed by a single strand nick in a heteroduplex and the mismatch correction is independent of whether the nick occurs 3’ or 5’ to the mispair (Fang & Modrich, 1993). This suggests that the human MMR possesses a
bidirectional capability. Polymerase δ and ε are responsible for lagging and leading DNA strand replication. The 3′ → 5′ proofreading exonuclease activity of both polymerase δ and ε has been proposed to be functionally redundant with Exo1 in *S. cerevisiae* (Tran *et al.*, 1999). Yeast strains harboring inactivation of *exo1* and the 3′ → 5′ proofreading exonuclease function of polymerase ε led to an increase in the mutation rate of up to 55-fold over that found for either single mutant (Tran *et al.*, 1999). Furthermore, yeast strains with mutation in the 3′ → 5′ proofreading exonuclease of DNA polymerase δ in combination with a deletion of *exo1* or *msh2* are lethal. These findings indicate that 5′ → 3′ activity of Exo1 and the 3′ → 5′ exonuclease activity of DNA polymerase δ participate in a bi-directional MMR (Tran *et al.*, 1999).

### 1.4 Methods

We employed the yeast two-hybrid system in order to identify new MMR proteins. The yeast two-hybrid system is a genetic assay designed to detect protein-protein interactions *in vivo* and has been used with great success to identify new partners in multi-protein complexes (Fields and Song, 1989; Chien *et al.*, 1991).

**Principles of the two-hybrid system**

The yeast two-hybrid system relies on the structure of particular transcription factors that have two physically separable domains. One domain (the Binding Domain) interacts with the DNA at an upstream activation site. The second domain (the Activation Domain) binds to the basal transcription apparatus and activates transcription. The MATCHMAKER GAL4 two-hybrid system (CLONTECH) utilizes the yeast GAL4 transcriptional activator which is required for expression of genes encoding proteins involved in galactose metabolism. In the two-hybrid system, the two GAL4 domains are separately fused to proteins, and the recombinant hybrid proteins are expressed in yeast. If the two hybrid proteins interact, the two GAL4 domains (BD and AD) will be in close proximity and will be able to activate transcription of reporter genes (e.i. *HIS* and *lacZ*) (Bai & Elledge, 1997).
**Figure 1.2. The principle of the two-hybrid system.** The bait is cloned into the DNA-BD vector where it is expressed as a fusion to amino acids 1–147 of the yeast GAL4 protein. A second gene or cDNA library is cloned into the AD vector, where it is expressed as a fusion to amino acids 768–881 of the yeast GAL4 protein. When the fusion proteins interact, the DNA-BD and AD domains are brought into close proximity and can activate transcription of reporter genes. The DNA-BD target the transcription factor to a specific promoter sequence UAS (upstream activation sequence) whereas the DNA-AD domain facilitate assembly of the transcription complex allowing the initiation of transcription (Bai & Elledge, 1997).

The BD-plasmids GAL4-hMSH2 (pLJR105) or GAL4-hMLH1 (pACJ14) were transformed into *S. cerevisiae* strain Y190 as described in Rasmussen *et al.* (2000). The human fetal liver matchmaker cDNA library (CLONTECH, #HL4029AH) was sequentially transformed into Y190/pLJR105 or Y190/pACJ14.

<table>
<thead>
<tr>
<th>Table 1.2 • Summary of Two-hybrid screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of independent clones in human fetal liver cDNA library</td>
</tr>
<tr>
<td>Average insert size</td>
</tr>
<tr>
<td>Amount of library plasmids used</td>
</tr>
<tr>
<td>Cotransformation efficiency</td>
</tr>
<tr>
<td>Clones screened</td>
</tr>
<tr>
<td>Positive colonies</td>
</tr>
</tbody>
</table>

*Libraries with at least 1x10^6 independent clones are representative of the genomic DNA or mRNA population complexity (protocol #PT3061-1; www.clontech.com).
Interactors were selected on synthetic dextrose minimal medium (SD) lacking tryptophan (to maintain the GAL4 binding domain plasmids), leucine (to maintain the GAL4 activation domain plasmids), and histidine (to identify peptides capable of assembling a functional GAL4 transcription factor), and supplemented with 30 mM 3-amino-1, 2, 4-triazole (3-AT). The plates were incubated at 30°C for 7-10 days and approximately 700 positive clones (minimum size \(\sim 1\) mm) for each screen were picked from SD/-TRP/-LEU/-HIS + 3-AT plates and screened for \(\beta\)-galactosidase activity to verify positive interactions (for more method details see Rasmussen et al., 2000). The total number of positive interactions after \(\beta\)-galactosidase screening was therefore only \(\sim 30\) positive clones per screen (table 1.2).

1.5 Results & Discussion

Library screenings with BD-hMSH2 and BD-hMLH1 were done twice for each MMR protein. The results presented in table 1.3A and 1.3B are a summary of four independent screens. A hMSH2-hMSH6 binding domain vector was also constructed by inserting the \(hMSH2\) coding sequence into the \(SalI\) site of the pBridge binding domain vector (CLONTECH). The human \(hMSH6\) coding sequence was inserted into the \(NotI\) site. The \(hMSH6\) protein was in this way conditionally expressed from the \(P_{Met25}\) promoter. Because hMSH2 binds to a mismatch DNA sequence in complex with hMSH6, one could expect hMSH6 to work as a bridge protein that stabilizes a weak interaction between hMSH2 and another MMR protein, or as a modifier of hMSH2 or another MMR protein. However, a library screen using this construct gave no positive interactions.
Table 1.3A • Result of yeast two-hybrid screen

<table>
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<tr>
<th>hMSH2 as bait</th>
<th>Number of clones</th>
</tr>
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<tr>
<td>Human exonuclease 1 (hEXO1)</td>
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<tr>
<td>Human G/T mismatch binding protein (hMSH6)</td>
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<td>Human alpha1-antichymotrypsin</td>
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<td>Human alpha-1-microglobin</td>
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<td>Human alphaglobin</td>
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</tr>
<tr>
<td>Human Alu RNA binding protein</td>
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</tr>
<tr>
<td>Human colony stimulating factor 1 (CSF-1, FMS)</td>
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</tr>
<tr>
<td>Human complement component 3 (C3)</td>
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<tr>
<td>Human cofillin (F-actin depolymerizing protein)</td>
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<td>Human ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5)</td>
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<tr>
<td>Human gammaglobin</td>
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<tr>
<td>Human hemoglobin gamma G (HBG2)</td>
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<td>Human homeobox protein HOX-B6 (Hox-2.2)</td>
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<td>Human HU-K4</td>
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<td>Human mitochondrial COX3 (cytochrome C oxidase subunit III)</td>
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Table 1.3B • Result of yeast two-hybrid screen

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<tr>
<th>Protein Description</th>
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<td>Human alpha-1-antichymotrypsin</td>
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<tr>
<td>Human alpha-2-HS-glycoprotein (AHSG)</td>
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<td>Human homeobox protein HOX-B6 (Hox-2.2)</td>
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</table>

Two-hybrid screening with hMSH2 as a bait

Our screens identified 77 clones as interactors. Sequence analysis showed that the majority of the clones contained unidentified human cDNAs and extracellular proteins such as gammaglobin, fibrinogen and α-antichymotrypsin. Furthermore, we isolated two clones containing hMSH6, which is known to form a complex with hMSH2 and seven clones containing hEXO1 (Rasmussen et al., 2000). When full-length hEXO1 was fused to GAL4 activation domain in pACT2, we were unable to detect protein-protein interaction with any of our MMR (BD) constructs in the two-hybrid system. However, when full-length hEXO1 was
fused to the GAL4 binding domain in pAS2, we could detect interactions with MMR (AD) proteins in the two-hybrid assay. This could explain why we failed to isolate any full-length cDNAs of hEXO1 in our two-hybrid screen (Rasmussen et al., 2000).

Three clones containing COX1, COX3 and 16S ribosomal RNA were shown to form a complex with hMSH2. However, these interactions are probably artifacts as COX1 and COX3 are subunits of cytochrome c oxidase (Complex III) of the electron transport chain. It has not yet been determined if MMR is active in human mitochondria. In yeast a MutS homolog (Msh1) of the MMR pathway has been identified in mitochondria (Reenan & Kolodner, 1992). Our two-hybrid screenings with hMSH2 and hMLH1 did not indicate the presence of mitochondria specific MMR proteins, although we can not exclude that they can be found among the unidentified human cDNAs.

**Two-hybrid screening with hMLH1 as a bait**

The screening with hMLH1 was performed as described previously for hMSH2 (Rasmussen et al., 2000). The GAL4 DNA-BD was fused to full-length hMLH1 and verified by sequencing. Again the sequence analysis of the hMLH1 interactors showed that the majority of the clones contained unidentified human cDNAs and proteins such as fibrinogen and α-antichymotrypsin (table 1.3B). Furthermore, we isolated three clones containing hPMS1, which is known to form a complex with hMLH1. To find hPMS1 as the interacting partner with hMLH1 at first surprised us, as hPMS2 has been shown to be approximately 10-fold more abundant in HeLa nuclear extract than hPMS1 (Räschle et al., 1999). However, the same authors demonstrated that the affinity of hMLH1 for hPMS1, measured in the yeast two-hybrid system, was greater than for hPMS2 (Räschle et al., 1999). Therefore, a greater affinity for hPMS1 could explain why we only isolated this gene in our two-hybrid screen.

Given that hPMS2 might compete with hPMS1 for the available hMLH1, we decided to determine the relative amounts of hPMS1 in human tissues. We used a human RNA master blot (figure 1.3) and a human multiple tissue RNA master blot (figure 1.4) from CLONTECH to characterize the expression pattern of hPMS1 (as described in Rasmussen et al., 2000). We found that hPMS1 is predominantly expressed in fetal liver and adult liver, but also in pancreas, kidney, testis and appendix (figure 1.3). The hMLH1-hPMS1 complex could therefore predominate in fetal liver and account for why we isolate hPMS1 and not hPMS2 when using the two-hybrid screen. It is tempting to speculate that hPMS1 is tissue specific and that the hMLH1-hPMS1 complex plays an important role in DNA repair in liver.
Analysis of human DNA Mismatch Repair

Figure 1.3. Expression profile of hPMS1. The RNA master blot (CLONTECH #7770-1) was hybridized with hPMS1 or β-actin control probes.

Figure 1.4. Hybridization of multiple tissue Northern blot with hPMS1 probe. Each lane contains following human tissues. Lane 1: brain. Lane 2: heart. Lane 3: skeletal muscle. Lane 4: colon. Lane 5: thymus. Lane 6: spleen. Lane 7: kidney. Lane 8: liver. Lane 9: small intestine. Lane 10: placenta. Lane 11: lung. Lane 12: peripheral blood leukocyte (CLONTECH #7780-1).
**Importin α**

In the hMSH2 and the hMLH1 two-hybrid screens we found interaction with an importin α homolog and importin-α, respectively. Importin-α is also known as importin 58/Srp1/Rch1/Kap60/karyopherin α (Jans *et al.*, 1998).

All passive and active transport into and out of the nucleus occurs through the nuclear pore complexes (NPCs) present in the nuclear envelope. Molecules smaller than approximately 60 kDa may passively diffuse through the NPCs into the nucleus, but import of larger molecules requires specific transport signals (Görlich, 1998). Targeting of many proteins to the nucleus is determined by the presence of a Nuclear Localization Signal (NLS), a short sequence containing one or two clusters of basic amino acid residues. (Köhler *et al.*, 1999). An NLS-bearing protein, often termed the "cargo", is delivered to the nucleus by association with a heterodimer, formed by importin α and importin β (also called karyopherin-α and -β). Importin α recognizes the NLS, while importin β is responsible for docking to the NPC and translocation through the pore (Görlich, 1998; Jans *et al.*, 1998; 2000). Translocation into the nucleus is terminated at the nuclear side of the NPC by disassembly of the trimeric NLS-protein/importin α/β complex. Association of importin β with the protein Ran, a Ras-related GTPase may trigger the dissociation (Jans *et al.*, 2000).

Higher eukaryotes possess more than one form of importin-α (at least 6 forms for humans) (Köhler *et al.*, 1999). The larger number of distinct importin α isoforms in higher mammals implies that there is specialization in their cellular role, and that different isoforms could bind unique target proteins. Consistent with this is the fact that neither any single human importin-α nor any triple combinations of three analyzed importin-α homologues were able to complement a *S. cerevisiae* SRP1 mutant (*S. cerevisiae* has only one gene for importin-α) (Nachury *et al.*, 1998).

The hMSH2 human mismatch repair protein has a weak nuclear signal (Boulikas, 1997). A protein processing a single weak NLS is more likely to be retained in the cytoplasm after a mutation at the NLS peptide than a protein with two or more NLSs (Boulikas, 1997). Mutations at the weak NLS of the hMSH2 gene, or dysfunction of translocation proteins like importin-α may result in cytoplasmic retention that again could lead to dysfunction of the MMR pathway. Hampered translocation of the MMR proteins could thus be a possible explanation why so many HNPPCC families fail to display mutations in the known MMR genes. We hope to do more research regarding translocation of MMR proteins in the future.
Potential NLSs for MMR proteins are listed in table 1.4 by using the PSORT software programs (Nakai et al., 1992; http://psort.nibb.ac.jp/).

<table>
<thead>
<tr>
<th>MMR protein</th>
<th>Potential NLSs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSH2</td>
<td>EKHEGKHQKLL at 422</td>
<td>(Boulikas et al., 1997)</td>
</tr>
<tr>
<td>hMSH3</td>
<td>RRKP at 3, RRKK at 76, RKKR at 77, KKRKP at 78, KKRK at 704, KRKR at 1091, PVKKKVK at 87, PLIKKRK at 701, RRKKRPLENDGPVKKKV at 76, RKKRPLENDGPVKKKV at 77, KKRPLENDGPVKKKV at 78, KRPLENDGPVKKKV at 79</td>
<td>(PSORT; Nakai et al., 1992)</td>
</tr>
<tr>
<td>hMSH6</td>
<td>KKRR at 246, RRKR at 298, KRKR at 299, HRRR at 382, RRRP at 383, RKRKRMVTGNGSLKRKS at 298, KRKRMVTGNGSLKRKSS at 299, RKRMVTGNGSLKRKSSR at 300, KRMVTGNGSLKRRKSSR at 301</td>
<td>(PSORT; Nakai et al., 1992)</td>
</tr>
<tr>
<td>hMLH1</td>
<td>PRKR at 469, RKRH at 470, KRHR at 471, PRRR at 496, PRKRRHRE at 469, PRRRIIN at 496</td>
<td>(PSORT; Nakai et al., 1992)</td>
</tr>
<tr>
<td>hPMS1</td>
<td>RPRK at 869, KRAIEQESQMSLKDGRK at 636</td>
<td>(PSORT; Nakai et al., 1992)</td>
</tr>
<tr>
<td>hPMS2</td>
<td>PNTKRFK at 574</td>
<td>(PSORT; Nakai et al., 1992)</td>
</tr>
<tr>
<td>hEXO1</td>
<td>KRPR at 418, KRKH at 775, PIKRKLI at 290</td>
<td>(PSORT; Nakai et al., 1992)</td>
</tr>
</tbody>
</table>
2. Repair of Mitochondrial DNA

2.1 Introduction

Within all mammalian cells there are two distinct genomes, one located in the nucleus (nDNA) and the other in the mitochondria (mtDNA). Although each human somatic cell has several hundred to thousand mitochondria (and 1-10 mtDNA copies per mitochondrion) the amount of the mtDNA is only roughly 1% of the total DNA in the cell due to the small size of mtDNA compared to nDNA (Bestwick, 1982; Wallace et al., 1998). The mammalian mtDNA retains only 22 tRNAs, 12S rRNA and 16S rRNA genes necessary for the mitochondrial protein synthesis as well as 13 polypeptide genes (Anderson et al., 1981). All 13 polypeptides are part of the 87 structural polypeptide subunits, all of which are components of the respiratory chain. The respiratory chain is composed of five multisubunit enzymes whose components are encoded by both the nuclear and mitochondrial genomes (Wallace, 1999).

Unlike nDNA mammalian mtDNA contains very few noncoding sequences, no introns and it is unprotected by histones. Therefore, damage to mtDNA can be expected to have greater impact on cell function than damage to nuclear DNA, as the probability of damaging coding sequences in mtDNA is much higher. Thus, it has been reported that the rate of point mutations is higher in mtDNA compared to nDNA in human tissues (Khapko et al., 1997). In addition mtDNA point mutations and mtDNA rearrangement mutations (deletions and insertions) have now been recognized to play a critical role in numerous human disorders which prove the importance of maintaining the integrity of the mitochondrial genome (Kang, 1998; Kogelnik et al., 19982; Pulkes & Hanna, 2001; Wallace, 1999).

For this reason DNA repair in mitochondria could be expected to be very efficient. However, early investigations of removal of UV-induced pyrimidine dimers in mtDNA led to the conclusion that mitochondria accumulate DNA damage because these organelles are deficient in repair activity (Clayton et al., 1974; Prakash et al., 1975). This finding, in combination with very limited interest in this research area, led to the belief that rather than repairing damage in mtDNA, cells simply destroyed the injured genomes and replaced them by replicating existing, undamaged mtDNA.

2 Variations in the human mitochondrial genomes are updated on http://www.gen.emory.edu/mitomap.html (Kogelnik et al., 1998).
(LeDoux et al., 1999). It has later been confirmed that UV-induced pyrimidine dimers are
not repaired in mitochondria (LeDoux et al., 1992; Pascucci et al., 1997), which suggest
absence of nucleotide excision repair. However, recent evidence showed that mitochondria
are indeed able to repair their genomes (Croteau et al., 1999; Marcelino & Thilly, 1999).
Unfortunately the knowledge about the repair mechanisms operating in the mitochondria is
still limited.

To gain a better understanding of the molecular processes and components responsible for
DNA repair in the human mitochondrion we have investigated the sub-cellular localization
of \(O^6\)-methylguanine-DNA methyltransferase (MGMT) (Rasmussen et al., II).

2.2 Base Excision Repair
Several observations support that mitochondria have Base Excision DNA Repair (BER)
pathways that are responsible for the removal of simple lesions in DNA (Croteau et al.,
1999; Marcelino & Thilly, 1999). Removal of a damaged base by the BER pathway is
initiated by DNA glycosylase that cleave the N-glycosylic bond between the base and the
deoxyribose-phosphate backbone (Lindahl & Wood, 1999). The resulting abasic site is
cleaved 5’ by an apurinic/apyrimidinic (AP) endonuclease that generates a 3’OH group,
which can be extended by a DNA polymerase, but not ligated before a 5’ terminal
deoxyribose-phosphate residue is removed. The removal of the abasic sugar residues is done
by a lyase. Finally, the gap is filled by DNA polymerase and the ends rejoined by DNA
ligase (Lindahl, 2000). Several distinct DNA glycosylases have been identified both in
nuclei and mitochondria in human cells (table 2.1).

**Repair of uracil**
Uracil-DNA Glycosylase (UNG or UDG) removes uracil bases in DNA. An uracil base in
DNA can occur as a result of either misincorporation of dUTP instead of dTTP into a newly
synthesized DNA, or deamination of cytosine to uracil (Slupphaug et al., 1995).
Deamination of cytosine to uracil, unless repaired before the next round of replication, will
result in a GC \(\rightarrow\) AT transition mutation (Slupphaug et al., 1995). Studies have
demonstrated the presence of both nuclear- and mitochondrial-associated UNG (Anderson &
Friedberg, 1980; Slupphaug et al., 1993; Otterlei et al., 1998). The human mitochondrial
(hUNG1) and nuclear (hUNG2) forms have identical catalytic domains, but very different N-
terminal sequences. The two forms are both generated from the *hUNG* gene using two promoters, and making use of an exon specific for the N-terminal end of the nuclear form, and alternative splicing (Nilsen *et al*., 1997; Otterlei *et al*., 1998).

**Repair of oxidative damage**

Both purine and pyrimidine residues in DNA are sensitive to reactive oxygen species (ROS) (Croteau & Bohr, 1997). The most common purine lesion is 8-hydroxyguanine also called 7,8-dihydro-8-oxoguanine (8-oxoG) (Steenken & Jovanovic, 1997; Burrows & Muller, 1998). 8-oxoG is a highly mutagenic base derivative, which base-pairs with adenine as well as cytosine, causing G → T transversion mutations (Cheng *et al*., 1992). The mitochondrial respiratory chain produces superoxide (Wallace, 1999) which can be converted to hydroxyl radicals via hydrogen peroxide (Imlay & Linn, 1988). The hydroxyl radical is the main species of active oxygen that attacks the guanine base (Kasai *et al*., 1984). Because mtDNA is subjected to a relatively high amount of oxidative damage and because the strand containing 28 of the 37 mitochondrial genes is rich in guanine (Bianchi *et al*., 2001), it seems that mitochondria would need efficient DNA repair mechanisms to remove oxidative damage from its DNA.

**Removal of 8-oxoguanine**

An early study revealed that 8-oxoG can pair with all four normal bases (Kuchino *et al*., 1987) but it was later shown that 8-oxoG perferentially pairs with C or A during *in vitro* DNA synthesis (Shibutani *et al*., 1991). In *E. coli* two DNA glycosylases, encoded by *mutM* (*Fpg*) and *mutY* genes, function to prevent mutagenesis by removing 8-oxoG paired with cytosine and adenine (Bailly *et al*., 1989; Tchou *et al*., 1991; Michaels *et al*., 1992). The human MutM homolog, hOGG1 (8-OxoGuanine DNA Glycosylase) excise 8-oxoG preferentially when it is paired with C, followed by 8-oxoG:T and 8-oxoG:G, but it has no detectable 8-oxoG:A-specific strand cleavage (Hazra *et al*., 1998). The glycosylase activity of hOGG1 is accompanied with apurinic/apyrimidinic (AP) lyase that cleaves the AP site via β-elimination (Hazra *et al*., 1998).

Human OGG1 localizes both to the nucleus and mitochondria (Takao *et al*., 1998; Nishioka *et al*., 1999). Seven alternatively spliced forms of *hOGG1* mRNAs have been identified (Nishioka *et al*., 1999). All these splice forms of hOGG1 contain a putative mitochondrial targeting signal (MTS) at the common N-terminal region. A nuclear localization signal (NLS) was only found in the C-terminal end of hOGG1-1a (Nishioka *et al*., 1999). Human
OGG1-1a, which has been found to have a weak MTS, localized predominantly to the nucleus. When the NLS is deleted, the protein is targeted to mitochondria (Nishioka et al., 1999). When a strong MTS is fused upstream to the hOGG1-1a gene it is selectively targeted to the mitochondria (Dobson et al., 2000).

Interestingly, results from mice liver have revealed that mitochondrial 8-oxoG glycosylase activity increased with age. In contrast no age-associated changes were found for nuclear 8-oxoG glycosylase activity or mtUDG activity (de Souza-Pinto et al., 2001). This result suggests that the mitochondrial OGG1 glycosylase is up-regulated during the aging process.

**Removal of adenine paired with 8-oxoguanine**

The Human MutY Homolog, hMYH has been shown to excise adenine mispaired with guanine and 8-oxoG as well as 2-hydroxyadenine paired with 8-oxoG in double-stranded oligonucleotides (Ohtsubo et al., 2000). This glycosylase has been detected in nucleus and mitochondria (Takao et al., 1998; 1999; Ohtsubo et al., 2000; Boldogh et al., 2001). Boldogh et al. (2001) found that the levels of hMYH in the nucleus was increased 3- to 4-fold during progression of the cell cycle and reached maximum levels in S phase, suggesting a cell cycle-dependent regulation of expression and/or subcellular targeting. However, there was no evidence that the cytoplasmic or mitochondrial levels of hMYH decreased as nuclear levels increased (Boldogh et al., 2001).

It has been suggested that 8-oxoG:C is more efficiently repaired in mitochondria than 8-oxo:A. Miyako et al. (2000) detected inefficient cleavage of human mtDNA by the *E. coli* 8-oxoG:C specific MutM protein but could report that human mtDNA was cleaved by the 8-oxoG:A specific MutY protein, suggesting that 8-oxoG accumulates as an 8-oxoG:A pair but not as an 8-oxoG:C pair.

**Removal of oxidized pyrimidines**

Oxidized pyrimidines, such as thymine glycol, 5-hydroxycytosine and 5,6-dihydrouracil (DHU), are excised by the hNTH1 glycosylase (a homolog of *E. coli* endonuclease III, Nth) (Ikeda et al., 1998; Takao et al., 1998; Lindahl & Wood, 1999). Thymine glycol is only slightly mutagenic but it can block progression of both DNA and RNA polymerases (Stierum et al., 1999). The DHU DNA lesion derives from cytosine by deamination. Its opposite base should be guanine, but DHU is also able to mispair with adenine during DNA replication. hNTH1 cleaves A or G opposite DHU with the same rate (Ikeda et al., 1998). The human NTH1 has, similar to hOGG1, combined glycosylase and AP lyase activity and
this enzyme is located to both nucleus and mitochondria (Tomkinson et al., 1990; Takao et al., 1998; Mol et al., 1999).

**Removal of 3-methyladenine**

The N-methylpurine-DNA glycosylase, MPG (MDG, AAG, APNG) gene is coding for a human glycosylase, which removes 3-methyladenine (3-MeA) as well as 3-methylguanine (3-MeG), 7-methylguanine (7-MeG), N6-ethenoadenine, hydroxanthine, guanine, and 8-oxoG (Pendlebury et al., 1994; Wyatt et al., 1999; Bouziane et al., 2000). The MPG activity has so far only been reported from nuclear extracts.

**Table 2.1 • Enzymes identified in repair of mitochondrial DNA in human cells**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Substrates/Activity</th>
<th>AP lyase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hUNG1</td>
<td>Uracil-DNA glycosylase</td>
<td>U</td>
<td>no</td>
</tr>
<tr>
<td>hOGG1</td>
<td>8-oxoguanine DNA glycosylase 1</td>
<td>8-oxoG opposite C</td>
<td>yes</td>
</tr>
<tr>
<td>hMYH</td>
<td>MutY homolog (E.coli)</td>
<td>A opposite 8-oxoG or G</td>
<td>no</td>
</tr>
<tr>
<td>hNTH1</td>
<td>Endonuclease three homolog 1(E.coli)</td>
<td>T-glycol</td>
<td>yes</td>
</tr>
</tbody>
</table>

**Other BER factors**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>Substrates/Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMTH1</td>
<td>MutT homolog (E.coli)</td>
<td>Hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP</td>
</tr>
<tr>
<td>hLIG3</td>
<td>DNA Ligase III</td>
<td>Ligation</td>
</tr>
<tr>
<td>hPOLG</td>
<td>Polymerase-gamma</td>
<td>Replication/repair polymerase</td>
</tr>
</tbody>
</table>

**Other BER factors**

In cells, the deoxyribonucleotide pools are also subjected to oxidative damage. dGTP can be converted to 8-oxo-dGTP and incorporated into nascent DNA strands opposite adenine. The MutT enzyme in *E. coli* hydrolyzes 8-oxo-dGTP to 8-oxo-dGMP, and thereby prevents misincorporation of the damaged base into DNA (Maki & Sekiguchi, 1992). The human MutT homolog, hMTH1 (for *mutT* homologue) is present in the cytoplasm as well as in the mitochondrial matrix (Kang et al., 1995; Nakabeppu, 2001). The human MTH1 protein produces by alternative transcription initiation and splicing, seven different mRNAs. One of these mRNA’s is imported into mitochondria when fused to green fluorescent protein, GFP (Nakabeppu, 2001). The human MTH1 protein has wide substrate specificity as it has been
reported to hydrolyze 8-oxo-dGTP, 8-oxoG, 8-oxo-dATP and 2-OH-dATP (Nakabeppu, 2001).

A major human AP endonuclease, hAPE1 (alternative titles; human apurinic endonuclease 1 (HAP1), apurinic/apyrimidinic exonuclease (APEX), apurinic/apyrimidinic endonuclease/redox effector factor (APE/REF-1)) plays a central role in BER (Evans et al. 2000). It initiates repair by hydrolyzing AP sites in DNA produced either spontaneously or after removal of bases in DNA by DNA glycosylases (i.e. UNG). Alternatively, it can act as a 3'-phosphoesterase after the AP lyase reaction of DNA glycosylases/AP lyases (i.e. hOGG1) (Izumi et al., 2000). Takao et al. (1998) examined the subcellular localization of hAPE1 and found that it was only localized to the nucleus (Takao et al., 1998). Furthermore, Prieto-Alamo & Laval (1999) found no increased hAPE1 activity in the mitochondrial fraction of Chinese hamster ovary (CHO-9) cells transfected with a plasmid expressing the human APE1 compared to a 4.5 fold activity increase in nuclear fractions. However, the APE1 homolog in S. cerevisiae, the Apn1 enzyme has been shown to localize in both nucleus and mitochondria. The Pir1 (a cell wall protein) is required for the localization of Apn1 to mitochondria (Vongsamphanh et al., 2001). Results of two different studies have also shown that APE1 is present in mitochondria of rat thyroid FRTL-5 cells and rat pleural mesothelial cells (Fung et al., 1998; Tell et al., 2001). However, the contribution of the mitochondrial AP lyase activity, to repair of abasic sites, and the processing of 3'-unsaturated sugar-phosphate generated by e.i. hOGG1 AP lyase activity, still remain to be established in human mitochondria.

Human mitochondrial DNA is replicated by polymerase γ. Polymerase γ (POLG) is a heterodimer composed of a 140-kD catalytic subunit (POLG1) and a smaller accessory subunit (POLG2) (Schmitt & Clayton, 1993). Because polymerase γ is the only known DNA polymerase in human mitochondria, it is expected to participate in DNA replication and repair in this organelle (Longley et al., 1998). The human polymerase γ has been shown to possess both 3'→5' exonuclease proofreading activity and lyase activity (Schmitt & Clayton, 1993; Longley et al., 1998). The human polymerase γ fills single nucleotide gaps and produces a substrate that can be ligated after action of uracil-DNA glycosylase and AP-endonuclease (Longley et al., 1998).
A mitochondrial DNA ligase has been identified. The human DNA ligase III gene encodes both a nuclear and a mitochondrial protein and DNA ligase III plays an essential role in the maintenance of mtDNA in mammalian cells (Lakshmipathy & Campbell, 1999; 2001).

**Mismatch repair**

The DNA mismatch repair (MMR) removes errors in the newly synthesized DNA strand that are missed by the polymerase proofreading (see Analysis of human DNA Mismatch Repair). It is not yet known if there is an active post-replication MMR system in human mitochondria but it has been indicated that hMSH6 is not involved in repair of mismatches in the mtDNA. Human hMSH6 deficient lymphoblastoid cells have a higher spontaneous mutation rate in the nuclear genome but a similar spontaneous mutation rate in mitochondrial DNA when compared to a parental mismatch repair proficient cell line (Marcelino et al., 1998; Marcelino & Thilly, 1999).

In yeast, a component of the MMR pathway (Msh1) has been identified in mitochondria (Reenan & Kolodner, 1992). The Msh1 protein is a homolog of the *E. coli* MutS mismatch binding protein (Reenan & Kolodner, 1992). Inactivation of the *MSH1* gene resulted in large scale mtDNA rearrangements suggesting that Msh1 is involved in repair of mtDNA (Reenan & Kolodner, 1992).

**Direct repair**

We have investigated the sub-cellular localization of O\(^6\)-methylguanine-DNA methyltransferase (MGMT) in human mitochondria (Rasmussen et al., II). Therefore, the MGMT protein will be described in greater detail in the next section.

**2.3 Direct Repair: O\(^6\)-Methylguanine-DNA Methyltransferase**

O\(^6\)-methylguanine (O\(^6\)-MeG) is generated endogenously by reactive cellular catabolites and S-adenosylmethionine, which normally acts as a methyl donor in the synthesis of 5-methylcytosine but occasionally causes methylation at other sites (Rossmann & Goncharova, 1998; Lindahl & Wood, 1999).

Alkylating agents produce various kinds of alkylated purine and pyrimidine bases in DNA, 3-methyladenine (3-MeA), O\(^6\)-methylguanine (O\(^6\)-MeG) and O\(^4\)-methylthymine (O\(^4\)-MeT), among other adducts (Lindahl & Sedgwick, 1988). 3-MeA has been shown to block DNA replication (Larson et al., 1985) and is excised by 3-methyladenine glycosylase (MPG). O\(^6\)-
MeG and $O^1$-MeT DNA lesions are mutagenic and carcinogenic (Loveless, 1969; Swann, 1990). However, $O^1$-MeT is formed less frequently than $O^6$-MeG. The proportion of alkylation at each site is $\sim 0.1\%$ for $O^1$-MeT and $3-6\%$ for $O^6$-MeG of total alkylation after reaction with Dimethylnitrosamine (DMN), Methylnitrosourea (MNU) or 1,2-Dimethylhydrazine (SDMH) (Singer & Dosanjh, 1990).

During DNA replication, $O^6$-MeG can pair with thymine leading to G:C $\rightarrow$ A:T transition mutations while $O^1$-MeT can pair with guanine, generating A:T $\rightarrow$ G:C transition mutations (Singer & Dosanjh, 1990; Dosanjh et al., 1991; 1993, Altshuler et al., 1996). Both prokaryotic and eukaryotic DNA alkyltransferases remove methyl groups from the $O^6$ position of the guanine. cDNAs coding for the repair protein from *E. coli* (ada, Sedgwick, 1983; *ogt*, Potter et al., 1987), *S. cerevisiae* (*MGT1*, Xiao et al., 1991), mouse (Shiota et al., 1992), rat (Rahden-Staron & Laval, 1991) and human (*MGMT*, Tano et al., 1990; Rydberg et al., 1990; Hayakawa et al., 1990) have all been cloned and characterized. Furthermore, DNA methyltransferase has been identified in 28 different species (Pegg, 2000). Although the amino acid sequences of these proteins are homologous, there are only 8 absolutely invariant residues in these sequences (Gly$^{109}$, Asn$^{137}$, Pro$^{144}$, Cys$^{145}$, His$^{146}$, Arg$^{147}$, Lys$^{165}$, and Glu$^{172}$, numbered according to the human MGMT sequence) (Xu-Welliver et al., 2000).

**MGMT function**

Human $O^6$-MethylGuanine-DNA MethylTransferase (MGMT, also known as AGT) repairs $O^6$-alkylguanine ($O^6$-MeG, $O^6$-ethylguanine & $O^6$-butylguanine) in double-stranded DNA in a single step. MGMT removes alkyl groups from the $O^6$ position of guanine to a cysteine acceptor residue in MGMT without removing the damaged base (Pegg, 2000). The cysteine residue is located at position Cys$^{145}$ in MGMT in a highly conserved sequence -PCHR- (Pegg, 2000). It has been shown that mutation of the cysteine acceptor site leads to a complete loss of MGMT activity (Harris et al., 1992; Crone & Pegg, 1993; Hazra et al., 1997; Edara et al., 1999). Once bound to the cysteine acceptor site, the alkyl group permanently inactivates MGMT. Therefore, the number of $O^6$-alkylG that can be repaired is equal to the number of active MGMT molecules – MGMT is “suicidal” in the repair of $O^6$-alkylG residues in DNA (Pegg, 1990; 2000). Even though MGMT is not specific for methyl groups, the rate of the reaction decreases as the size of the adduct increases (Pegg & Byers, 1992).

Mutational analysis of the MGMT protein showed that deletion of 7 amino acids from the N terminus (codons 1-7) or 28 amino acids from the C terminus (codons 180-207) result in
specific activity comparable to that found for the full-length MGMT (Hazra et al., 1997). Mutant MGMT with deletion of codon 1-10 or 1-19 were shown to be active in protecting cells from a methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) suggesting that they are able to repair methylated DNA in vivo (Crone et al., 1996). However, the mutant MGMT proteins were less effective compared to the wild-type MGMT and highly unstable with half-lives of 48-90 min compared to a half-live of > 720 min for wild-type MGMT. Interestingly, we found that deletion of codons 2 to 18 resulted in a complete absence of MGMT in nucleus and accumulation in cytoplasm (Rasmussen et al., II). This suggests that deletion of the first 18 codons interfere with stability and translocation to nucleus. However, our results indicate that this mutant MGMT protein provide the same level of protection to killing by MNNG as MGMT wild-type (Rasmussen et al., II). It has been found by site-directed mutagenesis that the fully conserved residues Asn\textsuperscript{137}, His\textsuperscript{146}, Arg\textsuperscript{147} and Glu\textsuperscript{172} are critical for maintaining the MGMT stability. Proteins with mutations in these residues were highly unstable with half-lives of 15-60 min (Crone et al., 1996). Furthermore, the residues Arg\textsuperscript{128} and Tyr\textsuperscript{114} seemed to be involved in DNA binding and catalytic activity (Kanugula et al., 1995; Goodtzova et al., 1998).

**Repair of O\textsuperscript{4}-methylthymine**

Although *E. coli* Ogt has been shown to repair O\textsuperscript{4}-MeT it is still not fully established if the human MGMT can repair O\textsuperscript{4}-MeT (Sassanfar et al., 1991). The rate constants for MGMT acting on O\textsuperscript{6}-MeG is much higher than O\textsuperscript{4}-MeT (O\textsuperscript{6}-MeG = 1x10\textsuperscript{9} M\textsuperscript{-1} min\textsuperscript{-1} and O\textsuperscript{4}-MeT = 1.8x10\textsuperscript{5} M\textsuperscript{-1} min\textsuperscript{-1} (Zak et al., 1994). Repair of O\textsuperscript{6}-MeG by MGMT in vivo is 1000-5000-fold greater than for O\textsuperscript{4}-MeT (Samson et al., 1997; Encell & Loeb, 2000). However, one study has shown that human MGMT expressed in *E. coli* suppresses MNNG-induced A:T \rightarrow G:C mutations indicating that MGMT repairs O\textsuperscript{4}-MeT DNA lesions (Kooistra et al., 1999). Other studies showed that expression of MGMT in a methyltransferase-deficient *E. coli* strain (lacking Ade and Ogt) failed to suppress A:T \rightarrow G:C mutations, but actually increased O\textsuperscript{4}-MeT mutations (Samson et al., 1997, Edara et al., 1999). Samson et al. (1997) suggested that MGMT binds to O\textsuperscript{4}-MeT lesions but repairs it so slowly that it interferes with the repair of this lesion by NER and consequently increases the frequency of A:T \rightarrow G:C mutations. The mammalian NER pathway has been shown to repair both O\textsuperscript{6}-MeG and O\textsuperscript{4}-MeT lesions (Bronstein et al., 1991; 1992, Huang et al., 1994, Kein et al., 1994). However, the ability to serve as substrates for NER increases with the size of adduct and is poor for methyl groups.
(Pegg & Byers, 1992). It is still unknown whether NER is the predominant pathway for removal of O\(^4\)-MeT lesions in human cells or serves as a back-up repair system for removing O\(^6\)-MeG lesions in cells with limited MGMT activity.

The methylation tolerant phenotype

Even though MGMT is widely expressed, the intracellular MGMT levels vary between different tissues and also between different individuals. The liver and spleen have the highest MGMT activities and the nervous system and mammary gland the lowest (Myrnes et al., 1983; Yarosh et al., 1985; Pegg & Byers, 1992; Karran & Bignami, 1994). Most tumor cells express high level of MGMT, while about 15-20% of human tumor cell lines are deficient in MGMT expression (Sklar et al., 1981; Tsujimura et al., 1987; Yu et al., 1999). Cells lacking MGMT activity (Mex- or Mer- cells) are more sensitive to mutagenesis and killing by alkylating agents (Karran & Bignami, 1994).

Alkylating agents were among the first cytotoxic drugs used in the treatment of cancer and are still used clinically against a number of tumors (Kleibl & Margison, 1998). A class of anti-tumor alkylating agents as the chloroethylnitrosourea (CENU) is clinically used to treat a number of tumors, including brain neoplasms, malignant melanoma, advanced lymphomas, and gastrointestinal carcinomas (Krekla et al., 1999). However, certain tumors are resistant to alkylating chemotherapeutic agents and this resistance are known to be correlated with the level of MGMT in the tumors (Pegg & Byers, 1992). Another mechanism of cellular resistance to methylating agents is an ability to ignore the presence of O\(^6\)-MeG lesions in DNA. This tolerance of DNA damage is due to the loss of MMR, even if MGMT is not expressed or expressed at low levels (Carethers et al., 1996; Karran & Hampson, 1996; Hampson et al., 1997; Umar et al., 1997; Dosch et al., 1998; Kawate et al., 1998, Humbert et al., 1999).

There is increasing evidence that tumor cells could acquire resistance to methylating drugs by loss of MMR activity. A lack of MMR results in poor ability of the cell to detect DNA damage and activate apoptosis, thus increasing the mutation rate throughout the genome (Fink et al., 1998). This implies that genotoxic and cytotoxic effects of O\(^6\)-MeG are mediated by MMR. In vitro studies have demonstrated that the mammalian hMSH2-hMSH6 complex can recognize O\(^6\)-MeG: T and O\(^6\)-MeG: C (Duckett et al., 1996; Cecotti et al., 1996; Mu et al., 1997; Christmann & Kaina, 2000). Although, the mechanisms of MMR-mediated response to base damage are not known, two models have been proposed as explanations.
1. In one model, the MMR system recognizes the mismatched bases opposite the damaged base but since there is no perfect complementary match for O\(^6\)-MeG, the polymerase will again fail to find an ideal partner and incorporate thymine or cytosine opposite O\(^6\)-MeG on the newly synthesized strand. The repeated attempts to repair the mismatch will result in a methylated template strand that is single-stranded for much of the time and a newly synthesized strand that contains persisting DNA termini. The killing effect of the lesion is expected to come during the following cycle of DNA replication, when the replication fork arrives at a discontinuity and thus generates a double-strand break that will then trigger apoptosis (Galloway et al., 1995; Karran & Hampson, 1996; Kaina et al., 1997).

2. In the second model, MMR proteins function as the sensors of DNA damage, and generate a signal capable of activating apoptosis (Modrich, 1997; Fink et al., 1998). The p53 protein functions as a transcription factor that regulates the expression of several genes involved in cell cycle control, DNA damage repair, and apoptosis. Therefore, p53 is the classic signal transduction molecule thought to respond to DNA breaks and gaps (Nelson & Kastan, 1994; Huang et al., 1996; Vogelstein et al., 2000). However, MNNG induced apoptosis seems to be p53-independent (Hickman & Samson, 1999). Moreover, Christmann & Kaina (2000) have shown that after exposure of cells to MNNG, hMSH2, hMSH6 and hPMS2 proteins are translocated from the cytoplasm into the nucleus, leading to an increased nuclear MMR protein level immediately after exposure. This translocation was independent of the p53 status of the cells and was first observed at high dosage level of the mutagen in cells expressing MGMT (>25 \(\mu\)M MNNG; 750 fmol/mg protein MGMT; Christmann & Kaina, 2000). In order to explore a possible link between p53 and MGMT we expressed the MGMT-GFP fusion protein in cell lines with different p53 status. One cell line was wild type for p53, one expressed very low levels of the p53 protein, and one cell line was completely deficient for p53 activity. We found no difference in subcellular localization of MGMT suggesting that MGMT translocation is independent of the p53 status. In all three cell lines the MGMT-GFP fusion protein was exclusively present in the nucleus (Rasmussen et al. II).

Treatment of human cells with MNNG concentrations that increased the mutation rate of nuclear genes showed no induction of mitochondrial mutations (Mita et al., 1988). Marcelino et al. (1998) observed that to induce MNNG mutations above a very high spontaneous background in mtDNA, one needed to treat MNNG-resistant (MT1) cells with an amount of MNNG (4 \(\mu\)M) that would kill all normal parental cells. Therefore, they
concluded that it is unlikely that in vivo mutations are induced in mtDNA by exogenous mutagens in normal human cells (Khrapko et al., 1997; Marcelino et al., 1998).

**Mitochondrial MGMT activity**

Two groups have reported mitochondrial MGMT activity in rat liver (Myers et al., 1988; Satoh et al., 1988). The kinetics of removal of \( O^6\)-MeG was similar in nuclear and mitochondrial DNA (Myers et al., 1988). Furthermore, they found very slow removal of \( O^6\)-butyl-2'-deoxyguanine in mtDNA but fast removal in nDNA which is consistent with the absence of NER in mitochondria (Myers et al., 1988). Satoh et al. (1988) reported the removal of \( O^6\)-ethyl-2'-deoxyguanosine (\( O^6\)-EtG) from mtDNA and nDNA in rat liver following exposure to \( N\)-ethyl-\( N\)-nitrosourea in vivo. Longer alkyl groups including ethyl- is known to be repaired by MGMT and NER (Pegg, 2000), indicating, that \( O^6\)-EtG is removed by an alkyltransferase mechanism in the mitochondria. Additionally, \( N\)-methyl-\( N\)-nitrosourea (MNU) induced mutations were repaired in mitochondria from Chinese hamster ovary (CHO) cells (LeDoux et al., 1992).

These results obtained in rodents suggest that an alkyltransferase mechanism is operating within the mitochondrion in mammalian species. However, extrapolation of results obtained with rodents to human cells may not be straightforward. Even MGMTs from mammalian species have different substrate specificity. For example rat MGMT repairs \( O^6\)-EtG much better than human MGMT (Liem et al., 1994). Mitochondrial MGMT activity has not yet been reported from human cells and the intracellular localization seems ambiguous.

**MGMTs intracellular localization**

Most immunostaining data have shown that MGMT is a nuclear protein (Ayi et al., 1992, Lim & Li, 1996; Belanich et al., 1996). However, there are reports on cytosolic appearance indicating that MGMT could be present in nucleus, cytoplasm and mitochondria (Ayi et al., 1992; Ishibashi et al., 1994a,b; Belanich et al., 1996). To access the actual localization of the MGMT protein we studied exogenous MGMT-GFP expressed as fusion protein in a human breast epithelial cell line (MCF12A) and detected the subcellular localization by fluorescence microscopy. We found that MGMT localized mainly in the nucleus. We also observed a weak cytoplasmic staining but no mitochondrial localization. Our results therefore suggest that MGMT is not present in mitochondria of human cells (Rasmussen et al., II). In order to test whether nuclear MGMT was recruited to mitochondria upon DNA damage of mtDNA by alkylating agents we treated cells expressing MGMT-GFP with
MNNG. The MGMT-GFP fusion protein was detected in nucleus but not in mitochondria indicating that nuclear MGMT is not recruited to mitochondria after DNA damage by MNNG (Rasmussen et al., II). This suggests that the alkyltransferase mechanism which seems to be operate within the mitochondrion in mammalian species is not MGMT in human breast epithelial cells.
3. Mitochondrial Dysfunction versus Genetic Stability of the Nuclear DNA

3.1 Introduction

Mitochondrial diseases are severely debilitating and characteristically complex in nature. They can affect any organ in the body at any age. Usually the mitochondrial diseases are inherited through the mother but they can also be sporadic or induced by the environment. Mitochondrial dysfunction is found in diseases as diverse as cancer, infertility, diabetes, heart diseases, blindness, deafness, kidney disease, liver disease, stroke, and migraine. Mitochondrial dysfunction is also involved in aging and neurodegenerative diseases such as Parkinson and Alzheimer dementia (Wallace, 1999; DiMauro & Schon, 2001). To date, there is no cure for mitochondrial diseases.

We used *Saccharomyces cerevisiae* as a model system to explore the role of mitochondrial dysfunction on genetic stability of the nuclear DNA (Rasmussen *et al.*, III) as well as the role of mitochondrial activity in oxidative DNA damage and repair of the nuclear genome (Rasmussen *et al.*, IV).

3.2 Mitochondrial Biology and Genetics

The mitochondria are surrounded by a double membrane, a smooth outer membrane, and a "highly folded" inner membrane (termed cristae). Depending on source and conformational state, cristae can vary from simple tubular structures to more complex lamellar structures that are connected to the inner boundary membrane (Frey & Mannella, 2000; Perkins & Frey, 2000). The inner membrane of the mitochondrion contains the proteins and enzymes of the electron transport chain (Complexes I-IV) and Complex V that are responsible for phosphorylation of ADP to ATP (adenosine diphosphate to adenosine trisphosphate). The inner compartment is called the matrix, which contains the many enzymes of Krebs cycle (the citric acid cycle), the pathway of fatty acid oxidation, and the mitochondrial DNA (Scheffler, 1999).

In mammalians, electron transfer from NADH donated to Complex I (NADH dehydrogenase or NADH:ubiquinone oxidoreductase) or electrons from succinate (donated to Complex II, succinate dehydrogenase) are transferred to coenzyme Q, also called ubiquinone or CoQ.
Regardless of whether CoQ receives its electrons from Complex I or II, it shuttles them to Complex III (ubiquinol:cytochrome c oxidoreductase). From Complex III the electrons move through cytochrome c and Complex IV (cytochrome c oxidase or COX), which passes the electrons directly to oxygen to form water. As electrons transverse complex I, III, and IV, protons (H\(^+\)) are moved from the mitochondrial matrix across the inner membrane into the inter-membrane space. This creates an electrochemical proton gradient (\(\Delta \Psi\)) that is positive and acidic on the outside and negative and alkaline on the mitochondrial matrix side. The proton gradient across the inner-membrane is used to drive the condensation of ADP and P\(_i\) (inorganic phosphate) to generate ATP by allowing the protons to flow back through Complex V (ATP synthase or F\(_1\)F\(_0\)ATPase). Under normal circumstances, the inner membrane is impermeable to protons, leaving Complex V as the only route whereby protons can return to the matrix (figure 3.1). The ATP is exchanged for ADP across the mitochondrial inner membrane by the Adenine Nucleotide Translocator, ANT (Wallace, 1999; Saraste, 1999). In fungi, Complex I is very similar to its counterpart in other eukaryotes except for \(S\). \textit{cerevisiae}, \textit{Saccharomyces carlsbergii} and \textit{Kluyveromyces lactis} which do not contain Complex I.

The mammalian Complex I is a NADH dehydrogenase involved in the oxidation of intra-mitochondrial NADH produced by the citric acid cycle, and its NADH-binding site faces the mitochondrial matrix. \(S\). \textit{cerevisiae} has three NADH dehydrogenases; Nde1 and Nde2 which both face the intermembrane space (referred to as external) and Ndi1 an internal NADH dehydrogenase like the mammalian Complex I (Joseph-Horne \textit{et al.}, 2001). Ndi1 is believed to be attached to the inner membranes on the matrix side (de Vries & Grivell, 1988; de Vries \textit{et al.}, 1992). It has been shown that when Ndi1 was expressed in a Complex I-deficient Chinese hamster cell, it restored the capacity for respiration and oxidative phosphorylation in these cells (Seo \textit{et al.}, 1998). Furthermore Ndi1 was incorporated into mitochondria in human embryonic kidney cells where Complex I was still present. Overexpression of Ndi1 in cells led to decreased coupling of NADH oxidation to ATP synthesis (ADP/Oxygen consumption ratio down from 2.4 to 1.8), while succinate oxidation was unchanged. These results suggest that the Ndi1 protein from \(S\). \textit{cerevisiae} complements the mammalian Complex I consisting of 43 peptides (Wallace, 1999). However, Ndi1 does not translocate protons, but only feeds electrons to the electron transport chain (Joseph-Horne \textit{et al.}, 2001). Apart from the lack of Complex I in \(S\). \textit{cerevisiae} the electron transport and proton transfer proceed via the same complexes as in mammals (figure 3.1).
3.3 Reactive Oxygen Species

Mitochondria generate more than 80% of cellular energy in the form of ATP and are therefore regarded as the "powerhouse" of the cell (Kang et al., 1998). However, mitochondrial respiration is also the major endogenous source of Reactive Oxygen Species (ROS), including superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (HO$^\cdot$) (Cadenas & Davies, 2000). Under normal physiological conditions, in vitro evidence indicates that electrons "leak" from the electron transport chain, converting about 1-2% of oxygen molecules into O$_2^-$, a more reactive form of oxygen (Boveris & Chance 1973; Boveris, 1977; Papa, 1996; Loft & Poulsen, 1996). Research in ROS production from mitochondria indicate that Complex I (Robinson, 1998) and Complex III (Turrens et al., 1985; Nohl & Jordan 1986) play a major role in production of superoxides however O$_2^-$ may also arise from Complex II (Ishii et al., 1998) in mammalian cells. Under normal metabolic conditions, Complex III seems to be the main site of O$_2^-$ production (Finkel, 2001).

Superoxide itself can attack enzymes containing the catalytically iron-sulfur [4Fe-4S] tetranuclear cluster, such as Complex I, Complex II and the Krebs cycle enzyme aconitase, resulting in release of iron and decreased mitochondrial ATP production (Flint et al., 1993; Gardner, 1995, Melov et al., 1999; Cadenas & Davies, 2000). Hence, mitochondria are particularly sensitive to oxidative stress. As we are using S. cerevisiae as model system it should be noted that the Ndi1 is a polypeptide enzyme with no iron-sulfur cluster and therefore not attacked by O$_2^-$ (de Vries & Grivell, 1988).

ROS is reduced by intracellular antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase. Superoxide is simultaneously reduced and oxidized (dismutated) to form hydrogen peroxide and oxygen by superoxide dismutase (SOD). Eukaryotes, including S. cerevisiae, hold a manganese containing superoxide dismutase (MnSOD, product of the SOD2 gene) in the matrix of the mitochondria, and a copper-and zinc containing form (CuZnSOD, product of the SOD1 gene) in the nuclear and cytoplasmic matrixes. Further, mammals have an extracellular CuZn superoxide dismutase (SOD3) (Crapo et al., 1992; Lindenau et al., 2000; Srinivasan et al., 2000).
Figure 3.1. Mitochondrial respiratory chain and reactions involved in removal of ROS in mitochondria

The respiratory enzyme complexes involved in oxidative phosphorylation are NADH-dehydrogenase (Complex I in mammalian and Ndi1 in S. cerevisiae), succinate dehydrogenase (Complex II); ubiquinol:cytochrome c oxidoreductase (Complex III); cytochrome c oxidase (Complex IV); ATP synthase (Complex V) in both mammalian and S. cerevisiae. Complex I and II in mammalian cells and Complex II in S. cerevisiae contains 4Fe-4S centers. When the respiratory chain is inhibited, the electrons accumulate in the early stages of the respiratory chain (Complex I and coenzyme Q), were they can be donated to molecular oxygen to give superoxide. Superoxide (O$_2^-$) is converted to hydrogen peroxide (H$_2$O$_2$) by mitochondrial superoxide dismutase (SOD2). H$_2$O$_2$ is converted to water (H$_2$O) by glutathione peroxide (GPx1 in mammalian). Ferrous ions (Fe$^{2+}$), released e.i. during O$_2^-$ attack of enzymes with 4Fe-4S centers, can participate in a Fenton reaction and convert H$_2$O$_2$ into hydroxyl radical (HO$^-$) (Wallace, 1999; Joseph-Horne et al., 2001). GSH (reduced glutathione); GR (glutathione reductase); GSSG (oxidized glutathione).

Hydrogen peroxide produced by SOD2 in the mitochondria is reduced to water (H$_2$O) by glutathione peroxidase (GPx), in a reaction that converts reduced glutathione (GSH) to oxidized glutathione (GSSG) (figure 3.1). Five GPx isoenzymes have been found in mammalian cells. GPx1, which are located in the mitochondria and cytosol and Gpx4 (or PHGPx, a phospholipid hydroperoxide) are found in most tissues. Cytosolic Gpx2 and extracellular Gpx3 are poorly detected in most tissues except for the gastrointestinal tract and kidney, respectively (de Haan et al., 1998). Hydrogen peroxide can also be reduced to
H$_2$O by catalase (CAT), located primarily in peroxisomes (especially in liver) and in cytoplasm (erythrocyte) (Bai & Cederbaum, 2001). Catalase has only been found in mitochondria of rat heart (Radi et al., 1991). In the presence of transition metal ions (Fe$^{2+}$, Cu$^+$) hydrogen peroxide can be converted by the Fenton reaction to the highly damaging hydroxyl radical, which can cause degradation of most biological macromolecules, e.g. peroxidation of lipids, oxidation of sugars and of protein thiols, DNA base damage, and strand breakage of nucleic acids (Bai et al., 1999).

### 3.4 Effects of Mitochondrial ROS Production

**Inhibition of mitochondrial oxidative phosphorylation**

Inhibition of mitochondrial oxidative phosphorylation in mice not only reduces energy production, but also increases mitochondrial ROS production. Mitochondria from normal mice have an increased H$_2$O$_2$ production after antimycin A inhibition (Esposito et al., 1999). Antimycin A is not a direct producer of H$_2$O$_2$ but an inhibitor of Complex III. Thus, the inhibition results in a substantial increase in superoxide production (Raha et al., 2000) which is converted into H$_2$O$_2$ by SOD2.

Also, mitochondria from adenine nucleotide translocator Ant1 (-/-) mice were shown to have the same high level of H$_2$O$_2$ as normal mitochondria treated with antimycin A. This suggests that the absence of ANT1 blocks the exchange of ADP and ATP across the mitochondrial inner membrane, thus inhibiting oxidative phosphorylation which is resulting in increased oxidative stress. Furthermore, tissues with high oxidative stress (skeletal muscle and heart) were showed to increase the level of ROS detoxification enzymes, SOD2 and GPx protein in Ant1 (-/-) mice. Although the antioxidant activities were increased, a very high level of mtDNA rearrangement was observed in the Ant1 (-/-) mice heart but not in skeletal muscle. It was suggested that the different levels of mtDNA rearrangement in the two tissues were a result of lower induction of SOD2 in the heart than in skeletal muscle (Esposito et al., 1999).

**Cytosolic superoxide dismutase (sod1) mutants**

By analyzing null SOD1 and SOD2 mutations in different species it has been suggested that ROS could be involved in limiting life span. Yeast cells deficient in the SOD1 gene are viable but grow poorly in oxygen and the mutation frequencies are increased in nDNA in sod1Δ mutant cells compared to the wild type parental strain (Gralla & Valentine, 1991;
Longo et al., 1997, Rasmussen et al., IV). It has been shown that SOD1 activity in motor-neurons is an important factor in ageing and lifespan of Drosophila (Parkes et al., 1998). Sod1 null mutants of Drosophila have a very short lifespan (the adult lifespan is shortened by 85-95%). Overexpression of human SOD1 in motor neurons of a wild type Drosophila extended the adult lifespan by up to 40% (Parkes et al., 1998).

Mutations in the SOD1 gene are also linked to Familial Amyotrophic Lateral Sclerosis (FALS) a disorder resulting from degeneration and death of motor neurons. 15-20% of FALS patients have been found to harbour mutations in SOD1. Transgenic mice that express a mutant SOD1 allele develop a motor neuron degeneration that parallels most aspects of ALS (Gurney et al., 1994; Bruijn et al., 1997). Whereas, knockout mice deficient in the entire SOD1 gene live to adulthood and show no overt motor defect (Reaume et al., 1996). This result demonstrates that the loss of SOD1 function is not, by itself, sufficient to kill motor neurons in vivo. It has been suggested that FALS is a consequence of reduction in SOD1 dismutation activity leading to oxidative damage (loss-of-function) or a gain-of-function as the mutant SOD1 protein seems to increase the ability to generate hydroxyl radicals (Yim et al., 1996; Bogdanov et al., 1998; Liu et al., 1999). The results obtained with SOD1/- knockout mice suggest that FALS SOD1 acquires or enhances toxic property.

Mitochondrial superoxide dismutase (sod2) mutants

Yeast sod2Δ mutants have a less dramatic phenotype than sod1Δ mutants when grown in glucose media although sod2Δ mutants are oxygen-sensitive and grow poorly in carbon sources that require respiration for their metabolism (Liu et al., 1992; Longo et al., 1996). In contrast to sod2Δ mutants in yeast, an acute toxicity of mitochondrial O2·− has been found in SOD2-/- mice. While mice, deficient in cytosolic SOD1 activity were viable (Reaume et al., 1996), inactivation of the mitochondrial SOD2 was lethal early in life. Two models of SOD2 knockout mice have been reported. SOD2tm1Cje mutant mice in which exon 3 has been deleted (Li et al., 1995) and SOD2m1BCM mutants which are missing exon 1 and 2 (Lebovitz et al., 1996). The homozygous SOD2tm1Cje mutant mice die within the first 10 days of life with a dilated cardiomyopathy, accumulation of lipid in liver and skeletal muscle, and metabolic acidosis (Li et al., 1995). The homozygous SOD2m1BCM mutant mice survived up to 18 days and showed motor disturbance, central nervous system
Injury, and extensive mitochondrial injury within cells with requirements of high levels of oxidative metabolism, including cardiac myocytes, neurons, hepatocytes, and hematopoietic cells (Lebovitz et al., 1996).

In the SOD2<sup>tm1Cje</sup> (-/-) mutant mice, the mitochondria's O<sub>2</sub><sup>-</sup> toxicity was shown to result in dramatic reductions in the activities of mitochondrial enzymes containing iron-sulfur centers, including the Krebs cycle enzyme aconitase and respiratory chain enzymes of Complex I and II. Hence, the dilated cardiomyopathy observed in SOD2 (-/-) mice (Li et al., 1995) was caused by blocking of the Krebs cycle and respiratory chain giving energy starvation in the heart (Melov et al., 1999). The increased mitochondrial oxidative stress of the SOD2 (-/-) mice also resulted in high levels of oxidative damage to DNA. However, it was not determined whether it was nDNA, mtDNA damage or both (Melov et al., 1999).

Measurement of the lipid peroxidation levels in heterozygous SOD2<sup>tm1Cje</sup> (+/-) mutants and SOD2 (+/+) control mice revealed that young SOD2 (+/-) and control animals had the same levels of lipid peroxidation in the liver mitochondria. However, lipid peroxidation peaked for SOD2 (+/-) at middle age and declined in old SOD2 (+/-) animals, whereas lipid peroxidation of SOD2 (+/+) mice first peaked at old age (Kokoszka et al., 2001). Apoptosis seemed to be able to explain the loss of mitochondrial lipid peroxidation from middle-age to old age in SOD2 (+/-) mice. TUNEL staining showed that old SOD2 (+/-) mouse livers had three times more apoptotic hepatocytes than old SOD2 (+/-) mouse livers. It was therefore suggested that cells with a high number of damaged mitochondria was destructed by apoptosis (Kokoszka et al., 2001).

It has also been demonstrated that Caenorhabditis elegans life span was increased 54% by treatment with SOD/catalase mimetics. In addition, treatment with these compounds could restore a mev-1 mutant life span to normal. The mev-1 gene, encodes the cytochrome b subunit of succinate dehydrogenase (Complex II) and the mutant increases mitochondrial ROS production which shortens the worms life span by 37% (Melov et al., 2000).

The just mentioned models showed that ROS generation by mitochondria can be damaging to mitochondria themself but also to the length of life span of the animal. High ROS production can increase the percentage of mutant mtDNA, which decreases the mitochondrial energetic capacity and increases ROS production as well as the propensity for apoptosis. The tissues most sensitive to mitochondrial dysfunction are brain, heart, skeletal muscle, endocrine system and kidney (Wallace, 1999).
ROS generates a variety of DNA lesions including modified bases, abasic sites, and single strand breaks. If left unrepaired, these damages may contribute to a number of degenerative processes, including aging and cancer. Association to cancer has been established because one of the major base lesions formed upon oxidative attack to DNA, 7,8-dihydro-8-oxoguanine (8-oxoG), was present at higher level in both lung and breast tumor tissue compared to normal tissue (Malins et al., 1991; Olinski et al., 1992). However, an important question is whether ROS generated in mitochondria contributes to induce mutations in nDNA?

### 3.5 Mitochondrial Dysfunction Contributes to nDNA Mutations

Hydroxyl radicals are extremely unstable with an estimated half-life of only $10^{-9}$ seconds (Pryor, 1986) whereas H$_2$O$_2$ is freely diffusible and relatively long-lived and may therefore efflux out of the mitochondria, and into the cytoplasm (Finkel & Holbrook, 2000; Bai & Cederbaum, 2001). Diffusion of H$_2$O$_2$ across the mitochondrial membrane has been suggested by Bai et al. (1999). They showed that catalase overexpressed in the mitochondria of HepG2 cells protected the cells from cytotoxicity of H$_2$O$_2$. Similarly, overexpression of cytosolic catalase protected HepG2 cells from cytotoxicity of antimycin A. Bai et al. (1999) showed that H$_2$O$_2$ diffuses into the mitochondria and that damage to the mitochondria could be an important factor contributing to H$_2$O$_2$ toxicity. Similarly, cytosolic catalase protected the cells against antimycin A induced H$_2$O$_2$ toxicity, suggesting that mitochondrial produced H$_2$O$_2$ diffused into the cytosol.

We used the yeast S. cerevisiae as a model system to investigate a potential link between mitochondrial activity and genomic instability. We generated strains that were either impaired in mitochondrial activity due to mutations in the mitochondrial genome (rho−) or strains absolutely deficient in mitochondrial activity due to lack of mitochondrial genome (rho$^0$). We assayed these strains for spontaneous mutations and found that spontaneous mutation frequencies, measured as nuclear mutational events, were significantly higher in both rho− and rho$^0$ strains compared to the wild type strains (Rasmussen et al., III)

To investigate whether the increased spontaneous mutation frequencies observed in the rho$^0$ and rho− strains were a result of defective respiratory function in these cells, we measured the effect of disrupting the respiratory chain function in S. cerevisiae using various
mitochondrial inhibitors. We demonstrated that all drugs caused an increase in nuclear spontaneous mutation frequencies but that antimycin A had the most profound impact. This indicates that mitochondrial ROS production can cause nuclear mutations. Further, our results show that mitochondrial function and more specifically complex III activity is critical for maintaining genomic integrity (Rasmussen et al., 2011). The results correspond with in vitro studies indicating that Complex III may be responsible for more than 80% of ROS produced in *S. cerevisiae* (Chance et al., 1979). However, not all types of inhibition of the respiratory chain result in mutations in nDNA. We observed that mutations in *COQ3* and *COX6* did not affect spontaneous mutation frequencies and we therefore concluded that inhibition of these specific gene products is not involved in mitochondrial-mediated mutagenesis (figure 3.2).

![Spontaneous mutation frequency](image)

**Table**: Spontaneous mutation frequency

<table>
<thead>
<tr>
<th>Strain</th>
<th>can' (×10^-8)</th>
<th>Fold increase</th>
</tr>
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<tbody>
<tr>
<td>wild type</td>
<td>11.2 ± 5.6</td>
<td>1</td>
</tr>
<tr>
<td>Hansen BY4741</td>
<td>16.7 ± 4.2</td>
<td>1.5</td>
</tr>
<tr>
<td>coq3∆</td>
<td>11.9 ± 6.2</td>
<td>1.1</td>
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</tbody>
</table>

**Figure 3.2.** Mutation frequencies in *S. cerevisiae* Hansen BY4741 wild type, coq3∆ and cox6∆ (Research Genetics, Inc., Huntsville, AL). The *COQ3* gene encodes an O-methyltransferase required for two steps in the biosynthetic pathway of ubiquinone (coenzyme Q) - coq3 mutants do not synthesize coenzyme Q ( Jonassen & Clarke, 2000). The *cox6* gene encodes subunit VI of Complex IV. *S. cerevisiae* cytochrome c oxidase is composed of nine different subunits. The three largest (cox1, cox2, and cox3) are encoded by mitochondrial genes the other six are encoded by nuclear genes (de Vries and Marres, 1987; Foury et al., 1998). Strains deficient in cox6 activity show residual cytochrome c activity corresponding to 7% of the activity found in wild type mitochondria (de Vries and Marres, 1987).

It has been demonstrated that respiration plays a role in ROS production as well as for viability in SOD deficient yeast strains. *S. cerevisiae* deficient in Sod2 activity failed to grow normally in hyperoxia (doubling time 10.8 h.). In contrast, sod2 null mutants with complete absence of electron transport (rho0) grew normally in hyperoxia (doubling time 2.8 h.). In addition, the viability was partially restored by mutations (cox6) which disrupt the electron transport chain (doubling time 5.6 h.). Lack of respiration (coq3 mutant) also prevents viability loss of sod1 null mutants and sod1sod2 double null mutants (Guidot et al., 1993; Longo et al., 1996).
We examined *S. cerevisiae sod* mutant strains and found that deficient sod activity increases nuclear spontaneous mutation frequencies and that this mutator phenotype is suppressed by inactivation of mitochondrial activity (*sod1Δ rho^0^ or *sod2Δ rho^0^*). These data indicate that mitochondrial dysfunction either decreases oxidative damage to nuclear DNA by preventing formation of ROS and/or increase repair of oxidative DNA damage (Rasmussen *et al.* IV). Measurement of intra-cellular levels of O_2^- and H_2O_2 in strains deficient in mitochondrial activity (rho^0^ and rho^-) showed lower levels of both O_2^- and H_2O_2 in rho^0^ and rho^- cells (Rasmussen *et al.* III). These results showed that inactivation of mitochondrial function decreases intracellular levels of ROS and suggest decreasing mutagenesis caused by ROS.

Furthermore, gene expression analysis showed that mitochondrial dysfunction did not increase repair gene expression in *sod1rho^0^* cells compared to *sod1* cells (Rasmussen *et al.* IV). Gene expression analysis also revealed that genes involved in detoxification are repressed in rho^0^ compared to wild type cells (Table 3.1).

### Table 3.1 • Expression of genes involved in detoxification in rho^0^ and rho^- cells

<table>
<thead>
<tr>
<th>Probe Set Name</th>
<th>ORF</th>
<th>Gene name</th>
<th>Abs Call</th>
<th>Fold Change in rho^0^</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes repressed in rho^0^</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7907_at</td>
<td>YPL163C</td>
<td>SVS1</td>
<td>P</td>
<td>2.4</td>
<td>Serine and threonine rich protein.</td>
</tr>
<tr>
<td>7432_at</td>
<td>YBL064C</td>
<td></td>
<td>P</td>
<td>2.7</td>
<td>Homolog to thiol-specific antioxidant</td>
</tr>
<tr>
<td>10634_at</td>
<td>YKL062W</td>
<td>MSN4</td>
<td>P</td>
<td>2.8</td>
<td>zinc finger protein</td>
</tr>
<tr>
<td>4074_at</td>
<td>YIR038C</td>
<td>GTT1</td>
<td>P</td>
<td>2.9</td>
<td>Glutathione transferase</td>
</tr>
<tr>
<td>8410_at</td>
<td>YOR163W</td>
<td>DDP1</td>
<td>P</td>
<td>3.1</td>
<td>Strong similarity to S.pombe SPAC13G6.14 protein</td>
</tr>
<tr>
<td>4924_at</td>
<td>YGR088W</td>
<td>CTT1</td>
<td>P</td>
<td>5.9</td>
<td>Cytoplasmic catalase T</td>
</tr>
<tr>
<td>10581_at</td>
<td>YKL026C</td>
<td>GPX1</td>
<td>A</td>
<td>5.3</td>
<td>Strong similarity to glutathione peroxidase</td>
</tr>
<tr>
<td>Genes induced in rho^-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8723_at</td>
<td>YOL156C</td>
<td>ENB1</td>
<td>P</td>
<td>2.2</td>
<td>Similarity to subtelomeric encoded proteins</td>
</tr>
<tr>
<td>4483_s_at</td>
<td>YHR053C</td>
<td>CUP1-1</td>
<td>P</td>
<td>2.5</td>
<td>Copper-binding metallothionein</td>
</tr>
<tr>
<td>8400_at</td>
<td>YOR1539W</td>
<td>PDR5</td>
<td>P</td>
<td>3.5</td>
<td>Multidrug resistance transporter</td>
</tr>
<tr>
<td>7109_at</td>
<td>YBR244W</td>
<td>GPX2</td>
<td>P</td>
<td>7.9</td>
<td>Probable glutathione peroxidase (EC 1.11.1.9)</td>
</tr>
</tbody>
</table>

The Abs Call = the Absolute Call; The transcript is present (P) or absent (A) for rho^0^. When the transcript is absent, is the fold change an approximation and calculated using the noise level.

We used DNA microarray hybridization to analyse the expression of genes known to be involved in detoxification of ROS in rho^0^ and wild type cells (table 3.1). We found that the cytoplasmic catalase (*CTT1*), glutathione transferase (*GTT1*), and glutathione peroxidase (*GPX1*) genes were repressed in rho^0^ cells. In contrast, glutathione peroxidase (*GPX2*) was induced. It has previously been shown that several oxidative agents such as H_2O_2 and O_2^- generating agents (Inoue *et al.*, 1999) induce the expression of the GPX2 gene. Our gene expression analysis showed that the expression of the transcription factor Msn4 encoded by
the MSN4 gene was repressed in rho^0^ cells. Msn4 and Msn2 proteins regulate the expression of gene products that are induced by stress conditions through the stress response element (STRE) promoter sequence (Gasch et al., 2000; Causton et al., 2001). Msn4 and Msn2 proteins have been shown to bind upstream of the STRE element (Görner et al., 1998). A known target for this transcription factor is the CTT1 gene (Gasch et al., 2000). Our results indicate that rho^0^ cells experience stress although the nature of this stress is unknown at this time.

These data indicate that absence of mitochondrial respiration is an important factor for the viability as well as for suppression of nuclear spontaneous mutations in sod1 rho^0^ mutant cells. Our results suggest that rho^0^ cells have a lower production of ROS which results in lower nuclear spontaneous mutation frequency even when Sod1 or Sod2 are absent.

Since the level of ROS is lower in rho^0^ cells one would expect that rho^0^ cells had lower spontaneous mutation frequencies than wild type but this is not the case. Interestingly we show that rho^0^ cells had (2.6 fold) statistically significant higher spontaneous mutation frequencies than wild type. Gene expression analysis showed that REV1 expression is upregulated in rho^0^ strains. The REV1 gene encodes a deoxycytidyl transferase activity involved in error-prone translesion DNA synthesis (TLS) that inserts dCMP opposite an abasic site or a strand break in the template, thereby producing a terminus that can be extended by polymerase zeta (Pol ξ). Polymerase ξ is composed of two subunits encoded by REV3 and REV7. TLS is mutagenic because Rev1 preferentially inserts cytosine opposite an abasic site in yeast (Lawrence and Hinkle, 1996; Nelson et al., 1996; Lawrence and Maher, 2001). We examined rev1 rho^0^, rev3rho^0^ and rev7rho^0^ strains to determine if inactivation of TLS could eliminate the mutator phenotype observed in rho^0^ cells. Our results show that mitochondrial-mediated mutator phenotype depend on functional Rev1, Rev3 and Rev7 proteins and that the mutator phenotype of rho^0^ cells is suppressed by preventing Rev1/Rev3/Rev7-dependent translesion synthesis (Rasmussen et al., III).

It has been shown that 3-methyladenine DNA glycosylase encoded by MAG1 in S. cerevisiae, which removes a variety of alkylated bases and generates abasic sites, increases spontaneous mutation frequency by 600-fold when overexpressed in S. cerevisiae. This mutator phenotype is also dependent on the Rev1/Rev3/Rev7 TLS bypass pathway (Glassner et al., 1998).
Our genetic evidence therefore suggests that mitochondrial dysfunction contributes to spontaneous DNA damage, which is fixed in the genome as mutation by error-prone Rev1/Rev3/Rev7 TLS pathway, which results in cell survival but genetic instability.

Interestingly, one of the profound features of cancer cells is their defective mitochondrial function. A role for mitochondria in tumorigenesis was hypothesized when it was found that most tumors up-regulate glycolysis and therefore seems to be more dependent upon glycolysis for energy production, than mitochondrial oxidative phosphorylation (Warburg, 1956).

Furthermore, cancer cell lines (bladder, head, neck, colorectal and pancreatic) and lung primary tumors have been observed to have high frequency of homoplasmic mutations of mtDNA. The mitochondrial mutations are present in each of the hundreds of mitochondrial genomes in the tumor cell and in virtually all of the cells of the tumor (Polyak et al., 1998; Fliss et al., 2000; Jones et al., 2001). Homoplasmic mutations of mtDNA indicate that a single cell with a mutant mitochondrial genome has acquired a selective growth advantage during tumor evolution, allowing it to become the predominant cell type in the tumor cell population. Homoplasmy also indicates that each mutant mitochondrial genome has a replicative advantage in the particular mitochondria in which it occurred, and that this mitochondrion has selectively proliferated over other mitochondria in the same cell (Polyak et al., 1998; Jones et al., 2001).

Our studies using *S. cerevisiae* with depleted mitochondria suggest that cancer cells by losing their mitochondrial function create a mutator phenotype (Rasmussen et al., II). Figure 3.3 provides a model for how mitochondrial dysfunction generates a mutator phenotype in two different ways:

1. Inhibition of the respiratory chain increases mitochondrial ROS production. Mitochondrial reactive oxygen species damage the mtDNA and the nDNA if mitochondrial H$_2$O$_2$ diffuses to the nucleus and is converted to HO$^*$ which can cause DNA damage.
2. Mitochondrial dysfunction leads to nuclear DNA damage. The DNA damage activates the Rev1/Rev3/Rev7 error-prone translesion DNA synthesis pathway that contributes to cell survival, but at the expense of higher spontaneous mutation frequency.
Figure 3.3. Mitochondrial dysfunction creates a mutator phenotype. Our results suggest that mitochondrial dysfunction can create a mutator phenotype in at least two ways. 1) Mitochondrial dysfunction caused by inhibition of the respiratory chain increases mitochondrial ROS production and if the ROS level is higher than the endogenous antioxidant capacity, ROS might diffuse as H$_2$O$_2$ to the nucleus. In nucleus H$_2$O$_2$ can be converted to HO$^*$ which causes DNA damage. If the DNA damage is left unrepaired mutations in nDNA will occur and may lead to mutations that enhances cancer risk. 2) Mitochondrial dysfunction leads to nuclear DNA damage. The nature of this spontaneous nuclear DNA damage is unknown at this time, but might be caused by imbalanced repair as seen when Mag1 is overexpressed (Glassner et al., 1998). However, the DNA damage activates the Rev1/Rev3/Rev7 TLS pathway, which contributes to cell survival, but at the expense of higher spontaneous mutation frequency, which may lead to enhanced cancer risk.
Section two

Paper I

Acknowledgements

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Identification of factors interacting with hMSH2 in the fetal liver utilizing the yeast two-hybrid system
In vivo interaction through the C-terminal domains of hEXO1 and hMSH2 and comparative expression analysis

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Abstract
Mutations in DNA mismatch repair (MMR) genes have been shown to segregate with Hereditary Nonpolyposis Colorectal Cancer (HNPCC). However, because many HNPCC families fail to display mutations in known MMR genes, we argued that changes in other components of the MMR pathway may be responsible. The increasing number of proteins reported to interact in the MMR pathway suggests that larger complexes are formed, the composition of which may differ among cell types and tissues. In an attempt to identify tissue-specific MMR-associated factors, we employed the yeast two-hybrid system, using the human hMSH2 as bait and a human fetal liver library as prey. We demonstrate that hMSH2 interacts with a human 5′−3′ exonuclease 1 (hEXO1 / HEX1) and that this interaction is mediated through their C-terminal domains. The hMSH6 protein does not interact with hEXO1 in the two-hybrid system. Dot-blot analysis of multiple tissue RNA revealed that hMSH2 and hEXO1 are coexpressed at high levels in fetal liver as well as in adult testis and thymus. Northern blot analysis also revealed that hEXO1 / HEX1 is highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Mismatch repair; hMSH2; hEXO1; Two-hybrid; Cancer

1. Introduction
Changes in the efficiency of DNA repair and recombination activities can be associated with predisposition to cancer [1]. The finding that Hereditary
Nonpolyposis Colorectal Cancer (HNPCC) families frequently harbor mutations in DNA mismatch repair (MMR) genes has generated widespread interest in this research area [2–4]. At the time of writing, germline mutations in at least four genes, hMSH2, hMLH1, hPMS1 and hPMS2, all homologs of bacterial components involved in MMR, have been found in HNPCC patients. Although no biochemical role has to date been proposed for hPMS1, the other three proteins have been shown to participate in MMR [3]. In addition, MMR proteins might play important roles in other pathways of DNA metabolism such as DNA recombination and cell cycle checkpoint signaling [4].

The development of an assay to study MMR in cell-free extracts of bacteria has led to a detailed understanding of the mechanism underlying the repair process and, on the basis of these in vitro studies, a model for MMR has been proposed [5], reviewed in Refs. [4,6]. Based on homology between eukaryotic and prokaryotic MMR proteins, it has been hypothesized that the mechanism underlying human MMR is similar. Subsequently, it has been shown that eukaryotic MMR proteins interact with polypeptides involved in DNA replication, DNA recombination, and DNA nucleotide excision repair (NER) [7–17]. A number of these genes have been inactivated giving rise to complex phenotypes, such as defects in MMR and recombination or in NER and recombination [18–20]. These findings suggest that DNA repair proteins are components of larger protein complexes, and that at least some of these proteins function in several distinct processes. The increasing number of polypeptides reported to interact with proteins in the MMR pathway suggests that these complexes are composed of a larger number of subunits, and also that the composition of such complexes differs among cell types and tissues.

In the present communication, we have employed the yeast two-hybrid system in an attempt to identify proteins that interact with the human MMR protein hMSH2 in vivo, using hMSH2 as bait. We chose a human fetal liver cDNA library as prey since we expected MMR-associated proteins to be highly expressed in an organ containing rapidly proliferating cells. We report that hMSH2 interacts with the gene products of both hMSH6 and hEXO1 / HEX1. We were unable to detect binding between hMSH6 and hEXO1 in the two-hybrid assay. Our results suggest that the interaction between hMSH2 and hEXO1 is mediated via the C-terminal domains of these proteins. Expression analysis showed that high levels of this exonuclease are indeed coexpressed with hMSH2 in fetal liver as well as in adult testis and thymus. Coexpression, although at lower levels, is also found in colon, small intestine, bone marrow, placenta, fetal kidney, fetal spleen and fetal thymus. Further expression analysis revealed high levels of hEXO1 transcripts in liver cancer cell lines and in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue.

2. Material and methods

2.1. Human cell lines

The cell lines HepG2 (human hepatocellular carcinoma), SK-Hep1 (human liver adenocarcinoma), Huh-7 (human hepatocellular carcinoma), Chang (human liver), and WRL68 (human liver embryo) were purchased from European Collection of Cell Cultures (Wiltshire, UK). All cell lines were maintained as monolayer cultures in DMEM (Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies).

2.2. Plasmids

Plasmid pLJR105 was constructed by inserting the human hMSH2 coding sequence into the Neol site of the pAS2 binding domain vector (CLONTECH). The hMSH2 gene was obtained on a 2.9-kbp fragment from pCite-1-hMSH2 [21] kindly provided by Dr. Josef Jiricny (Institute of Medical Radiobiology of the University of Zurich, Switzerland). The hMSH2 bait was sequenced to confirm that the hMSH2 protein was in frame with the GAL4 binding domain. Plasmid pLJR112 contains a C-terminal truncation of the hMSH2 gene and was constructed by deleting a 1-kb fragment between the Blp1 and Sal1 sites in the vector sites in pLJR105. This truncation eliminates the C-terminal 374 amino acids of the hMSH2 protein and includes elimination of the conserved region. The HEX1 (C-terminal) and HEX1 (N-terminal) plasmids
were constructed by inserting either a 1182- or a 1355-bp fragment into the *NcoI* and *BamHI* sites of the pACT2 activation domain vector (CLONTECH). The fragments were amplified by PCR using primers containing additional *NcoI* or *BamHI* sites. Primer pairs 5'-GGGCCATATGGGATACAAGGAT-3′, 5'-CGGGATCTTCATTCATTGCGGATCT-3′ and 5'-GGCCCATGGCCCTGCCCTTCCAGAAGT-3′, 5'-CGGGATCTTCAGAATTTCATTTTGAAAATCGA-3′ were used for amplification of the N-terminal and C-terminal parts of the hEXO1α/HEX1 protein, respectively.

### 2.3. Two-hybrid techniques

The GAL4-hMSH2 (pLJR105) bait plasmid was transformed into *S. cerevisiae* strain Y190 (MATα, *ura3*-52, *his3*-200, *lys2*-801, *ade2*-101, *trpl*-901, *leu2*-3, 112, *gal4Δ, gal80Δ, cyhΔ2, *LYS2::GAL1*Δ*HIS3*TATA-HIS3, *URA3::GAL1*Δ*HIS3*ΔTATA-lacZ [22]. The resulting strain was subsequently transformed with a human fetal liver matchmaker cDNA library (CLONTECH, #HL4029AH) and hMSH2 interactors were selected on synthetic dextrose minimal medium (SD) [23] lacking tryptophan (to maintain pLJR105), leucine to maintain the GAL4 activation domain plasmids, and histidine (to identify peptides capable of assembling a functional GAL4 transcription factor) and supplemented with 25 mM 3-amino-1, 2, 4-triazole (3-AT) (SD-HIS-TRP-LEU + 25 mM 3-AT). 3-AT is a competitive inhibitor of the *S. cerevisiae* His3 protein and is used to lower the background growth of the reporter strain due to leaky expression of the *HIS3* gene. The plates were incubated at 30°C for 7–10 days and a total of 53 positive clones, Y1–Y53, were screened for β-galactosidase activity on SD-TRP-LEU + 60 μg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal) plates to verify positive interactions. These clones were rescued for the GAL4 binding domain plasmid (pLJR105) by repeated growth on SD-LEU plates. The rescued clones were tested for lack of lacZ expression on SD-LEU + X-gal plates to eliminate false positive clones.

### 2.4. DNA sequencing

DNA sequencing was performed directly on yeast colonies grown on SD-HIS-TRP-LEU plates by touching the colony with a sterile pipette tip. The pipette tip was rinsed in incubation buffer (1.2 M sorbitol; 100 mM sodium phosphate, pH 7.4; 2.5 mg/ml zymolase) and incubated at 37°C for 5 min. Sequence inserts were amplified by PCR using the MATCHMAKER AD LD-Insert Screening Amplimer Set (CLONTECH). The PCR amplified fragments were purified using a Qiagen PCR purification kit (Qiagen) and sequenced. Database searches were performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

### 2.5. Northern blot analysis

Human RNA Master Blot containing 100–500 ng poly(A)^+RNA/dot (Human RNA Master Blot) was purchased from CLONTECH. For detection of transcripts in human cells aliquots (20 μg) of total RNA (RNAqueous™, Ambion) were run on 1% agarose/0.2 M formaldehyde gels and immobilized onto nylon membranes. All probes were labeled by random primer extension (Rediprime DNA labeling system, Amersham Pharmacia Biotech) and [α-32P]dCTP (Amersham Pharmacia Biotech) using a PCR product as template. For the detection of exonuclease homologs, the blots were probed with either a 914-bp fragment containing the N-terminal region of hEXO1α/HEX1 (N-terminal probe) or a 946-bp fragment containing the C-terminal region of hEXO1α/HEX1 (C-terminal probe). These probes were generated by PCR primers 5’-GGTGCCA-CATCGGATCTCTGAG-3′ and 5’-TCAAATCC-CACGCAGTGTGATGAG-3′ (HEX1 N-terminal probe) and 5’-GAGTGTAAGCATACTCCACATGG-3′ and 5’-CCCAGCTTGTTCTCCGCGCATT-3′ (HEX1 C-terminal probe). For the detection of hMSH2, the blots were probed with a 1054-bp fragment containing the N-terminal region of hMSH2, which shows limited homology to other known MutS homologs. This probe was generated by PCR primers 5’-GTGCGGTCTTCTCAGGAGCCATGC-3′ and 5’-TTATTCAAGGAG-CAGCCAGG-3′ (hMSH2 probe). All membranes were hybridized with 1 × 10^6 cpm/ml of 32P-labeled probes. The Human RNA Master Blot was prehybridized for 30 min at 65°C and hybridized overnight at 65°C in ExpressHyb® solution according to the manufacturer (CLONTECH). All other membranes were prehybridized for 30 min at 68°C and hy-
bridized for 90 min at 68°C in QuikHyb® Hybridization Solution (Stratagene). Blots were washed twice at room temperature for 15 min and once at 60°C with wash solution (1 × SSC, 0.1% SDS). Images were obtained using autoradiography. GAPDH and β-actin probes provided by the manufacturer (CLONTECH) were prepared as described above and used to correct for differences in loading.

2.6. β-galactosidase assays

β-galactosidase specific activity was assayed on three separate transformants of each strain. In brief, colonies were inoculated into 5 ml of SD-LEU-TRP medium and grown overnight at 30°C with vigorous shaking. The next day, 2 ml of overnight cultures were inoculated into 2 ml of YPD medium and the cultures grown for 3–5 h at 30°C with vigorous shaking. Samples were prepared and assayed for β-galactosidase activity essentially as described by CLONTECH Protocol PT1020-1, www.clontech.com.

3. Results

3.1. Identification of hMSH2-interacting protein partners

To define the mechanism of human MMR and to identify missing components of this complex process, we set up a two-hybrid screen in S. cerevisiae, using as bait GAL4 fused to the entire open reading frame of human hMSH2 and as prey GAL4 activation-tagged human fetal liver matchmaker cDNA library. Our initial screen identified 53 (Y1–Y53) clones as interactors. Sequence analysis showed that the majority of the clones contained either yet unidentified human cDNAs or proteins such as γ-globulin, fibrinogen, albumin, haptoglobin, repeat regions, ferritin, and α-chymotrypsin but that Y5, Y10, Y47 and Y50 contained a carboxyl-terminal region of the human homologue of S. cerevisiae exonuclease EXO1, termed hEXO1b [10, 11] (Fig. 1). We also isolated two clones containing hMSH6, which is known to form a complex with hMSH2 [4], confirming to us that hMSH2 is able to form a complex with human MMR proteins in yeast.

The DNA sequences of the hEXO1 clones we isolated (Y5, Y10, Y47 and Y50) showed a perfect match to the 3'-end of hEXO1b, which is dissimilar to the hEXO1a/HEX1 sequence (Fig. 1). Furthermore, we observed several DNA sequence polymorphisms in the hEXO1 clones that could reflect human population variation (Fig. 1). The C to T base pair change (bp 2488) in Y5, Y10 and Y47 causes a proline (P) to leucine (L) codon change, whereas the G to A (bp 1279) in Y50 causes an arginine (R) to histidine (H) codon change. The Y50 clone contains also an A to G base pair change (bp 1983), which results in a lysine (K) to glutamic acid (E) codon change. Furthermore, Y50 contains a deletion of CAG (bp 1486) resulting in a deletion of an alanine (A) residue. All the clones we isolated appear to interact equally well with hMSH2 in the two-hybrid assay, indicating that these amino acid residues are not crucial for hMSH2-hEXO1 interaction. The Y5, Y10 and Y47 clones contained an additional 127-bp sequence (Fig. 1), which corresponds to 59171–59048 bp of the genomic DNA sequence Genbank AC004783. This sequence is located upstream of the first exon (Exon 1) in hEXO1a/HEX1 [12]. For simplicity, we have named this 127-bp DNA sequence for Exon 0. We do not think that Y5, Y10 and Y47 represent true splice variants of hEXO1b since we were only able to detect transcripts corresponding to full-length hEXO1 using Northern blot analysis (data not shown).

3.2. Interaction domains of hMSH2 and hEXO1

It has been shown that purified full-length hEXO1b interacts with hMSH2, in vitro, in an immunoprecipitation assay [10]. We used two-hybrid analysis to characterize the interaction between hMSH2 and hEXO1 in vivo and to define the region of association. We fused the full-length hEXO1a/HEX1 and hEXO1b cDNAs to the GAL4 activation domain in pACT2 and tested for interaction with our GAL4–hMSH2 binding domain plasmid (pLJR105). We were unable to detect protein–protein interactions with any of our full-length exonuclease 1 constructs in the two-hybrid assay (Fig. 2A). However, when we switched the inserts around and instead fused hMSH2 to the GAL4 activation domain in pACT2, and hEXO1b and
Fig. 1. Exonuclease 1 gene structures. The full-length hEXO1α/HEX1 and hEXO1β [10–12] coding regions comprise 13 exons (II–XIV). The shorter variants named Y5, Y10, Y47 and Y50 comprise eight exons (VII–XIV). These clones contain only a part of exon VII that we refer to as exon VII′. Solid lines represent exon sequences containing the N and I regions thought to be required for exonuclease activity. Shaded boxes in hEXO1α/HEX1 and hEXO1β represent the predicted coding regions. ΔGA to stop indicates the two base pairs deletion of GA that causes a frameshift in hEXO1α/HEX1 compared with hEXO1β. The Y5, Y10 and Y47 clones contain an additional 127-bp DNA sequence (exon 0) corresponding to 59171–59048 bp of the genomic DNA sequence Genbank AC004783.

hEXO1α/HEX1 to the GAL4 binding domain in pAS2, we could detect an interaction in the two-hybrid assay (Fig. 2A, Table 1). One reason for this discrepancy could be that the GAL4 (activation domain)-hEXO1 fusion proteins are structurally different from the native proteins and therefore unable to interact with the GAL4 (binding domain)–hMSH2 fusion protein. This would also explain why we failed to isolate any full-length cDNAs of hEXO1β or hEXO1α/HEX1 in our initial two-hybrid screen.

We constructed truncated peptides of hEXO1α/HEX1 such that the protein was divided into two regions containing either the 451 amino acids N-terminal or the 388 amino acids C-terminal part of full-length hEXO1α/HEX1 (Fig. 1). We also constructed a C-terminal truncation of hMSH2 to delete the conserved region of the protein that is necessary for interaction with hMSH3 and hMSH6 [24]. We did not observe any interaction between hMSH2 and the N-terminal region of hEXO1 in the two-hybrid test (Fig. 2B). Neither did we observe interaction between the hMSH2 N-terminal region and any of the exonuclease clones we isolated in the two-hybrid screen (data not shown). However, we cannot rule out that the N-terminal region of hEXO1 makes contact to hMSH2 in the native protein. Interestingly, we did observe an interaction between hMSH2 and Y5 (hEXO1β C-terminal), as well as...
Fig. 2. Interaction of hMSH2 with hEXO1a and hEXO1b (Y5). (A) Strains containing various plasmids were streaked in horizontal rows on either SD-LEU-TRP + X-gal (upper panel) or SD-LEU-TRP-HIS + 45 mM 3-AT (lower panel) plates. (B) Strains containing various plasmids were streaked on SD-LEU-TRP X-gal plate. The plasmids contained in each strain tested are indicated at the top of each column and at the left of each row. No insert: pAS2, SNF4: pACT2 containing SNF4 control, and SNF1: pAS2 containing SNF1 (control). BD: binding domain. AD: activation domain.

Table 1
Specific β-galactosidase activity assay for protein interactions in the two-hybrid assay

<table>
<thead>
<tr>
<th>Protein Interaction</th>
<th>Specific β-galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y5 (AD) + hMSH2 (BD)</td>
<td>6.3</td>
</tr>
<tr>
<td>Y5 (AD) + hMSH2-N (BD)</td>
<td>0</td>
</tr>
<tr>
<td>HEX1-N (AD) + hMSH2 (BD)</td>
<td>0</td>
</tr>
<tr>
<td>HEX1-N (AD) + hMSH2-N (BD)</td>
<td>0</td>
</tr>
<tr>
<td>HEX1-C (AD) + hMSH2 (BD)</td>
<td>0.3</td>
</tr>
<tr>
<td>HEX1-C (AD) + hMSH2-N (BD)</td>
<td>0</td>
</tr>
<tr>
<td>hMSH2 (AD) + HEX1 (BD)</td>
<td>17.7</td>
</tr>
<tr>
<td>hMSH2 (AD) + hEXO1b (BD)</td>
<td>12.8</td>
</tr>
<tr>
<td>SNF4 (AD) + SNF1 (BD)</td>
<td>3.1</td>
</tr>
<tr>
<td>pACT2 (AD) + pAS2 (BD)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Specific β-galactosidase activity was determined as described in Materials and methods.

between hMSH2 and hEXO1a/HEX1 (C-terminal region) (Fig. 2B, Table 1). Our Y5 (hEXO1b C-terminal) clone did not contain the full-length hEXO1b cDNA, but instead contained an insert encoding the carboxyl half of hEXO1b protein beginning at amino acid 302. These data argue that the carboxyl-terminal region of hMSH2 contacts the carboxyl-terminal domains of both hEXO1a/HEX1 and hEXO1b. Thus, the hMSH2-interacting region is outside the N and I regions (Fig. 1) thought to be required for exonuclease activity [12,25,26]. Our results complement the finding that S. cerevisiae yMSH2 interacts with the carboxyl-terminal region of yEXO1 outside the N and I regions [16]. hEXO1a/HEX1 differs from hEXO1b by two nucleotides (2616-AG-2617) at the boundary of exon 14 (Fig. 1). This frame-shift results in the truncation of the hEXO1a/HEX1 polypeptide by 43 amino acids. We show that hMSH2 interacts with both forms of human exonuclease 1, suggesting that the interacting domain is located between exons 8 and 13 (Fig. 1). Interestingly, from data shown in Fig. 2 it appears that hMSH6 does not interact with hEXO1 in our two-hybrid assay.

3.3. Expression of exonuclease and hMSH2 in human tissues

We used a human RNA master blot to characterize the expression pattern of hEXO1 and to look for
tissues predominately expressing the C-terminal transcript of \textit{hEXO1}. One of the DNA probes contained only the C-terminal region of \textit{hEXO1a/HEX1} and should detect all \textit{hEXO1} splice variants identified to

\begin{figure}
\centering
\includegraphics[width=\textwidth]{expression_profiles.png}
\caption{Expression profiles of human exonuclease 1 and \textit{hMSH2}. The RNA master blot was hybridized with \textit{hMSH2}, \textit{hEXO1a/HEX1} N-terminal, \textit{hEXO1a/HEX1} C-terminal or \textit{\beta}-actin control probes.}
\end{figure}
date. The second probe contained the N-terminal region of \( h\text{EXO1a/HEX1} \), which would be specific for full-length \( h\text{EXO1a/HEX1} \) and \( h\text{EXO1b} \) mRNAs, but would not detect the C-terminal transcripts (Fig. 1). We reasoned that by comparing the expression patterns obtained with these two probes, we would be able to identify tissues that express only C-terminal fragment. Since we did not observe any differences in expression results, it would appear that no tissue preferentially expresses the C-terminal part of \( h\text{EXO1} \) (Fig. 3). We do not know whether the C-terminal part of \( h\text{EXO1} \) is naturally present and has a specific function in the cell or whether its occurrence is a result of inefficient DNA synthesis during the construction of the matchmaker cDNA library, but presume the latter to be true based on the fact that shorter transcripts were not observed. Both probes detected transcripts prominently expressed in fetal liver, adult testis and thymus, but we also observed significant expression in adult colon, small intestine, bone marrow, placenta, fetal kidney, fetal spleen and fetal thymus (Fig. 3). Interestingly, we did not detect any expression of \( h\text{EXO1} \) mRNA in adult liver, spleen, and kidney, indicating a specific role for \( h\text{EXO1} \) in the development of these tissues.

Since \( h\text{MSH2} \) interacts with exonuclease 1 (Fig. 2), we compared the expression patterns of \( h\text{MSH2} \) with that of \( h\text{EXO1} \) to identify tissues where the exonuclease could play a role in DNA repair and/or recombination. We found that \( h\text{MSH2} \) is expressed in all tissues investigated (Fig. 3). Fig. 3 shows that \( h\text{MSH2} \) and \( h\text{EXO1} \) are coexpressed at higher levels in fetal liver, adult testis, thymus, and at lower levels in bone marrow, placenta, fetal spleen, fetal kidney and fetal thymus.

### 3.4. Expression of exonuclease and \( h\text{MSH2} \) in human liver cancer cell lines

We have shown that \( h\text{EXO1} \) is expressed in the fetal liver but not in the adult liver (Fig. 3). We thus examined the expression of the \( h\text{MSH2} \) and \( h\text{EXO1} \) genes in various human liver cell lines (Fig. 4): HepG2 (human hepatocellular carcinoma), SK-HeP1 (human liver adenocarcinoma), Huh-7 (human hepatocellular carcinoma), Chang (human liver), and WRL68 (human liver embryo). As expected, both \( h\text{EXO1} \) and \( h\text{MSH2} \) were highly expressed in the fetal liver but not in the adult liver (Fig. 4). One reason for failing to detect any \( h\text{MSH2} \) expression in the adult liver in this experiment could be that the blot shown in Fig. 3 contains poly(A)\(^+\) mRNA while the blot in Fig. 4 contains total RNA. However, since \( h\text{MSH2} \) is preferentially expressed in proliferating cells [27–29], high transcript levels in the normally quiescent adult liver would be unexpected. When the \( h\text{EXO1} \) probes were used, we could only detect a single transcript of approximately 3 kb, which corresponds to full-length \( h\text{EXO1} \) (Fig. 4 and data not shown). This result suggests that \( h\text{EXO1} \) mRNA is unlikely spliced to any of the shorter forms identified in the two-hybrid screens and that the C-terminal clones likely do not exist. A 3-kb transcript was detected using the \( h\text{MSH2} \) probe, which corresponds to the full-length \( h\text{MSH2} \) cDNA. Inter-
Interestingly, we find that both \textit{hEXO1} and \textit{hMSH2} are highly expressed in the liver cancer cell lines HepG2, SK-Hep1 and Huh7 as well as in the fetal-like Chang and WRL68 cells.

3.5. Expression of exonuclease and hMSH2 in tumor tissue

Our results revealed that \textit{hEXO1} is expressed preferentially in tissue containing proliferating cell populations as well as in cell lines established from human liver tumors (Figs. 3 and 4). This led us to investigate the expression of \textit{hEXO1} in human tumors from colon and pancreas. Interestingly, we found that \textit{hEXO1} expression is indeed upregulated in samples isolated from colon and pancreas adenocarcinomas compared to normal tissue (Fig. 5). These results support the idea of \textit{hEXO1} being preferentially expressed in proliferating cells. Interestingly, we found that \textit{hMSH2} expression is upregulated in the colon tumors but not in the pancreas tumors (Fig. 5). These results may imply a defect in MMR activity in the pancreas tumors due to the low levels of \textit{hMSH2} protein.

4. Discussion

Eukaryotic proteins are often components of larger complexes. It is therefore easy to imagine that the disruption of a particular protein–protein interaction might affect several different cellular processes. In addition, many eukaryotic genes show tissue-specific expression and it is conceivable that the composition of protein complexes varies among different cell types and tissues. In agreement with this model, it has been shown that mice carrying disruptions of specific MMR genes display a variety of phenotypes ranging from tissue-specific tumorigenesis to sterility [20,30–35]. This is consistent with the evidence that implicates MMR proteins in processes other than postreplicative MMR. Homologous and homeologous recombination, transcription coupled repair or cell cycle control following DNA damage are examples of such pathways [2,4,6]. It is possible, even likely, that MMR proteins will interact with different partners in each of these alternative pathways. Identification of these partners will help elucidate the mechanisms of these complex processes.

In an attempt to identify tissue-specific MMR-associated factors, we employed the yeast two-hybrid system, using the human \textit{hMSH2} as bait and a human fetal liver library as prey. We demonstrate that \textit{hMSH2} interacts with \textit{hEXO1} through their C-terminal domains. Another MutS homolog \textit{hMSH6} does not interact with \textit{hEXO1} in the two-hybrid system. It has previously been shown that the human exonuclease 1 is expressed in a wide variety of tissues [11]. Our comparative expression analysis revealed that \textit{hMSH2} and \textit{hEXO1} are coexpressed in the fetal liver, adult testis and thymus as well as in colon, small intestine, and in the fetal tissues of kidney and spleen but not in the corresponding adult tissues. \textit{hEXO1}/\textit{HEX1} is highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue.
4.1. Interaction between hMSH2 and human exonuclease 1

The human MMR protein hMSH2 forms heterodimers with hMSH3 or with hMSH6, which function in the substrate recognition step during MMR. In the subsequent step, these bound heterodimers interact with the hMLH1/hPMS2 complex. Although the following events of the MMR process are not clear, it is anticipated based on the bacterial model that the four-protein complex will recruit DNA helicase and exonuclease activities, which mediate the displacement and the degradation of the mismatch-containing strand, respectively. The position of the strand break and the initiation site of the exonucleolytic process with respect to the mismatch would determine the nature of the exonuclease. Thus, repair of mismatches with upstream-positioned strand-discrimination nicks will require a 5'-3' exonuclease such as EXO1 [16]. Conversely, MMR involving downstream nicks would require 3'-5' exonucleases to mediate the degradation process. In the present study, we show that hMSH2 interacts with the human 5'-3' exonuclease hEXO1. We are currently investigating whether the hMSH2/hEXO1 complex functions in MMR or other pathways such as recombination, where hEXO1 has already been shown to play a role [11,16].

4.2. Comparative expression analysis of hEXO1

Tumorigenesis in HNPCC patients is primarily restricted to the proximal colon and endometrium. We found that the hMSH2 and hEXO1 genes are coexpressed, although at low levels, in colon and small intestine. Due to the likely redundancy between exonucleases in the MMR process, it would appear unlikely to anticipate that mutations in the hEXO1 gene will segregate with HNPCC. Human population studies monitoring the association of hEXO1 gene defects with cancer or the construction of a mEXO1 knockout mouse should clarify if this exonuclease plays any role in carcinogenesis. Our expression profiles of hMSH2 and hEXO1 also revealed that these genes are coexpressed in human testis and bone marrow, suggesting a role in meiosis and hematopoiesis likely due to their function in recombination [36]. Along these lines, we have also shown that both hEXO1 and hMSH2 are highly expressed in the fetal liver, a site of hematopoiesis during fetal development. However, in addition to supporting hematopoiesis during embryonic development, the fetal liver is also characterized by rapid division of fetal hepatocytes. We have shown that hEXO1 and hMSH2 are not expressed in normal adult liver where cell turnover is normally very slow. However, both genes are highly expressed in the liver cancer cell lines HepG2, SK-Hep1 and Huh7 as well as in the fetal-like Chang and WRl68 cells. Furthermore, we showed that hEXO1 is highly expressed in colon and pancreas adenocarcinomas but not in the corresponding non-neoplastic tissues. These results suggest a role for hEXO1 in cellular processes other than meiosis and hematopoiesis [36]. Such processes could be MMR, recombinational repair or replication, which are expected to be active in proliferating cells such as fetal hepatocytes and cancer cells. Current studies are examining for potential associations of hEXO1 with factors from these cellular activities. The fact that hEXO1 is specifically expressed in liver cancer cells as well as in colon and pancreas adenocarcinomas could make the expression of this gene a useful marker to diagnose these cancers.

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References


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Characterization of $O^6$-MeG DNA Methyltransferase (MGMT) protein in repairing human mitochondrial DNA

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Abstract

DNA repair is essential for maintaining the integrity of the genetic material. A number of DNA repair mechanisms have been well characterized for the nuclear DNA. However, little is known about DNA repair in mitochondria. Mutations in mitochondrial DNA (mtDNA) are involved in the pathogenesis of a variety of diseases including cancer, heart disease, diabetes, and a number of degenerative diseases. Furthermore, accumulation of mutations in mtDNA is a common feature of aging cells. Human mtDNA is extremely rich in guanine, therefore, repair of modified guanine is important for proper mitochondrial function. Certain intracellular or exogenous DNA damaging agents are known to frequently modify guanine to $\text{O}^{6}$-MeG in DNA. Generation of $\text{O}^{6}$-MeG is mutagenic because it can mispair with thymine resulting in G:C $\rightarrow$ A:T transition mutations. The DNA repair protein $\text{O}^{6}$-Methylguanine-DNA Methyltransferase (MGMT) removes methyl groups from the $\text{O}^{6}$ position of guanine ($\text{O}^{6}$-MeG) in the nuclear DNA. In order to gain understanding of the role of MGMT in mitochondria, we investigated (1) whether MGMT contains mitochondrial localization signals and is transported to mitochondria, (2) whether MGMT, when expressed in mitochondria can repair $\text{O}^{6}$-MeG DNA lesions in this organelle, and (3) whether MGMT when expressed in mitochondria can protect cells from apoptosis induced by the DNA damaging agents which modify guanine in mtDNA.
**Introduction**

Mammalian cells contain two distinct genomes: a nuclear (nDNA) and a mitochondrial (mtDNA). Despite each human somatic cell contains 100 to 1000 mitochondria, that each contains 1 to 10 mitochondrial genomes, the amount of the mtDNA is only estimated to account for 1% of total DNA in the cell (Wallace *et al*., 1998; Bestwick, 1982).

In contrast to nDNA the mammalian mitochondrial genome contains few noncoding sequences, no introns and it is not protected by histones. Thus, damage to mtDNA is expected to have great impact on the function of this organelle. Accordingly, it has been reported that the rate of point mutations is elevated in mtDNA compared to nDNA (Khrapko *et al*., 1997). Point mutations in and genomic rearrangements of mtDNA have been recognized to play a critical role in numerous human disorders underscoring the importance of maintaining the integrity of the mitochondrial genome (Pulkes and Hanna, 2001; Wallace, 1999; Kogelnik *et al*., 1998).

Given the importance of maintaining the mitochondrial genome intact for organelle activity, efficient DNA repair systems in mitochondria would be expected. Even so, early investigations showed that UV-induced pyrimidine dimers are not removed from mtDNA, a result that led to the conclusion that mitochondria are deficient in DNA repair (Clayton *et al*., 1974; Prakash *et al*., 1975). These findings encouraged the model that rather than repairing DNA damage in mtDNA, cells simply eliminate damaged genomes and replace them by replicating existing undamaged mitochondrial genomes (LeDoux *et al*., 1999). It was later confirmed that UV-induced pyrimidine dimers are not repaired in mitochondria (LeDoux *et al*., 1992; Pascucci *et al*., 1997) suggesting that mitochondria do not have a functional nucleotide excision repair (NER) pathway. However, recent research has shown that at least base excision repair (BER) activities are operational in mitochondria showing that this organelle does indeed repair damaged mtDNA (Croteu *et al*., 1999; Sawyer *et al*., 1999). But the knowledge about the mechanisms underlying DNA repair in the mitochondria is still limited.

DNA repair alkyltransferases, responsible for removing methyl groups from the $O^6$ position of guanine, have been identified in both prokaryotic and eukaryotic organisms. The genes coding for these DNA repair alkyltransferases have been cloned from *Escherichia coli* (*ada*, Sedgwick, 1983; *ogt*, Potter *et al*., 1987), *Saccharomyces cerevisiae* (*MGT1*, 1983).
Xiao et al., 1991), mouse (MGMT, Shiota et al., 1992), rat (MGMT, Rahden-Staron and Laval, 1991) and human (MGMT, Tano et al., 1990; Rydberg et al., 1990; Hayakawa et al., 1990). To date most research has focused on the role of DNA repair alkyltransferases in repair of nDNA but mitochondrial MGMT activity has been reported in rat liver (Myers et al., 1988; Satoh et al., 1988).

A mitochondrial DNA repair alkyltransferase has been partially purified from rat liver mitochondria and it was shown that this protein is similar in size to the 23 kDa DNA repair protein found in nuclei (Myers et al., 1988). DNA damage caused by the methylating agent N-methyl-N-nitrosourea (MNU) is repaired in mitochondria from Chinese Hamster Ovary (CHO) cells (LeDoux et al., 1992) suggesting that an alkyltransferase repair mechanism is active in mammalian mitochondria. However, mitochondrial MGMT activity has not been identified in human cells and the intracellular localization seems ambiguous.

To gain a better understanding of the molecular processes and components responsible for DNA repair of methylation damage in the human mitochondrion we have investigated the localization of human MGMT. We found that full-length MGMT localized to the nucleus when expressed as a GFP fusion protein. This nuclear localization was independent of p53 status and we did not observe any MGMT protein in mitochondria in cells treated with MNNG. Our results suggest that the N-terminal region (codons 2-18) is required for directing the MGMT protein to nucleus. Expression of the MGMT protein lacking codons 2-18 in the mitochondria protected the cells from the cytotoxic effect of MNNG but had no effect on survival of cells treated with the oxidizing agent Menadione.

3 Variations in the human mitochondrial genomes are updated on [http://www.gen.emory.edu/mitomap.html](http://www.gen.emory.edu/mitomap.html) (Kogelnik et al., 1998).
Materials & Methods

Cell Cultures

Human MCF12A (breast, mammary gland, normal, non-tumorigenic epithelial) cells were obtained from ATCC. MCF12A cells were maintained in DMEM/F12 growth medium (Gibco/Life Technologies) supplemented with 10% Horse Serum (Gibco/Life Technologies), 1% Penicillin-Streptomycin (Gibco/Life Technologies), 100 ng/ml Cholera Toxin (Sigma), 500 ng/ml Hydrocortisone (Sigma), 10 µg/ml Insulin (Sigma), and 20 ng/ml EGF (Epidermal Growth Factor, PeproTech) at 37°C in a humidified 95% air-5% CO₂ atmosphere. HCT116 p53-/- (colorectal carcinoma) human cells were obtained from Dr. Bert Vogelstein (Johns Hopkins University) and maintained in McCoy's 5A medium (Gibco/Life Technologies) supplemented with 10% fetal bovine serum (Gibco/Life Technologies), 1% Penicillin-Streptomycin (Gibco/Life Technologies) at 37°C in a humidified 95% air-5% CO₂ atmosphere. HeLa (HSL2, cervical carcinoma) cells were maintained as previously described (Singh et al., 1999) in DMEM (Gibco/Life Technologies) supplemented with 10% FBS (Gibco/Life Technologies), 1% Penicillin-Streptomycin (Gibco/Life Technologies) and 4 ng/ml uridine (Gibco/Life Technologies) at 37°C in a humidified 95% air-5% CO₂ atmosphere.

Construction of plasmids

A DNA sequence of MGMT lacking the terminal 23 amino acids (codon 185 to 207) was cloned into the Eco47III and SacI sites of pEGFP-N2 (Clontech). Oligonucleotides were used to PCR amplify a MGMT fragment (ΔMGMT) lacking codons 2 to 18 and 185 to 207. The 5' primer: 5'GAAGATCTATGCTGGAG CTGTCTGGTT3' contains a BglII site and the 3' primer, 5'TTGAGCTC CCTCCCAAGCCTG3' contains a SacI site. The ΔMGMT fragment was cloned into the BglII and SacI sites of the pEGFP-N2 vector. The pShooter vectors pEF/myc/mito and pEF/myc/mito/GFP (control) (Invitrogen) containing the mitochondrial targeting sequence (MSVTPLLRLRGLTSARRLPVPRAKIHS) are designed to express and target a recombinant protein to the mitochondria in mammalian cells. Plasmid pEF/myc/mito-MGMT plasmid was constructed by inserting the complete coding sequence of human MGMT into the XhoI and NotI sites of pEF/myc/mito. The fragment containing full-length MGMT was amplified by PCR using the primers: (5' primer) 5'CCGCTCGAGATGGACAAGGATTGTGA3' containing a XhoI site and (3'
primer) $5'$AAGCGGCCGCTCAGTTTCGCCAGCA$<sup>3'</sup> containing a NotI site. The DNA sequences of all constructs were confirmed by DNA sequencing.

**Transfection**

Cell cultures were transfected using either Lipofectamine2000 or Lipofectin (Gibco/LifeTechnologies) according to manufacturer’s guidelines. For fluorescence microscopy exponentially growing cells were counted and diluted, and $3 \times 10^5$ cells were seeded in 2 ml culture medium in glass bottom dishes (BioSoft International, Amsterdam, The Netherlands) one day prior to treatment. On the day of treatment cells were transfected with 3.5 $\mu$g DNA of each construct and incubated at 37°C in a humidified 95% air-5% CO<sub>2</sub> atmosphere for 24 hours before they were assayed for MGMT-GFP expression. Alternatively, exponentially growing cells were counted and diluted, and $7 \times 10^4$ cells were seeded in 2 ml culture medium without serum in tissue culture dishes containing coverslips one day prior to treatment. On the day of treatment cells were transfected with 2 $\mu$g DNA and incubated at 37°C in a humidified 95% air-10% CO<sub>2</sub> atmosphere for 48 hours before they were assayed. In these experiments the growth medium was changed every 18 hours.

**MitoTracker staining**

For detection of mitochondria, MitoTracker Red (CMXros, Molecular Probes, Eugene, OR) was added to the growth medium of the transfected cells. Cells grown in glass bottom dishes were stained with 500 nM of MitoTracker Red for 20 min at 37°C in a humidified 95% air-5% CO<sub>2</sub> atmosphere. Cells grown on coverslips were stained with 50 nM of MitoTracker Red for 30 min in a humidified 95% air-5% CO<sub>2</sub> atmosphere before fixation with 3.7% formaldehyde in Hank's balanced salt solution. Cells were treated with 20 $\mu$M MNNG (N-methyl-N’-nitro-N-nitrosoguanidine) (Sigma) for 2 or 3 hours. After 1 hour and 45 min of MNNG treatment the growth medium was changed to medium containing both MNNG and MitoTracker Red and incubated further. The cells were visualized using a LSM 510 confocal laser scanning microscope (Zeiss) or a Zeiss-Axiovert 135 TY Inverted microscope equipped with PXL camera (SENSYS Phptometrics).

**Cell proliferation assay**

The Cell Titer 96TN AQueous nonradioactive cell proliferation assay kit (Promega) was used to assay cell survival. Exponentially growing cells were counted, diluted, and seeded
in 100 µl of culture medium in 96-well microtiter plates at a cell density of 1 x 10^4 cell/well one day prior to treatment. On the day of treatment cells were transfected with 0.32 µg DNA/well in 125 µl growth medium. Cells were transfected with either pEF/myc/mito/GFP (control), pEF/myc/mito-MGMT, pEGFPN2 (control), MGMT-GFP or ΔMGMT-GFP. Cells were incubated for 2-3 days at 37°C in a humidified 95% air-5% CO_2 atmosphere before treatment with 0, 13.6, 54.4, 108.8, and 217.6 µM MNNG or 100, 400 or 1000 µM Menadione (Sigma). Cells were incubated for 3 days and on the third day cell survival was evaluated by the cell proliferation assay according to manufacturer’s guidelines. The data represent an average of two experiments where each data point represents an average of ten independent measurements.
Results

The human MGMT protein localizes to nucleus

DNA repair alkyltransferases have been identified in both nuclear and cytoplasmic fractions of eukaryotic cells (Ayi et al., 1992; Lee et al., 1992; Brent et al., 1993; Ishibashi et al., 1994a,b; Lim and Li, 1996). Cytoplasmic appearance of MGMT indicates that this repair protein could be present in mitochondria. To access the actual localization of the MGMT protein we studied exogenous MGMT-GFP expressed as fusion protein in human breast cell line MCF12A and detected the subcellular localization by fluorescence microscopy. We found that MGMT protein is mainly localized in the nucleus (Figure 2), which is supported by most of the immunostaining analysis reported (Ayi et al., 1992, Lim and Li, 1996; Belanich et al., 1996). However, we observed no mitochondrial localization concluded from the results of Mito Tracker Red staining of the cells (Figure 2). We did not observe any difference in subcellular localization of MGMT when we compared fixed cells (Figure 2B) with viable cells (Figure 2C). Our control experiment expressing the GFP alone showed the expected diffuse localization (Figure 2A). These results reveal that the human MGMT, when expressed as a GFP fusion protein, localizes in the nucleus. The results presented in Figure 2 also show that the codons 184 to 207 of the C-terminal part of MGMT is not required for proper nuclear localization of the protein as these were deleted in our construct. We deleted codons 2 to 18 of MGMT in order to investigate the importance of this region in subcellular localization of MGMT. The ΔMGMT-GFP construct, lacking codons 2 to 18 of the N-terminal region and codons 184 to 207 of the C-terminal region of MGMT, was exogenously expressed in MCF12A cells. Interestingly, we found that deletion of codons 2 to 18 resulted in weak nucleus staining and an accumulation of ΔMGMT in cytoplasm (Figure 2D). We observed weak fluorescence with several fluorescence forming dots within the cytosol. However, the granular dots was not identical with the mitochondrial Mito Tracker Red staining, although we can not exclude that any cytoplasmic ΔMGMT protein is located in mitochondria because of the uniform weak fluorescence around the granular dots (Figure 2D). Our data suggest the N-terminal domain or more specifically codons 2 to 18 are essential for proper nuclear localization of MGMT.
The localization of MGMT is independent of p53 status

The p53 protein has been detected inside the mitochondria but the majority of mitochondrial-associated p53 is found on the surface of this organelle (Sansome et al., 2001). Furthermore, the p53 protein has been shown to reduce transcription of the MGMT gene in human fibroblasts (Harris et al. 1996). Both p53 and MGMT play roles in DNA damage recognition and repair. The p53 protein is believed to detect DNA damage and activate one or more cell cycle checkpoints that allow repair of damaged DNA. In contrast, there has been no evidence so far that MGMT is involved in activation of cell cycle checkpoints but a role in repair of DNA damage is well established. Previous studies by Balanich et al. (1996) have shown that MGMT in HT29 colon cancer cells were localized to nucleus and cytoplasm (cell lines defective in p53, Gaye et al., 2001), indicating that MGMT could be present in nucleus, cytoplasm and mitochondria in p53 defective cells. In order to explore a possible link between p53 and MGMT we expressed the MGMT-GFP fusion protein in two cell lines differing in p53 status. One cell line is completely deficient in p53 activity (HCT116 p53-/-) and the other expresses very low levels of the p53 protein (HeLa HSL2). We found no difference in subcellular localization of MGMT (Figure 3) suggesting that MGMT translocation is independent of the p53 status. In both cell lines the MGMT-GFP fusion protein was exclusively present in the nucleus.

Alkylating agents have no effect on the localization of MGMT

It has been shown that mtDNA is damaged by alkylating agents and that O\(^6\)-Me-G DNA lesions are repaired in mitochondria (Satoh et al., 1988; LeDoux et al., 1992). However, our data suggest that MGMT locates to the nucleus and no MGMT protein is detected in mitochondria of untreated cells (Figure 2). In order to test if nuclear MGMT is recruited to mitochondria upon DNA damage of mtDNA by alkylating agents we treated cells expressing MGMT-GFP with MNNG. MCF12A cells were treated with 20 \( \mu \)M MNNG for 3 hours and cells were analyzed by fluorescence microscopy. The MGMT-GFP fusion protein was detected in nucleus but not in mitochondria (Figure 4) indicating that nuclear MGMT is not recruited to mitochondria after DNA damage by MNNG.

Effect of MGMT expression in protecting cells from alkylating agents

Research from other groups has shown that O\(^6\)-Me-G and O\(^6\)-Et-G lesions are repaired in mitochondria (Satoh et al., 1988; LeDouxi et al., 1992). Our data suggest that MGMT does
not play a role in repair of O\textsuperscript{6}Me-G DNA lesions in mitochondria. We constructed a set of plasmids in order to (1) investigate the cytotoxic effect of alkylation damage in cells expressing MGMT in mitochondria and (2) investigate the role of the cytoplasmic ΔMGMT protein in protecting cells after alkylation damage. MCF12A cells were transfected with the pEF/myc/mito-MGMT construct, which directs the fusion protein to mitochondria, as well as the pEF/myc/ΔMGMT-GFP and pEF/myc/MGMT-GFP constructs. Surprisingly, expression of mito-MGMT increased sensitivity to killing by MNNG whereas expression of ΔMGMT-GFP provided the same level of protection to killing by MNNG as MGMT-GFP (Figure 5). We also treated cells with Menadione, which introduce oxidative damage to DNA. These lesions are not repaired by MGMT serving as a control for the effects on cell survival observed after transfection with the plasmids and MNNG treatment are specific for alkylation damage. We did not observe any difference in cell survival after treatment with Menadione (Figure 6). Our results indicate that the presence of MGMT in mitochondria increases sensitivity to MNNG. Conversely the cytoplasmic ΔMGMT protein protects cells from killing by MNNG at high doses.
Discussion

MGMT is a small protein of 207 amino acids and a molecular weight of 21.7 kDa (Hayakawa et al., 1990; Rydberg et al., 1990). It has been proposed that small non-nuclear proteins, with a molecular weight less than 40-60 kDa can passively equilibrate between the cytosol and the nucleus (Weis, 1998). The small size of the MGMT protein and its DNA binding property may allow apparent mobilization to nuclear DNA by passive diffusion and DNA binding. However, when bacterial methyltransferase (ada; 39 kDa) is expressed in mammalian cells it fails to accumulate in the nucleus of NIH-3T3 cells (Dumenco et al., 1989). Therefore, it seems unlikely that the “diffusion” model of the entry into the nucleus for this protein operates. It has been suggested that the sequence -KLLKVVK- (codons 101-107) shares some homology to the nuclear location signal PKKRRKV (Ayi et al., 1992; Weis, 1998). However, mutants of this basic –KLLKVVK- region of MGMT were DNA binding and repair deficient but entirely nuclear (Lim and Li, 1996). MGMT expressed as β-gal fusion protein, which is too large (130 kDa) for the passive diffusion into the nucleus, was shown to be present in the nucleus (Lim and Li, 1996). We show that a MGMT-GFP fusion protein localizes to the nucleus suggesting that MGMT can target the GFP into the nucleus. We also show that the MGMT protein does not localize to mitochondria when fused to GFP.

Comparison of the structures and sequences of bacterial (Ada) with the human MGMT proteins reveals that the similarity between these proteins is highest in the C-terminal region (MGMT amino acids 92 to 174 and Ada amino acids 94 to 175)(Wibley et al., 2000). A divergent N-terminal region could therefore explain nuclear-targeting differences between Ada and MGMT. It has been suggested that the residues Glu45 to Gly55 form a "hydrophobic handle" that may serve to anchor MGMT to other proteins (Wibley et al., 2000). The conformation of residues Glu45 to Gly55 is stabilized by the first β-strand (residues Lys8 to Ser14) in MGMT. We showed that a deletion of the first 18 amino acids is sufficient to keep MGMT in the cytoplasm. Mutant proteins of MGMT carrying deletions of either codons 1 to 10 or 1 to 19 were active in protecting cells from killing by MNNG (Crone et al., 1996). These results suggest that these mutant proteins are active in repair of alkylation DNA damage in vivo. However, it was shown that the mutant MGMT proteins were less effective in repair compared to wild type MGMT as well as highly unstable with half-lives of 48-90 min compared to a half-live of > 720 min for the
wild type MGMT. We found that the ΔMGMT-GFP construct lacking codons 2 to 18 of the N-terminus resulted in weak fluorescence from nuclear and several bright granular dots in the cytosol, indicating degradation in lysosome and therefore a shorter half-live than MGMT-GFP. Interestingly, it has been shown that both N- and C-terminal deletions of the MGMT protein (deletion of codons: 1-30; 1-60; 1-90; 1-120; 1-150; 1-180; 165-180; 59-207 or 31-207) affect the nuclear localization of MGMT (Lim and Li, 1996). In summary these observations indicate that the structure of MGMT is of great importance for subcellular localization.

We re-evaluated possible targeting signal for nucleus and mitochondria by using the PredictNLS, MitoProt and PSORT software programs. PredictNLS finds experimentally known nuclear localization signals in a given protein. MitoProt calculates the N-terminal protein region that can support a Mitochondrial Targeting Sequence (MTS) and the cleavage site (Claros and Vincens, 1996) and PSORT finds both MTS and NLS sequences (Nakai et al., 1992). Neither of these programs identified any nuclear localization signal or Mitochondrial Targeting Sequence (MTS) in the MGMT protein (data not shown).

The majority of proteins, which are synthesized in the cytoplasm and imported to the mitochondria, contain a MTS in the N-terminal region of the protein. The sequence is diverse in both length (20 to 60 amino acid residues) and composition. However, the major feature appears to be residues with abundant positive charges, very little if any negative charges, and frequent hydroxylated residues which can form an amphipathic a-helix (Neupert, 1997). A fraction of mitochondrial proteins lacks this targeting sequence, for instance, APE1 which has been shown to locate to rat mitochondria (Tell et al., 2001). In fact a large number of outer and inner membrane proteins, as well as proteins that reside in the intermembrane space, have internal targeting signals and no MTS (Neupert, 1997). The conclusion of the re-evaluation of possible targeting signals is, therefore, that MGMT seems to lack both MTS and experimentally known nuclear signals.

Overexpression of the simple glycosylase MPG (AAG) increases sensitivity to alkylating agents as a result of accumulation of cytotoxic AP sites (Limp-Foster and Kelley, 2000; Coquerelle et al., 1995). This is supported by the finding that overexpression of MPG in the human breast carcinoma cells does not alter the activities of the proteins immediately downstream in the BER pathway (i.e. APE1) (Kreklaau et al., 2001). Increased sensitivity to alkylating agents is also observed when MPG is overexpressed in the mitochondria.

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4 PredictNLS (http://cubic.bioc.columbia.edu/predictNLS) & MitoProt (www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter) & PSORT (http://psort.nibb.ac.jp)
(personal communication with Dr. Mark R. Kelley, Department of Pediatrics - Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN, USA, submitted to JBC). It was concluded that overexpression of MPG results in imbalance of the proteins involved in BER. MGMT is a direct repair protein and it is unlikely to cause imbalance in downstream components of a pathway. However, it has been suggested that MGMT binds to $O^4$-MeT DNA lesions, but the repair is slow, resulting in hindrance of NER of this lesion (Samson et al., 1997). This could explain the increased sensitivity to MNNG treatment in cells overexpressing the mito-MGMT protein in mitochondria (Figure 6). However; so far no NER activity has been identified in mitochondria. Alternatively, MGMT blocks the mitochondrial DNA replication by binding to $O^4$-MeT DNA lesions.

In summary, we have shown that MGMT when fused to GFP does not localize to mitochondria but to the nucleus. This nuclear localization is independent of the p53 status of the cells. We show that deletion of the first 18 amino acids is sufficient to keep MGMT in the cytoplasm and overexpression of MGMT in mitochondria increases sensitivity to the alkylating agent MNNG.
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Manuscript II


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Legends to figures

Figure 1. Nucleotide sequence of human $MGMT$ cDNA. Letters in bold indicate the sequences deleted in full-length and truncated MGMT expression plasmids. A highlighted box marks the cysteine acceptor residue surrounded by the 3 conserved amino acids.

Figure 2. Subcellular localization of MGMT in MCF12A cells. (A) GFP-control, MCF12A transfected with pEGFP-N2 vector. (B) Detection of fixed cells transfected with MGMT fused to GFP. (C) Detection of viable cells transfected with MGMT fused to GFP. (D) Detection of viable cells transfected $\Delta$MGMT-GFP construct lacking codon 2-18 of the N-terminus.

Figure 3. Subcellular localization in HeLa and HTC116 (p53-/-) cells. (A) Detection of HeLa cells transfected with fusion product of MGMT and GFP. (B) Detection of HTC116 (p53-/-) cells transfected with MGMT-GFP.

Figure 4. Localization of MGMT in MCF12A cells after 20 $\mu$M MNNG treatment for 3 hours.

Figure 5. Survival following methylation. MCF12A cells transfected with Mito-MGMT, MGMT-GFP and $\Delta$MGMT-GFP were treated with different doses of MNNG (0, 13.6, 54.4, 108.8 or 217.6 $\mu$M) before the viability of the cells were determined. Each data point represents an average number of ten measurements from one representative experiment.

Figure 6. Survival following oxidative damage. MCF12A cells transfected with Mito-MGMT, MGMT-GFP and $\Delta$MGMT-GFP were treated with different doses of Menadione (0, 100, 400 or 1000 $\mu$M) before the viability of the cells was determined. Each data point represents an average number of ten measurements from one representative experiment.
Figure 1

Codons 2-18 are deleted from MGMT-GFP

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Codons 185-207 are deleted from MGMT-GFP and ∆MGMT-GFP
Figure 2

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Figure 4

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Figure 5

![Graph showing survival against MNNG (µM)]

Figure 6

![Graph showing survival against Menadione (µM)]
Manuscript III

Mitochondria as determinant of genetic stability in

Saccharomyces cerevisiae

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Accumulation of mitochondrial mutations is a consistent phenotype of cancer cells (1-2). Another hallmark of cancer cells is the generation of a mutator phenotype, which results in a rapid accumulation of mutations that, drives tumor development (3). To date, it is not clear how mitochondrial dysfunction impacts on the genetic stability of nuclear genome. Using *Saccharomyces cerevisiae* as a model organism we analyzed the consequences of disrupting mitochondrial function on genetic stability of the nuclear genome. In strains of yeast lacking mitochondrial DNA (rho<sup>0</sup>), and those with mitochondrial mutations (rho<sup>-</sup>), we tested the instability of the nuclear genome by measuring the frequency of canavanine resistant colonies. We demonstrate that mitochondrial dysfunction induces mutations in the nuclear genome. We also demonstrate that *REV1, REV3* or *REV7* gene products, implicated in error-prone translesion DNA synthesis (TLS) (4) mediate the genetic instability of the nuclear genome arising as a result of mitochondrial dysfunction. Our results in the yeast model provide a direct link between mitochondrial dysfunction and genomic instability, which has important implications in human cancer and aging.
Besides generating ATP, mitochondria perform multiple functions that are essential to the cell (5). Mitochondrial dysfunction is one of the most common and profound phenotypes of many human cancers (6-14). Mitochondrial dysfunction is also involved in aging, adaptive mutation, pathogenesis of many childhood and adult mitochondrial diseases (7, 16-20). Aging studies and studies conducted in human cancer cells have reported the accumulation of mutations in mitochondrial DNA (mtDNA) (2, 5, 11-12, 16-19). In this paper, we investigated the effect of mitochondrial dysfunction on the integrity of the nuclear genome. We generated yeast strains that were either impaired in mitochondrial activity due to mutations in the mitochondrial genome (rho\(^-\)) or strains deficient in mitochondrial functions due to the absence of the entire mitochondrial genome (rho\(^0\)) (Figure 1). To these cells, we applied a mutation detection assay that measures the stability of the nuclear genome (21). The CAN1 gene of *S. cerevisiae* encodes a transmembrane amino acid transporter that renders the cell sensitive to lethal arginine analogue, canavanine. Any inactivating mutation in this gene results in a canavanine resistant phenotype (CAN\(^R\)) (15). We calculated the frequency of canavanine resistant colonies as a measure of spontaneous nuclear mutational events in rho\(^0\) and rho\(^-\) strains. We found that, compared to wild type cells, nuclear mutational events were significantly higher in both rho\(^-\) and rho\(^0\) strains (Figure 2A).

Both rho\(^0\) and rho\(^-\) cells lack proper mitochondrial function. Therefore, one possible explanation for the increased spontaneous mutation frequencies observed in the rho\(^0\) and rho\(^-\) strains is that these cells exhibit defective respiratory function. To test if respiratory dysfunction played a role in increased rate of mutagenesis in rho\(^-\) and rho\(^0\) cells, we disrupted mitochondrial respiration with Oligomycin, Antimycin A, and potassium cyanide, inhibitors of mitochondrial function (22). These inhibitors influence different steps in mitochondrial-mediated electron transport. Oligomycin binds directly to mitochondrial ATP synthase and inhibits both electron transfer and ATP production (complex V). Antimycin A is a specific inhibitor of the quinone reduction site, binds to the bc1 complex and blocks electron flow at complex III. Potassium cyanide blocks electron flow at complex IV (22). Analysis of nuclear mutational events in the strain treated with
each of these inhibitors showed that mutation frequency increased in cells exposed to the mitochondrial inhibitors (Figure 2B). Of all the mitochondrial inhibitors used in this study, Antimycin A had the most profound effect indicating that complex III activity is critical for maintaining genomic integrity (Figure 2B). It is noteworthy, that of all the mitochondrial inhibitors, only Antimycin A treatment induces many of the same genes induced in rho\(^0\) cells (23). Together, our results provide evidence that mitochondrial dysfunction generates a mutator phenotype in the nuclear genome.

It has been hypothesized that mitochondrial dysfunction could increase the level of reactive oxygen species (ROS) (1, 18-19). Elevated level of reactive oxygen species could lead to loss of genomic integrity. Therefore, we measured superoxide and hydrogen peroxide levels in rho\(^0\) and rho\(^-\) cells. Our results show that both complete loss of the mitochondrial genome and mutations in mtDNA result in lower levels of superoxide (O\(_2^\cdot\)) and H\(_2\)O\(_2\) (Figure 3 A and B). Thus, ROS does not appear to play a major role in the mitochondria-mediated mutagenesis. Mitochondria are intimately involved in pyrimidine biosynthesis (24). It is conceivable that impairment of nucleotide biosynthesis due to mitochondrial dysfunction contributes to mutagenesis of the nuclear genome.

Yeast cells are known to modulate the expression of nuclear genes in response to mitochondrial dysfunction (25). In fact, mitochondria-to-nucleus communication has been studied extensively in yeast cells lacking mitochondrial function (25-26). Previous studies have demonstrated that up-regulation of certain nuclear genes involves activation and nuclear translocation of heterodimeric basic helix-loop-helix factors Rtg1 and Rtg3p in response to mitochondrial dysfunction. A related protein Rtg2 is also involved in mitochondria-to-nucleus communication (25). To identify genes, whose expression profiles change as a result of impairment of mitochondrial function, we conducted microarray analysis (data not shown). We compared gene expression of cells lacking mitochondrial function (rho\(^0\)) with that of wild type cells (rho\(^+\)). Interestingly we found that among other genes, the expression of REV1, which is involved in error-prone translesion DNA synthesis (TLS) (27), was upregulated. TLS enables cells to repair DNA lesions that escape the vigilance of the generally
efficient DNA repair systems (27). TLS occurs when the replication machinery, upon encountering a lesion, has, or some how acquires, the ability to copy the damaged template directly by incorporating a nucleotide opposite the modified base. TLS is potentially mutagenic because it often incorporates incorrect nucleotides (27). TLS has significance in tumorigenesis since mutations induced during replication of damaged nucleotides are believed to cause cancer (3).

In *S. cerevisiae*, the three proteins, Rev1, Rev3 and Rev7 constitute the major components of TLS. The *REV1* gene product possesses deoxycytidyl transferase activity whereas Rev3 and Rev7 proteins are the subunits of DNA polymerase zeta (27). We examined whether these three gene products play a role in determining the mitochondria-mediated mutator phenotype. We determined mitochondria-mediated mutator phenotype in strains with null mutations in *REV1, REV3 or REV7* genes. Our results clearly showed that the mutator phenotype is eliminated in cells lacking *REV1, REV3, or REV7* genes (Figure 4). These results strongly suggest that the mitochondria-mediated mutator phenotype is dependent on functional Rev1, Rev3 and Rev7 proteins. The *REV1, REV3* and *REV7* genes are conserved between yeast and humans. Based on our observations in yeast, it is conceivable that human Rev1p, Rev3p and Rev7p proteins are involved in mitochondria-mediated mutagenesis.

The data presented in this paper supports the following conclusion for the role of mitochondrial dysfunction in genetic instability. Mitochondrial dysfunction leads to spontaneous nuclear DNA damage. The nature of this spontaneous nuclear DNA damage is unknown at this time. In order to avoid cell death due to spontaneously damaged nuclear DNA, cells activate the error-prone TLS pathway that contributes to cell survival but genetic instability.

Mitochondrial dysfunction and genetic instability are both characteristic features of cancer cells (1-3, 32). Mitochondrial dysfunction due to accumulation of mutations in mtDNA is also a key feature of aging (16-19). Our studies using a yeast model system suggest that mitochondria contain some intrinsic properties that control the mutator phenotype associated with cancer cells. Our study provides, for the first time, a link between cancer and aging and mitochondrial dysfunction.
Methods

Media and Strains. Growth media were prepared as described in (29). The *S. cerevisiae* strains used in this study are derived from RKY3109 (15) provided by R. D. Kolodner (Ludwig Institute for Cancer Research, La Jolla, CA).

Generation of rho⁻ and rho⁰ strains. Strains with mitochondrial mutations (rho⁻) or strains lacking mtDNA (rho⁰) were generated by treatment of strains with ethidium bromide (29). The mutants were selected as cells unable to form colonies on yeast extract-peptone-glycerol (YPG) plates. In rho⁰ cells the loss of mtDNA was verified by DAPI (4,6-diamidino-2-phenylindole) staining and rho⁻ status was verified by genetic crossing with rho⁰ cells.

Generation of yeast rev null mutants. Mutations in *REV1*, *REV3* and *REV7* genes (rho⁰*rev1*, rho⁰*rev3* and rho⁰*rev1*) were introduced by one-step gene deletion/replacement in YAKR144 (table 1). The plasmids pSF3-*REV1Δ::URA3*, pYPG101-*REV3Δ::URA3* and pYPG102-*REV7Δ::URA3* used to generate the *S. cerevisiae* REV null mutants. The null plasmids were obtained from Dr. C. Lawrence (Department. of Biochemistry and Biophysics, University of Rochester, NY).

Determination of spontaneous mutation frequency. Mutation frequencies were determined using the fluctuation test method essentially as described in (30). Briefly, for each strain five to seven independent cultures were grown in yeast extract-pentose-dextrose (5 ml, YPD). Each experiment was repeated at least twice. Appropriate dilutions were plated on YPD plates to determine the number of viable cells. The frequency of forward mutation at the *CAN1* gene locus was determined from the number of canavanine resistant colonies that grew on selective minimal dropout plates (SD/-Arg + 60 µg/ml canavanine). The colonies were counted after incubation at 30°C for 3-5 days. The data were analyzed by using the method of the median (30).
Inhibition of respiratory activity. Wild type strain (RKY3109) was grown in YPD media overnight before plating the cells on YPD plates and SD/-Arg + canavanine plates containing either water (control), oligomycin (5 µg/ml), Antimycin A (0.02 mM), potassium cyanide (0.5 mM). After incubation at 30°C for 3-5 days the colonies were counted and the data were analyzed by the method of the median (30).

Flow cytometric analysis of intracellular levels of free radicals. To determine superoxide anion generation, dihydroethidium (DHE, Molecular Probes, Eugene, OR) was added to early exponential phase cultures of either wild type (RKY3109), rho⁻ (YAKR145) or rho⁰ (YAKR144). The cultures were grown in YPD medium to A₆₀₀ = 0.4 - 0.5. The cells were washed twice in water and the cells were incubated in 1 ml 50 mM Tris pH 7.5 containing 40 µg/ml DHE for 2 hrs before they were analyzed by flow cytometry. The H₂O₂-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, Molecular Probes) was used to measure peroxide production in the cells. CM-H₂DCFDA staining was conducted by adding the dye (10 µM) to the growth medium (1 ml) and incubated for 20 hrs. For sample analysis, an aliquot (100 µl) of the CM-H₂DCFDA-stained yeast cultures were suspended in 900 µl of 50 mM Tris pH 7.5 before they were analyzed by flow cytometry (31).
Acknowledgement

We thank Dr. Richard Kolodner for the yeast strain RKY3109. Research in our laboratories is supported by grants from National Institutes of Health and American Heart Association (KKS) and from Danish Cancer Society, Danish Research Council, Carlsberg Foundation (LJR). We thank the members of our laboratory for critical reading of this manuscript.
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<td>RKY3109 $MAT_a$, $ura3\Delta52$, $leu2\Delta1$, $trp1\Delta63$, $his3\Delta200$, $lys2\Delta Bgl$, $hom3\Delta10$, $ade8$, $ade2\Delta1$</td>
</tr>
<tr>
<td>rho-</td>
<td>YAKR145 RKY3109 rho-</td>
</tr>
<tr>
<td>rho0</td>
<td>YAKR144 RKY3109 rho0</td>
</tr>
<tr>
<td>rho0 rev1</td>
<td>YAKR184-188 YAKR144 $REV1\Delta::URA3$</td>
</tr>
<tr>
<td>rho0 rev3</td>
<td>YAKR189-193 YAKR144 $REV3\Delta::URA3$</td>
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<tr>
<td>rho0 rev7</td>
<td>YAKR194-198 YAKR144 $REV7\Delta::URA7$</td>
</tr>
</tbody>
</table>


**Figure Legends:**

**Figure 1:**
Strains with mtDNA mutations (rho⁻) or strains lacking mtDNA (rho⁰) were generated by treatment of strains with ethidium bromide (29). The mutants were selected as cells unable to form colonies on yeast extract-peptone-glycerol (YPG) plates. Wild type and rho⁻ cells show mtDNA after cells were stained with DAPI (4,6-diamidino-2-phenylindole). DAPI stains both the nuclear and mtDNA. The rho⁰ cells show nuclear DNA staining but they do not show punctuate staining indicating loss of mtDNA. The rho⁻ status was verified by genetic crossing with rho⁰ cells. Fluorescence was examined using a DAPI optimized filter on Zeiss-Axiovert 135 TV inverted microscope equipped with a PXI camera (SEBSYS photometrica).

**Figure 2 A and B:**
Strains were tested in CAN1³ assay as described in Methods. The average median frequency in each assay is presented. CAN1⁵ mutation assay detects frequency of CAN1⁵ to CAN1³ forward mutations. A: Frequency of mutation in CAN1 gene in yeast strains defective in mitochondrial function. B: Frequency of mutation in CAN1 gene after respiratory inhibition. Inhibition of respiration was carried out as described in material methods. The data presented are an average of at least 10 independent cultures.

**Figure 3 A and B:**
Production of reactive oxygen species in yeast strains lacking mitochondrial function. A) superoxide and B) hydrogen peroxide. Free radical production was measured as described in Methods. Superoxide was measured by using dihydroethidium (DHE) and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) was used to measure H₂O₂.

**Figure 4:**
Mutation frequency in yeast strain lacking rev1, rev3 or rev7 gene expression. Strains were tested in CAN1³ assay as described in Methods. The average median frequency in each assay is presented. CAN1⁵ mutation assay detects frequency of CAN1⁵ to CAN1³ forward mutations. The data presented are an average of at least 10 independent cultures.
**Figure 1**

Wild type  \[\text{rho}^0\]  \[\text{rho}^0\]

**Figure 2 A**

Mutation frequency per 10^8 cells

WT  \[\text{rho}^-\]  \[\text{rho}^0\]
Figure 2 B

![Bar chart showing mutation frequency per 10^8 cells for different treatments: Water, Oligomycin, Antimycin A, K cyanide.](image)

Figure 3 A

![Fluorescence (O_2^-) histograms for Rho^+, Rho^-, and Wild-type.](image)
Figure 3 B

Figure 4
Manuscript IV

Mitochondrial dysfunction suppresses the mutator phenotype of Saccharomyces cerevisiae superoxide dismutase deficient cells

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Abstract

Oxidative stress and accumulation of mutation are associated with increased risk of cancer and aging. Alterations of oxidative phosphorylation affect intracellular levels of reactive oxygen species (ROS) which induce oxidative stress. DNA lesions introduced by ROS are potentially cytotoxic and mutagenic. We used *Saccharomyces cerevisiae* to understand the mechanisms of mutagenesis in cells deficient in superoxide dismutases (encoded by the *SOD1* and *SOD2* genes). Our analysis reveals that *SOD1* gene inactivation leads to mutations in the nuclear genome and that mitochondrial dysfunction suppress this mutator phenotype. We also demonstrate that agents which introduce oxidative damage decrease cell survival and increase mutations in cells with mitochondrial dysfunction.
Introduction

Mitochondria generate more than 80% of cellular energy in the form of ATP. In addition, these organelles carry out other cellular functions, such as respiration and heme, lipid, amino acid and nucleotide biosynthesis (Kang et al., 1998). Mitochondrial respiration is a major endogenous source of reactive oxygen species (ROS), including superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (HO•) (Cadenas and Davies, 2000). These ROS also arise from external environmental factors such as drugs, radiation, and heavy metals (Cadenas, 1989). As a result, ROS cause damage to proteins, lipids, and nucleic acids and thereby compromise cell viability. Both prokaryotic and eukaryotic cells have developed defense mechanisms against the lethal and mutagenic effect of ROS. Superoxide dismutases (SOD) are enzymes that eliminate $O_2^-$ and thus protect cells from damage induced by free radicals (Halliwell and Gutterridge, 1985; Fridovich, 1995; Oberley and Buettner, 1979). ROS are also reduced by intracellular antioxidant enzymes such as glutathione peroxidase (GPx) and catalase (CAT) as well as some antioxidant molecules like glutathione (Raha & Robinson, 2001; Jamieson, 1998). Therefore, the balance between ROS production and antioxidant defenses determines the degree of oxidative stress.

Superoxide is the primary oxygen free radical produced by the mitochondrial respiratory chain. However, $O_2^-$ is rapidly dismutated to $H_2O_2$ by either Sod2 (mitochondria) or Sod1 (nucleus, cytoplasm, and lysosomes) (Srinivasan et al., 2000). Hydrogen peroxide is reduced to water by CAT and GPx (Wallace, 1998). *Saccharomyces cerevisiae* cells deficient in Sod1 activity show a variety of phenotypes such as growth inhibition under aerobic conditions, sensitivity to killing by menadione or freeze-thawing, decreased viability in stationary phase, auxothropy for the amino acids methionine and lysine as well as increased spontaneous mutation rates under aerobic growth conditions. In contrast, cells deficient in Sod2 activity have a less dramatic phenotype when grown in glucose media but are oxygen-sensitive and grow poorly on carbon sources that require respiration for metabolization. The phenotype of the sod1sod2 double mutant strain includes all phenotypes of the single mutants (Gralla and Valentine, 1991; Liu et al., 1992; Longo et al., 1996; Park et al., 1998).
Oxidative phosphorylation generates ROS which suggests a role for mitochondrial activity in cell survival and mutagenesis. Guidot et al. (1993) found that S. cerevisiae cells deficient in the mitochondrially-located superoxide dismutase (Sod2) failed to grow normally in hyperoxia. In contrast, strains deficient in both Sod2 and mitochondrial activity (rho\(^0\)) grew normally in hyperoxia. In addition, the viability was partially restored by mutations, which disrupted the electron transport chain. Lack of respiration also prevents viability loss of sod1 and sod1sod2 double mutant strains (Longo et al., 1996). These data imply that mitochondrial function and respiration play an important role in viability of sod mutant strains. Studies have shown that rho\(^0\) strains as well as strains carrying deletions in their mitochondrial DNA (rho\(^-\)) are more sensitive to killing by O\(_2\)\(^-\) and H\(_2\)O\(_2\) than wild type cells (Flatter-O'Brien et al., 1993; Jamieson, 1992; Collinson and Dawes, 1992; Grant et al., 1997).

Mammalian cells respond to oxidative stress by increasing antioxidant enzymes, which are capable of detoxifying reactive oxygen species (Akashi et al., 1995; Brambilla et al., 1997; Esposito et al., 1999; de Hann et al., 1998). However, there exists tissue specificity in the cellular response to oxidative stress. Skeletal muscle dramatically induced SOD2 expression whereas heart only increased SOD2 expression modestly upon oxidative stress. In contrast, expression of GPx1 was induced similarly in both tissues. In S. cerevisiae, the expression of both superoxide dismutases, SOD1 and SOD2, glutathione peroxidase GPX2, CTT1 encoding the cytosolic catalase T, and glutathione reductase GLR, which catalyses reduction of glutathione disulfide (GSSG) to glutathione (reduced form, GSH) are induced in response to oxidative stress (Galiazzo et al., 1987; Gasch et al., 2000; Grant et al., 1996; Inoue et al., 1999; Marchler et al., 1993).

In this paper we examined the role of Sod1 and Sod2 in mitochondria-mediated mutagenesis in S. cerevisiae. We show that cells deficient in Sod activity increase spontaneous mutation levels in the nuclear genome and that this mutator phenotype is suppressed by inactivation of mitochondrial activity. In order to characterize the role of mitochondrial activity in mutagenesis and cell survival we treated wild type and rho\(^0\) cells with various drugs that affect intracellular levels of ROS. We show that both H\(_2\)O\(_2\) and menadione increase spontaneous mutations in nuclei; but that rho\(^0\) cells are 10-20 fold more sensitive than wild type cells to mutations introduced by these agents. Furthermore, both H\(_2\)O\(_2\) and menadione decrease survival of rho\(^0\) cells indicating that cells deficient in
mitochondrial activity are impaired in repair of DNA damage introduced by these agents. We used DNA microarray hybridization analysis to further characterize the cellular response to oxidative stress. We compared gene expression patterns in \textit{sodl} and \textit{sodl}\textsubscript{rho}\textsuperscript{0} mutant strains. We did not detect any increase in the expression of the majority of DNA repair genes in \textit{sodl}\textsubscript{rho}\textsuperscript{0} cells compared to \textit{sodl} cells. However, gene expression analysis revealed that genes involved in DNA repair are induced in \textit{sodl} compared to wild type cells.
Materials and Methods

Media and strains. Growth media were prepared as described in Sherman et al., 1994. The S. cerevisiae strains used in this study are derived from RKY3109 (Ni et al., 1999) and provided by Dr. Richard Kolodner (Ludwig Institute for Cancer Research, La Jolla, CA) or strain DL1 (D273-10B) (Paul et al., 1989). The strains used in this study are listed in Table 1.

Generation of yeast strains. Mutations in the superoxide dismutase genes (sod1 and sod2) were introduced by one-step gene deletion/replacement in RKY3109. The plasmids pUC-SOD1::URA3-A and pG-SOD2::TRP1-A were provided by Dr. Gralla (Department of Chemistry and Biochemistry, University of California, Los Angeles, CA). The sod1 mutant strain YAKR129 was generated using the SOD1::URA3 deletion/disruption cassette as described in (Gralla and Valentine, 1991). The sod2 mutant strain was generated similarly using the SOD2::TRP1 deletion/disruption cassette (Marres et al., 1985). The sod deletion derivatives were verified by PCR analysis (data not shown).

Generation of rho⁻ and rho⁰ strains. Strains with mitochondrial mutations (rho⁻) or strains lacking mtDNA (rho⁰) were generated by treatment of wild type, sod1 and sod2 strains with ethidium bromide (Sherman et al., 1994). The mutants were selected as cells unable to form colonies on extract-pentose-glycerol (YPG) plates. The loss of mtDNA was verified by DAPI (4,6-diamidino-2-phenylindole) staining (data not shown).

Determination of spontaneous mutation frequency. Mutation frequency was determined using fluctuation test method described in (von Borstel, 1978). For each strain, five to seven independent cultures were grown in 5 ml yeast extract-pentose-dextrose (YPD). Appropriate dilutions were plated on YPD agar plates to determine the number of viable cell in the cultures. The frequency of forward mutation at the CAN1 gene locus was determined from the number of canavanine resistant colonies that grew on selective minimal dropout plates (SD/-Arg + 60 mg/liter canavanine). The colonies were counted after incubation at 30°C for 3-5 days for wild type and rho⁰ cells and for 11-14 days for sod mutant strains. The data were analyzed by the method of the median (Lea 1949, von Borstel, 1978). Each experiment was repeated at least twice.
**Drug treatment.** Overnight cultures grown in YPD medium were diluted into fresh growth medium at $A_{600}$ of 0.15 and incubated until cell density reached $A_{600}$ of 0.4 - 0.5. Aliquots of 5 ml were treated with various concentrations of drugs at 30°C with vigorous shaking for 1 hr. The concentrations of the drugs were: $H_2O_2$ (0, 2 and 4 mM), 4-NQO (4-Nitroquinole N-oxide) (0, 2 and 4 µg/ml), adriamycin (0, 20 and 40 µg/ml), menadione sodium bisulfite (0, 10 and 20 mM), and diamide (0, 25 and 50 mM). Relative cell survival was determined by immediately diluting the samples into sterile $H_2O$ and plating on YPD plates. Mutation frequency was determined from the number of canavanine resistant colonies by the method of the median (von Borstel, 1978). The yeast cells were collected by centrifugation, washed in water before plating on SD/-Arg + canavanine plates. All reagents were obtained from Sigma Chemical Co. (St Louis, MO) with the exception of adriamycin (Johns Hopkins Hospital, MD).

**Gene array analysis.** Total RNA was isolated from log-phase *S. cerevisiae* strains according to manufacturers guide lines using RNeasy (QIAGEN). Total RNA (5 µg) was converted into double stranded cDNA by GIBCO BRL's SuperScript Choice system for cDNA synthesis (LifeTechnologies) and a T7-(dT)$_{24}$ oligomer provided by Research Genetics (Huntsville, AL). Double stranded cDNA was purified by phenol/chloroform extraction and ethanol precipitation. *In vitro* transcription was performed with T7 RNA polymerase following the instructions from BioArray high yield RNA transcript Labeling kit from Enzo (distributed by Affymetrix). The Biotin-labeled cRNA was purified on an affinity resin (RNeasy mini cleanup, QIAGEN) and the amount of labeled cRNA was determined by measuring absorbance at 260 nm and using the convention that 1 OD at 260 nm corresponds to 40 µg/ml RNA. The Yeast Genome S98 Array (Affymetrix) containing approximately 6,400 open reading frames (ORFs) of *S. cerevisiae* genome was used for gene expression analysis. Hybridization, reading and analysis were performed by Research Genetics (Huntsville, AL). The classification of genes into functional groups was done as described in MIPS database, Munich Information Center for Protein Sequences (Mewes et al., 1997; http://mips.gsf.de).
Results and Discussion

Mitochondrial dysfunction prevents mutations in the nuclear genome in superoxide dismutase deficient cells (Sod1 and Sod2) deficient cells. *Saccharomyces cerevisiae coq3sod1* double mutant deficient in superoxide dismutase activity and oxidative phosphorylation show increased viability compared to the superoxide deficient *sod1* mutant alone (Longo *et al*., 1996). This indicates that free radicals generated during oxidative phosphorylation in mitochondria are cytotoxic and that superoxide dismutases protect the cell from the lethal effect of mitochondrial-mediated damage. In order to characterize the interaction between mitochondrial-mediated mutagenesis and repair in more detail we measured spontaneous mutation frequencies. We applied a mutation detection assay that measures the stability of the nuclear genome. The *CAN1* gene of *S. cerevisiae* encodes a transmembrane amino acid transporter that renders the cell sensitive to the lethal arginine analogue, canavanine. Any inactivating mutation in this gene results in a canavanine resistant phenotype (*CAN*R). We calculated the frequency of canavanine resistant colonies as a measure of spontaneous nuclear mutational events in *sod1* and *sod2* mutant strains (Table 2). As expected, based on earlier work (Gralla and Valentine, 1991; Longo *et al*., 1997), mutation frequencies are increased in the *sod1* mutant strain compared to the wild type parental strain (Table 2). Inactivation of the *sod2* gene increased mutation frequencies about 2-fold, which is similar to the mutator phenotype generated by inhibiting mitochondrial activity (*rho*0 or *rho*) (Rasmussen *et al*., (III)). The increased mutation frequencies observed in superoxide dismutase deficient cells were suppressed by inactivation of mitochondrial activity (*sod1rho*0 and *sod2rho*0) (Table 2). These data suggest that the mutator phenotype observed in superoxide dismutase deficient cells is dependent on mitochondrial activity.

Differential expression of repair genes in superoxide dismutase deficient strains. We used genome analysis by DNA microarray hybridization analysis to identify DNA repair transcripts differentially expressed as a result of mitochondrial dysfunction and superoxide dismutase deficiency (Table 3). We found that a large number of DNA repair genes acting in various DNA repair pathways are induced by the *sod1* mutation (Table 3). The differentially expressed genes included not only DNA repair genes known to repair oxidative DNA damage (*NTG2*), but also genes involved in repair of other kinds of DNA
damage such as base excision repair (MAG1), direct reversal repair (MGT1), nucleotide excision repair (RAD10, RAD14, RAD16, RAD26 and RAD28), recombinational repair (RAD50, RAD52, RAD59, SGS1, MUS81, and PSO1), mismatch repair (MSH3), and meiotic recombination (MSH4). It has been shown that the transcript of MAG1, encoding a 3-methyladenine DNA glycosylase, is induced after treatment with the methylating agent methylmethane sulfonate (MMS) as well as with the oxidizing agents H2O2 and menadione (Jelinsky and Samson, 1999; Gasch et al., 2001). The induction of DNA repair genes observed in the sod1 mutant strain suggests that one or more shared transcription factors responsible for expression of DNA repair genes are activated in response to endogenous DNA damage in superoxide dismutase deficient cells.

Further analysis of gene expression patterns revealed that several checkpoint genes were induced in the sod1 mutant strain (Table 3). The MEC3 gene is a checkpoint gene that together with RAD9, RAD17, RAD24 and DDC1 are required for detection of DNA damage and activation of the checkpoint pathways (Elledge, 1996, Zhou and Elledge, 2000). Induction of MEC3 and RAD9 suggest that sod1 mutant cells generate DNA damage that is detected by the checkpoint pathways.

We show in Table 2 that the mutator phenotype of the sod1 mutant strain is suppressed by inactivating mitochondrial activity (sod1rhol0). We compared gene expression patterns of the sod1 mutant strain with the sod1rhol0 mutant strain in order to identify DNA repair genes involved in this suppression. Interestingly, only seven DNA repair genes were differentially expressed (Table 4). One of these is the OGG1 gene encoding the 8-oxoguanine DNA glycosylase. Both purine and pyrimidine residues in DNA are sensitive to ROS-mediated DNA damage (Croteau and Bohr, 1997). However, the most common purine DNA lesion is the mutagenic 8-hydroxyguanine (8-oxoG) (Steenken and Jovanovic, 1997; Burrows and Muller, 1998). We have recently shown that complete loss of the mitochondrial genome (rhol0) results in lower levels of ROS (Rasmussen et al., 2000). Therefore, it is tempting to speculate that the sod1 mutant strain induces Ogg1 in response to high levels of ROS.
Cells deficient in mitochondrial activity are sensitive to $H_2O_2$ and menadione induced DNA damage.

Studies have shown that rho$^-$ and rho$^0$ cells, which are respiratorically deficient, are more sensitive to the cytotoxic effect of ROS (Flatter-O'Brien et al., 1993; Jamieson, 1992; Collinson and Dawes, 1992; Grant et al., 1997). We treated exponentially growing wild type, rho$^-$, and rho$^0$ cells with various drugs that affect intracellular levels of ROS to determine the degree of sensitivity and mutation frequencies for each drug (Figure 1). Adriamycin (Doxorubicin) is an anti-tumor drug that can inhibit DNA replication by direct intercalation between DNA base pairs (Singal et al., 1997). Single electron donors such as NADPH cytochrome P450 reductase can reduce the quinoid ring in adriamycin to semiquinone. In the presence of molecular oxygen the semiquinone radicals are rapidly oxidized to the parent compound in a process, which generate $O_2^-$ (Olson and Mushlin, 1990). NADPH-cytochrome P450 reductase catalyzes the reduction of menadione leading to the formation of semiquinones that is readily autooxidized generating $O_2^-$ (Lind et al., 1982). 4-nitroquinoline (4-NQO) is metabolized in vivo to the carcinogenic 4-hydroxyaminoquinoline 1-oxide (4-HAQO), which is oxidized generating $H_2O_2$ (Hozumi, 1969). However, 4-NQO also undergoes redox cycling to generate superoxide, which can be converted to $H_2O_2$ and form oxidative DNA damage such as 8-oxoG and DNA strand breaks (Nunoshiba and Demple, 1993; Yamamoto et al., 1993). In addition, acylated 4-HAQO reacts with DNA to form stable quinoline-purine monoadducts at the N$^2$ of guanine and N$^6$ of adenine and 4-NQO is therefore also known as a "UV-mimetic" (Ramotar et al., 1998). Finally, diamide induces oxidation of cysteine SH-residues of proteins present in mitochondrial membranes and this oxidation also induces formation of $O_2^-$ (de Souza and Geibel, 1999).

As expected, based on earlier work (Flatter-O'Brien et al., 1993; Jamieson, 1992; Collinson and Dawes, 1992; Grant et al., 1997), rho$^0$ cells are more sensitive to killing by $H_2O_2$ and menadione compared to wild type. We showed that these agents at the same time increased spontaneous mutations in rho$^0$ cells (Figure 1A and 1B, Table 5). In contrast, mitochondrial dysfunction mediated resistance to killing by 4-NQO and adriamycin as well as increased 4-NQO-induced mutations (Figure 1D and 1E, Table 5). A phenotype not only observed for yeast as human HeLa rho$^0$ cells have been shown to be more resistant to adriamycin than HeLa rho$^+$ cells (Singh et al., 1999). We did neither detect any difference in cell survival nor any difference in spontaneous mutations in rho$^0$ cells after treatment with diamide (Figure 1C, Table 5). These results suggest that antioxidant and/or repair
defense against damage generated by H$_2$O$_2$ and menadione is impaired in rho$^0$ cells. However, the data also indicate that the mutagenic and cytotoxic effect of 4-NQO and adriamycin can be separated in rho$^0$ cells.

The transcript of $REV1$ is induced in rho$^0$ cells compared to wild type cells (Rasmussen et al., III). The Rev1 protein is involved in error-prone translesion synthesis and possesses a transferase activity, which incorporate dCMP opposite abasic site in the DNA template (Lawrence and Maher, 2001). The Rev1 protein is also involved in bypass of a T-T (6-4) UV photoproduct (Nelson et al., 2000). Thus, the enzymatic nature of this Rev1p function is not yet fully understood. We are currently testing a Rev1-model to see if Rev1 can bypass DNA lesions introduced by 4-NQO (UV-mimetic) but not H$_2$O$_2$ and menadione.
Acknowledgements

We thank Dr. Richard Kolodner for the yeast strain RKY3109. Research in our laboratories is supported by grants from National Institutes of Health and American Heart Association (KKS), Danish Cancer Society (LJR), Danish Natural Science Council (LJR), Carlsberg Foundation (LJR), Denmark-America Foundation (AKR), Frimodt-Heineke Foundation (AKR), Frederik and Marie Beyers Fellowship (AKR), Christian and Ottilia Brorsons Fellowship (AKR), Grosserer L.F. Foghts Foundation (AKR), Julie von Müllens Foundation (AKR), Købmand Sven Hansen and Hustru Ina Hansens Foundation (AKR), and Anna and Dagny Hjerrilds Foundation (AKR). We thank the members of our laboratory for the critical reading of this manuscript.
References


Flattery-O'Brien J, Collinson LP, Dawes IW. Saccharomyces cerevisiae has an inducible response to menadione which differs from that to hydrogen peroxide. J Gen Microbiol. 1993;139:501-7.


**Table 1. Saccharomyces cerevisiae strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
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<td>wild type</td>
<td>RKY3109, MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade8, ade2Δ1</td>
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<td>YAKR145, RKY3109 rho−</td>
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**Table 2. Spontaneous mutation frequencies in superoxide deficient strains**

<table>
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<tr>
<th>Strain</th>
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<th>Fold increase</th>
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<tr>
<td>Wild type</td>
<td>15.3 ± 3.7</td>
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<tr>
<td>rho−</td>
<td>34.0 ± 5.0</td>
<td>2.2</td>
</tr>
<tr>
<td>rho0</td>
<td>39.9 ± 11.7</td>
<td>2.6</td>
</tr>
<tr>
<td>sod2</td>
<td>30.5 ± 5.6</td>
<td>2.0</td>
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<tr>
<td>sod2 rho0</td>
<td>6.4 ± 2.4</td>
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</tr>
<tr>
<td>sod1</td>
<td>165.5 ± 5.1</td>
<td>127.3</td>
</tr>
<tr>
<td>sod1 rho0</td>
<td>8.9 ± 1.8</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Spontaneous mutation frequency in rho−, rho0, sod2, sod2 rho0, sod1 and sod1 rho0 is significantly different (P < 0.01) from wild type, as determined by t-Test: Two-Sample Assuming Equal Variances.
<table>
<thead>
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<th>Gene</th>
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</tr>
</thead>
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<tr>
<td>MEC3</td>
<td>Checkpoint control and DNA repair</td>
<td></td>
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<tr>
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<td>NER</td>
<td>CSA</td>
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</tr>
<tr>
<td>LIF1</td>
<td>DSBR</td>
<td>X RCC4</td>
<td>2.6</td>
</tr>
<tr>
<td>ECM32</td>
<td>Unknown DNA repair function</td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>TFB3</td>
<td>NER</td>
<td></td>
<td>2.3</td>
</tr>
</tbody>
</table>

DNA mismatch repair (MMR), Base excision repair (BER), Nucleotide excision repair (NER) and Double strand break repair (DSBR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Human homolog</th>
<th>Fold induction in sod1</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD9</td>
<td>Checkpoint control and DNA repair</td>
<td>hRAD9</td>
<td>2.2</td>
</tr>
<tr>
<td>OGG1</td>
<td>BER</td>
<td>hOGG1</td>
<td>2.5</td>
</tr>
<tr>
<td>RAD26</td>
<td>NER</td>
<td>CSB/ERCC6</td>
<td>2.1</td>
</tr>
<tr>
<td>RAD28</td>
<td>NER</td>
<td>CSA</td>
<td>2.2</td>
</tr>
<tr>
<td>MUS81</td>
<td>DSBR</td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td>CDC2 (POL3)</td>
<td>BER, NER</td>
<td></td>
<td>2.1</td>
</tr>
<tr>
<td>FUN30</td>
<td>Unknown DNA repair function</td>
<td></td>
<td>2</td>
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</tbody>
</table>

Base excision repair (BER), Nucleotide excision repair (NER) and Double strand break repair (DSBR)
<table>
<thead>
<tr>
<th>Conc.</th>
<th>H$_2$O$_2$ (mM)</th>
<th>Menadione (mM)</th>
<th>Diamide (mM)</th>
<th>4-NQO (µg/ml)</th>
<th>Adriamycin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>wt</td>
<td>2.5</td>
<td>6.8</td>
<td>4.0</td>
<td>3.6</td>
<td>1.7</td>
</tr>
<tr>
<td>rho$^{0}$</td>
<td>3.8</td>
<td>87.6</td>
<td>85.4</td>
<td>6.1</td>
<td>11.6</td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1.** Cell survival of wild type and rho\(^0\) cells after drug treatment. Percent survival is expressed relative to the untreated control culture (100%). Cells were treated with various concentrations of (A) H\(_2\)O\(_2\), (B) menadione, (C) diamide, (D) 4-NQO, and (E) adriamycin. Each data point represents an average of duplicate measurements from one representative experiment. Closed symbols: wild type. Open symbols: rho\(^0\).

![Figure 1 Diagrams](image-url)
Supplemental materials

<table>
<thead>
<tr>
<th>Probe Set Name</th>
<th>ORF</th>
<th>Gene name</th>
<th>A C</th>
<th>Fold Change in DL1</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Damage Checkpoint</td>
<td>DNA Mismatch Repair</td>
<td>Meiotic recombination</td>
<td>Base Excision Repair</td>
<td>DNA Alkylation Repair</td>
<td>Direct Repair</td>
</tr>
<tr>
<td>10048_at</td>
<td>YLR288C</td>
<td>MEC3</td>
<td>P</td>
<td>2.8</td>
<td>Involved in checkpoint control and DNA repair.</td>
</tr>
<tr>
<td>6274_at</td>
<td>YDR217C</td>
<td>RAD9</td>
<td>P</td>
<td>3.4</td>
<td>Cell cycle arrest protein.</td>
</tr>
<tr>
<td>6787_at</td>
<td>YCR092C</td>
<td>MSH3</td>
<td>P</td>
<td>2.9</td>
<td>MutS homolog, forms a complex with Msh2p to repair insertion-deletion mispairs.</td>
</tr>
<tr>
<td>5385_at</td>
<td>YFL003C</td>
<td>MSH4</td>
<td>A</td>
<td>5.1</td>
<td>MutS homolog, localizes to discrete sites on meiotic chromosomes, meiosis specific protein.</td>
</tr>
<tr>
<td>8609_at</td>
<td>YOL043C</td>
<td>NTG2</td>
<td>P</td>
<td>2</td>
<td>Endonuclease III-like glycosylase 2.</td>
</tr>
<tr>
<td>5582_at</td>
<td>YER142C</td>
<td>MAG1</td>
<td>A</td>
<td>3.2</td>
<td>3-methyladenine DNA glycosylase.</td>
</tr>
<tr>
<td>6663_at</td>
<td>YDL200C</td>
<td>MGT1</td>
<td>A</td>
<td>6.3</td>
<td>6-O-methylguanine-DNA methylase.</td>
</tr>
<tr>
<td>9428_at</td>
<td>YMR201C</td>
<td>RAD14</td>
<td>P</td>
<td>2.4</td>
<td>Human xeroderma pigmentosum group A DNA repair gene homolog.</td>
</tr>
<tr>
<td>7250_at</td>
<td>YBR114W</td>
<td>RAD16</td>
<td>P</td>
<td>4.2</td>
<td>Radiation repair protein, putative DNA helicase.</td>
</tr>
<tr>
<td>9749_at</td>
<td>YML095C</td>
<td>RAD10</td>
<td>A</td>
<td>3.1</td>
<td>Endonuclease (with Rad1p) that degrades single-stranded DNA for repair.</td>
</tr>
<tr>
<td>9750_at</td>
<td>YML095C</td>
<td>RAD10</td>
<td>A</td>
<td>3.2</td>
<td>Questionable ORF</td>
</tr>
<tr>
<td>11009_at</td>
<td>YJR035W</td>
<td>RAD26</td>
<td>P</td>
<td>2.3</td>
<td>DNA-dependent ATPase, homologous to human Cockayne syndrome B gene ERC6, that is a putative helicase.</td>
</tr>
<tr>
<td>6444_at</td>
<td>YDR030C</td>
<td>RAD28</td>
<td>P</td>
<td>2.6</td>
<td>Protein involved in the same pathway as Rad26p, has beta-transducin (WD-40) repeats.</td>
</tr>
<tr>
<td>9099_at</td>
<td>YNL250W</td>
<td>RAD50</td>
<td>P</td>
<td>3.8</td>
<td>Protein contains a purine-binding domain, two heptad repeats and a hydrophobic tail, Rad50p interacts with Mre11p and Xrs2p in two-hybrid and immunoprecipitation analyses; it co-localizes to spots with Mre11p and Xrs2p in a rad50s background.</td>
</tr>
<tr>
<td>9678_at</td>
<td>YML032C</td>
<td>RAD52</td>
<td>A</td>
<td>3.1</td>
<td>Questionable ORF.</td>
</tr>
<tr>
<td>6539_at</td>
<td>YDL059C</td>
<td>RAD59</td>
<td>P</td>
<td>2.8</td>
<td>A mutation in this gene results in RADiation sensitivity and recombination defects, which are general properties of the RAD52 epistasis group mutants. rad59 is epistatic to rad52 for its repair and recombination defects. The RAD59 gene product has homology to the Rad52 protein.</td>
</tr>
<tr>
<td>9461_at</td>
<td>YMR190C</td>
<td>SGS1</td>
<td>P</td>
<td>2.2</td>
<td>Has DNA helicase signature motifs.</td>
</tr>
<tr>
<td>6083_at</td>
<td>YDR386W</td>
<td>MUS81</td>
<td>A</td>
<td>3.8</td>
<td>MMS and UV Sensitive; Mus81p and Rad54p are found together in a complex from whole-cell extracts.</td>
</tr>
<tr>
<td>9491_at</td>
<td>YMR137C</td>
<td>PSO2</td>
<td>P</td>
<td>2.2</td>
<td>Interstrand crosslink repair protein.</td>
</tr>
<tr>
<td>10528_at</td>
<td>YKR056W</td>
<td>RNC1</td>
<td>P</td>
<td>2.8</td>
<td>Endo-exonuclease yNucR.</td>
</tr>
<tr>
<td>4844_at</td>
<td>YGR144W</td>
<td>THI4</td>
<td>A</td>
<td>8.6</td>
<td>Component of the biosynthetic pathway producing the thiazole precursor of thiamine.</td>
</tr>
<tr>
<td>5107_at</td>
<td>YGL090W</td>
<td>LIF1</td>
<td>P</td>
<td>2.6</td>
<td>Ligase Interacting Factor 1; physically interacts with DNA ligase 4 protein.</td>
</tr>
<tr>
<td>5524_at</td>
<td>YER176W</td>
<td>ECM32</td>
<td>A</td>
<td>2.5</td>
<td>DNA Helicase I.</td>
</tr>
<tr>
<td>6019_at</td>
<td>YDR460W</td>
<td>TFB3</td>
<td>P</td>
<td>2.3</td>
<td>TFIIH subunit Tfb3 ; similar to mammalian CAK subunit.</td>
</tr>
</tbody>
</table>

AC = the Absolute Call; The transcript is present (P) or absent (A) for DL1. When the transcript is absent is the fold change an approximation and calculated using the noise level.
4. Concluding Remarks

Increased spontaneous mutation frequency is associated with higher risk of cancer. However, the relative contribution of spontaneous endogenous mutagenesis to carcinogenesis is not yet known. Among the normal cellular metabolic processes that lead to elevated spontaneous mutation rates are (Jackson et al., 1998; Rossman & Goncharova, 1998; Rasmussen et al., III):

- DNA polymerase errors,
- Depurination, which gives rise to abasic sites,
- Deamination of deoxycytidine residues (C → U and 5-methyl-C → T),
- Methylation of DNA (O6-methylguanine),
- Damage by oxygen free radicals,
- and mitochondrial dysfunction.

Thus, both a main repair pathway and one or more backup pathways can generally remove these lesions. For example the main repair pathway for eliminating mutations, caused by the deoxyguanosine oxidation product 8-oxoG, is BER (hOGG1, hMYH and hMTH). However, studies in S. cerevisiae have shown that MSH2-MSH6-dependent MMR is involved in repairing 8-oxoG:A mispairs and 8-oxoG:C base pairs. These results suggest that MMR is functioning as a backup pathway under high oxidative stress (Ni et al., 1999). The MMR pathway is also involved in repair of O6-MeG and O6-MeG-triggered cell death when MGMT repair capacity is saturated (see 2.3 Direct Repair: O6-methylguanine-DNA methyl-transferase). Furthermore, it has been suggested that the hMLH1 protein, from the MMR pathway, has an impact on the apoptosis pathway after oxidative stress. The mitochondria were suggested to be involved in this apoptosis pathway because increased mitochondrial permeability and cytochrome c release after H2O2 treatment were observed preferentially in hMLH proficient cell lines (Hardman et al., 2001).

Therefore, it seems like inactivation or dysregulation of the DNA protection system are important for generating a mutator phenotype. Its importance in human disease is supported by the discovery that defects in several kinds of DNA repair are known to raise spontaneous mutation frequency i.e. mutations in MMR genes cause hereditary non-polyposis colon cancer, HNPCC (Hoeijmakers, 2001).
In an attempt to identify new MMR genes, we employed the yeast two-hybrid system, using the human mismatch repair protein hMSH2 (Rasmussen et al., 2000) or hMLH1 as bait and a fetal liver cDNA library as prey. We demonstrated that hMSH2 interacts with exonuclease hEXO1 suggesting that hEXO1 plays a role in MMR. Furthermore, we showed that hMSH2 and EXO1 are co-expressed at high levels in fetal liver but not in adult liver where cell turnover is normally very slow. The hEXO1 protein was highly expressed in several liver cancer cell lines as well as in colon and pancreas adenocarcinomas, but not in the corresponding non-neoplastic tissue. These results support a role for hEXO1 in cellular processes such as MMR, recombination repair or replication, which are expected to be active in proliferating cells (Rasmussen et al., 2000).

The mismatch repair protein hMLH1 is known to interact with hPMS1. However, the exact biochemical role for the hMLH1-hPMS1 complex has not yet been determined. We showed that hMLH1 interacts with hPMS1 in the yeast two-hybrid system using hMLH1 as bait and fetal liver cDNA library as prey. Dot-blot analysis of multiple tissue RNA revealed that hPMS1 is predominantly expressed in fetal liver, adult liver, pancreas, kidney, testis and appendix. Unfortunately, we do not have the corresponding dot-blot analysis for hPMS2. However, it is tempting to speculate that hPMS1 is tissue specific and that the hMLH1-hPMS1 complex plays an important role in DNA repair in liver. Furthermore, it could be interesting to compare hPMS1 and hPMS2 expression levels in different cancer cell lines with non-neoplastic tissue to investigate if any up or down regulation will occur in the cancer cells.

Mutations in MMR genes are known to cause spontaneous mutations. Defects in translocation of repair proteins could be a way to inactivate the DNA protection system. We present preliminary data showing that hMSH2 and hMLH1 interact with an importin-α homolog and importin-α, respectively. Importin-α is part of the translocation system to the nucleus. Protein-protein interaction and nuclear localization studies with HNPCC-hMSH2 mutants or HNPCC-hMLH1 mutants together with importin-α and the importin-α homolog could be a way to determine if mutations in MMR mutant genes found in HNPCC patients interfere with the translocation system.
Another model to inactivate the DNA repair system is oxidative damage. The mitochondrial theory of aging postulates that accumulation of oxidative damage in the mtDNA leads to mitochondrial dysfunction that results in energy deficiencies in old cells. Declined mitochondrial energy capacity could in turn lead to lack in repair (Ames, 1989).

Our results in S. cerevisiae showed that mitochondrial dysfunction caused significantly higher spontaneous mutation frequencies in nDNA compared to wild type and this was not due to increased levels of ROS in the cells. However, we demonstrated that disruption of the respiratory chain function using various mitochondrial inhibitors caused an increase in spontaneous mutation frequencies, indicating that increased mitochondrial ROS production can diffuse to the nucleus where it causes DNA damage. Furthermore, in rho<sup>0</sup> cells we found an up-regulation of Rev1 compared to wild type and we showed that mitochondrial-mediated mutator phenotype of rho<sup>0</sup> cells is suppressed by preventing Rev1/Rev3/Rev7-dependent translesion synthesis (Rasmussen et al., III). Even though we do not know today how mitochondrial dysfunction leads to spontaneous nuclear DNA damage, mitochondrial dysfunction elevates the spontaneous mutation frequencies.

The nuclear (Sod1) and mitochondrial (Sod2) superoxide dismutases eliminate the superoxide anion and thus protect cells from damage induced by free radicals. We find that strains deficient in SOD activity increase spontaneous mutations and that this mutator phenotype is suppressed both in sod1Δrho<sup>0</sup> and sod2Δrho<sup>0</sup> mutant strains. Our results suggest that mitochondrial dysfunction either reduces oxidative damage to DNA by preventing formation of ROS and/or increases repair of oxidative DNA damage. We compared gene expression patterns of the sod1 mutant strain with the sod1rho<sup>0</sup> mutant strain in order to identify DNA repair genes involved in this suppression. This analysis suggested that sod1rho<sup>0</sup> cells did not increase repair response compared to sod1 cells. This infers that removal of mitochondrial function reduces intracellular levels of ROS and consequently decreases mutagenesis caused by oxidative damage to DNA (Rasmussen et al., IV).

We found that both H<sub>2</sub>O<sub>2</sub> and menadione treatments reduced cell survival of rho<sup>0</sup> cells but at the same time increased spontaneous mutations in these cells compared to wild type. Gene expression analysis showed that the induced antioxidant enzymes in rho<sup>0</sup> cells were different from the ones induced in the wild type. Implying that defense against oxidative
damage generated by $\text{H}_2\text{O}_2$ and Menadione is different in rho$^0$ cells. These results emphasize the significance of mitochondrial activity in controlling cell death as well as mutational events following DNA damage. Proteins involved in such control mechanisms have been identified and include checkpoint proteins such as the human tumor suppressor protein p53. It is therefore tempting to consider mitochondria as a new guardian of genomic stability.

Given the fact that the integrity of the mtDNA is important it could be beneficial to understand the molecular processes and components responsible for mtDNA repair. Most DNA glycosylases that have been identified in the nucleus have also been identified in mitochondria, which indicates that BER is operating in mitochondria.

It has not yet been determined if mitochondrial DNA is repaired by MMR. Our two-hybrid screenings with hMSH2 or hMLH1 as baits and a fetal liver cDNA library as prey gave no answers to this question. However, we found that full-length MGMT, when expressed as a GFP fusion protein in human MCF12A (breast) epithelial cells, were localized to the nuclei but not to the mitochondria (Rasmussen et al., II). These data suggest that the alkyltransferase mechanism, which seems to be operating within the mitochondrion in mammalian species, is not MGMT in human breast epithelial cells.

Expression of the mitochondrial genes are required to maintain proper function of the organelle, suggesting that even a slight alteration of mitochondrial function may have profound effects. Interestingly, one of the profound features of cancer cells is their defective mitochondrial function (Warburg, 1956; Polyak et al., 1998; Fliss et al., 2000).
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Human MCF12A breast epithelial cells transfected with MGMT-GPF