MMS1 and the Repair of Replication-Dependent DNA Damage in Saccharomyces cerevisiae

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Microbiology and Immunology University of Saskatchewan

Todd Hryciw, B.Sc.

Spring 2001

© Copyright Todd Hryciw, 2001. All rights reserved.
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L’auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author’s permission.

L’auteur conserve la propriété du droit d’auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-63878-2
PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or make other use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Microbiology and Immunology
107 Wiggins Road
University of Saskatchewan
Saskatoon, SK
Canada S7N 5E5
ABSTRACT

In an effort to characterize DNA alkylation repair, a series of yeast mutants were isolated that are sensitive to killing by the monofunctional DNA alkylation agent methyl methanesulfonate (MMS) but not by UV or X radiation (Prakash and Prakash, 1977, Genetics 86: 33-55). Our laboratory cloned and sequenced one of the corresponding genes, MMS1. The mms1Δ mutant is sensitive to MMS, and mildly sensitive to UV and γ-rays. The mms1Δ mutant displays a RAD9-dependent slow growth phenotype, as well as an abnormal cellular morphology shared by recombination-defective mutants.

Epistasis analysis showed that MMS1 does not belong to the base excision, nucleotide excision, or postreplication repair groups. MMS1 belongs to the RAD52 group of recombinational repair genes, as rad52Δ is epistatic to mms1Δ for MMS sensitivity. This was not anticipated, as mutants of all known RAD52-group genes are very sensitive to ionizing radiation, and mms1Δ is only slightly γ-sensitive. Like other RAD52-group mutants, mms1Δ affects certain recombination events. For an assay measuring recombination between two ade2 heteroalleles in an intrachromosomal inverted repeat, the observed recombination rate for the mms1Δ strain was ten-fold higher than that of the corresponding wild type strain. This observation suggests that single-strand gaps or double-strand breaks accumulate in the mms1Δ cells, and supports an hypothesis that Mms1 repairs these lesions. However, through pulsed field gel electrophoretic analysis of genomic DNA isolated from wild type, mms1Δ and rad52Δ cells treated with MMS, I was unable to show a defect in the repair of MMS-induced double-strand breaks in the mms1Δ mutant under conditions where rad52Δ mutants were
shown to be repair-defective. It is possible that this double-strand break assay lacks the sensitivity required to detect an Mms1 effect, especially if Mms1 is only required for the repair of a subset of MMS-induced double-strand breaks, as is suggested by the much greater MMS sensitivity of rad52Δ cells as compared to the mms1Δ strain.

Both mms1Δ and other RAD52-group mutants are sensitive to a variety of DNA damaging agents which are capable of inducing replication-dependent double-strand breaks. RAD52 group mutants are additionally extremely sensitive to ionizing radiation, which can directly induce double-strand breaks in DNA; mms1Δ cells, however, are only slightly more γ-sensitive than the wild type. Therefore hypothesized that Mms1 specifically protects cells from replication-dependent DNA damage. Three experiments were performed to address this issue: assaying sensitivity of the mms1Δ mutant to camptothecin, to cdc2-2-induced DNA damage, and to hydroxyurea. The results showed: first, the mms1Δ mutant was sensitive to killing by camptothecin in a Top1-dependent manner; second, deletion of either RAD52 or MMS1 in the cdc2-2 background dramatically decreased the survival of the cdc2-2 strain after a 4 hour incubation at the non-permissive temperature; third, the mms1Δ mutation also induces sensitivity to killing by hydroxyurea, an agent that inhibits DNA synthesis and induces DNA damage during S phase. Since Rad52 is required for the formation of Holliday junctions and recombinational rescue of the DNA template after cdc2-2-induced DNA damage, and for the repair of hydroxyurea-induced DNA damage, it is conceivable that the DNA lesions acquired by the cdc2-2 mutant at non-permissive temperature and by cells treated with hydroxyurea are mainly double-strand breaks. Together these results implicate Mms1 in the Rad52-dependent repair of replication-dependent DNA strand breaks.
Apart from its role in Rad52-dependent DNA repair, Mms1 was also required for the transcriptional upregulation of the DNA-damage-inducible gene \textit{MAG1}. Mms1 was not required for the induction of the divergently transcribed and also damage-inducible gene \textit{DDII}, nor for damage induction of \textit{RNR3} or \textit{RAD51}. The Dun1 protein kinase is also required for \textit{MAG1} induction in response to DNA damage, and results showed that \textit{mms1Δ} is epistatic to \textit{dun1Δ} for MMS sensitivity, suggesting that Mms1 and Dun1 belong to the same signal transduction pathway for \textit{MAG1} induction.
ACKNOWLEDGEMENTS

First I thank my supervisor, Dr. Wei Xiao, for being a good person, an excellent scientist, and a great advisor, who allowed me to let my imagination roam, but ensured I was always on track to finish my program. Thanks to my advisory committee, Drs. Hughes Goldie, Sean Hemmingsen, Lambert Loh, and Rob Warrington, for always being happy to help me out whenever I asked, and for providing excellent advice throughout my studies and during the editing of this text. Thank you too to my external examiner, Dr. Jack von Borstel, for agreeing to come to Saskatoon for my defense. Your presence set me at ease and made the experience enjoyable.

I truly appreciate the love and support I received throughout from Lynne Robinson. Thanks to my dad for letting me tag along with him to his lab at a very early age, and to my mom, who told me “I don’t care what you do with your life, as long as you use that brain of yours.”

Special thanks to the U of S Summer Noon-Hour Soccer League regulars. Best soccer in town. I’d also like to single out Nathan Peters, Carl Power, Roderick Slavcev, and Seema Madhavan.

Most important in my graduate school experience were my coworkers. I would often stick around when my work was done just to hang out with these people. Thank you Treena Swanston, Barb Chow, Yule Liu, Mahmood Chamankhah, Janelle Franko, Yu Zhu, Michelle Hanna, Parker Andersen, Landon Pastushok, and Leslie Barbour.

Finally, I’d like to extend my eternal gratitude to my labmate, fellow grad student, and best friend, Stacey Broomfield.

Financial support was provided by: College of Medicine Graduate Scholarship; University of Saskatchewan Graduate Scholarship; and Arthur Smyth Graduate Scholarship.
Dedicated to the pursuit of knowledge for pleasure
TABLE OF CONTENTS

PERMISSION TO USE i
ABSTRACT ii
ACKNOWLEDGEMENTS v
DEDICATION vi
TABLE OF CONTENTS vii
LIST OF TABLES xii
LIST OF FIGURES xiii
LIST OF ABBREVIATIONS xv

CHAPTER ONE - INTRODUCTION 1

1.1 DNA damage 1
  1.1.1 Exogenous DNA damaging agents and DNA damage 3
    1.1.1.1 Alkylating agents 3
    1.1.1.2 Ultraviolet radiation 5
    1.1.1.3 Ionizing radiation 6
  1.1.2 Spontaneous or endogenous DNA damage 7

1.2 DNA repair 9
  1.2.1 Base excision repair and alkylation damage 10
  1.2.2 Nucleotide excision repair and alkylation damage 15
  1.2.3 Postreplication repair and alkylation damage 18
  1.2.4 Recombination and alkylation damage 24
    1.2.4.1 Gene conversion 24
1.2.4.2 Single-strand annealing
1.2.4.3 Non-homologous end-joining
1.2.5 Substrate overlap for DNA repair pathways

1.3 **Cell cycle control, transcriptional regulation, and DNA damage**
1.3.1 Cell cycle arrest and DNA damage
1.3.2 Transcriptional response to DNA damage

1.4 **DNA replication, DSB production, and recombination**
1.4.1 Replication forks blocks and recombination in *E. coli*
1.4.2 Mutations in DNA replication genes are recombinogenic in *S. cerevisiae*
1.4.3 DNA topoisomerase I and DNA damage

1.5 **Rationale for the project**

**CHAPTER TWO - MATERIALS AND METHODS**

2.1 **Yeast genetics**
2.1.1 Yeast strains, culture, transformation, and storage
2.1.2 Total genomic DNA isolation
2.1.3 Recombination assays
2.1.4 Spontaneous mutagenesis
2.1.5 β-galactosidase assay
2.1.6 Cell killing by DNA damaging agents
2.1.7 Cell viability assay

2.2 **Molecular biology techniques**
2.2.1 Bacterial culture and storage
2.2.2 Bacterial transformation 69
2.2.3 Plasmid manipulation 69
2.2.4 Agarose gel electrophoresis and DNA fragment isolation 73
2.2.5 Radioactive labeling of DNA fragments 74
2.2.6 Southern transfer and hybridization 74
2.2.7 Pulsed field gel electrophoresis of chromosomal DNA 75

CHAPTER THREE - RESULTS 77

3.1 DNA sequence analysis of the MMS1 gene 77
3.1.1 DNA sequence of MMS1 77
3.1.2 Database-assisted analysis of the MMS1 open reading frame 77

3.2 Cloning and sequencing of the mms1-I mutation 83

3.3 Phenotypes of the mms1Δ mutant 85
3.3.1 DNA damage sensitivities of mms1Δ 85
3.3.2 The mms1Δ mutant has a slow growth phenotype 89
3.3.3 mms1Δ cells have an abnormal cellular morphology 91

3.4 Does MMS1 belong to any of the known DNA alkylation repair groups? 95
3.4.1 MMS1 is likely not a member of the base excision repair pathway 95
3.4.2 MMS1 does not likely represent a new member of the nucleotide excision or postreplication repair pathways 95
3.4.3 MMS1 represents a novel branch of the RAD52 group recombinational repair pathway 98

3.5 Evidence of elevated spontaneous DNA damage and genomic ix
3.5.1 Elevated spontaneous recombination in \( mms1\Delta \) cells

3.5.2 Basal level of expression of DNA damage inducible genes is elevated in the \( mms1\Delta \) background

3.6 The \( mms1\Delta \) mutation renders cells sensitive to replication-induced DNA damage

3.6.1 \( mms1\Delta \) cells are sensitive to killing by camptothecin

3.6.2 \( mms1\Delta \) cells are sensitive to DNA polymerase-induced DNA damage

3.6.3 The \( mms1\Delta \) mutation renders cells sensitive to hydroxyurea treatment

3.7 Spontaneous mutagenesis in the \( mms1\Delta \) background

3.8 Deleting the \( YKU80 \) gene encoding a DNA end-binding protein can partially rescue the DNA damage sensitivity of \( mms1\Delta \)

3.9 \( mms1\Delta \) cells are proficient in the repair of MMS-induced DSBs

3.10 MMS-induced DSBs are dependent on cellular metabolism but not on DNA replication

3.11 MMS induces recombination events in \( S. cerevisiae \)

3.12 \( MMS1 \) is involved in the transcriptional regulation of \( MAG1 \) following DNA damage

3.12.1 \( MMS1 \) is required for \( MAG1 \) induction following DNA damage

3.12.2 \( mms1\Delta \) is epistatic to \( dun1\Delta \)

CHAPTER FOUR - DISCUSSION
4.1 *mms1*-I is a nonsense mutation
4.2 *mms1Δ* is not sensitive exclusively to DNA alkylation damage
4.3 *MMS1* is a member of the *RAD52* epistasis group
4.4 Replication-dependent DNA strand breaks and *MMS1*
4.5 Spontaneous DNA damage in the *mms1Δ* background
4.6 Possible mechanism(s) of *Mms1* function in recombination
4.7 *Mms1* transduces the DNA damage signal for *MAG1*-induction
4.8 Could *Dun1* play a direct role in DNA repair?
4.9 MMS induces DNA DSBs that are dependent on cellular metabolism

REFERENCES
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Genetic diseases with defective DNA repair</td>
<td>2</td>
</tr>
<tr>
<td>1-2</td>
<td><em>Saccharomyces cerevisiae</em> radiation repair genes and groups</td>
<td>11</td>
</tr>
<tr>
<td>2-1</td>
<td>Yeast strains</td>
<td>58</td>
</tr>
<tr>
<td>3-1</td>
<td>DNA damaging agent sensitivity spectrum of the mms1A mutant</td>
<td>88</td>
</tr>
<tr>
<td>3-2</td>
<td>Viability of mms1A and rad52A cells in log phase culture</td>
<td>90</td>
</tr>
<tr>
<td>3-3</td>
<td>Abnormal cell morphology for mms1A cells</td>
<td>93</td>
</tr>
<tr>
<td>3-4</td>
<td>Intrachromosomal recombination between inverted ade2 heteroalleles</td>
<td>102</td>
</tr>
<tr>
<td>3-5</td>
<td>Sister chromatid exchange between ade3 heteroalleles</td>
<td>103</td>
</tr>
<tr>
<td>3-6</td>
<td>Measurement of ADE2 deletion by recombination within the rDNA array</td>
<td>104</td>
</tr>
<tr>
<td>3-7</td>
<td>Survival of the cdc2-2 strain derivatives after incubation at restrictive temperature</td>
<td>114</td>
</tr>
<tr>
<td>3-8</td>
<td>Spontaneous mutagenesis: trp1-289 reversion and CAN1 forward mutation rates</td>
<td>117</td>
</tr>
<tr>
<td>3-9</td>
<td>MMS-induced intrachromosomal recombination between inverted ade2 heteroalleles in strain B365-14c</td>
<td>128</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

1-1  Potential sites of alkylation by DNA methylating agents 4
1-2  UV-induced sister chromatid exchange: daughter strand gap repair without Holliday junction cleavage 23
1-3  UV-induced sister chromatid exchange: strand displacement, branch migration, and template switching to allow DNA replication beyond the lesion 25
1-4  Szostak model of DSB-induced gene conversion 28
1-5  Synthesis-dependent strand-annealing model of DSB-induced gene conversion 29
1-6  DSB-induced recombination requiring both leading and lagging strand DNA synthesis 31
1-7  Single-strand annealing 35
2-1  Graph of rO vs. rO/m, to interpolate m for use in the Lea and Coulson method of the median calculations 64
2-2  mms1Δ disruption cassettes 72
3-1  Complete DNA sequence of MMS1 78
3-2  Deletions used in gap-repair cloning of the mms1-1 mutation 84
3-3  Sequence of the mms1-1 mutation 86
3-4  Sensitivity of the mms1Δ mutant to various DNA damaging agents 87
3-5  rad9Δ rescues the slow growth of mms1Δ 92
3-6  Morphology of mms1Δ cells 94
3-7 Epistasis analysis of \( \textit{mms}1 \Delta \) with DNA repair mutations
96

3-8 \textit{recombination} \( \textit{mms}1 \Delta \) double mutants have a synergistic growth
phenotype in the DBY747 genetic background
99

3-9 \( \textit{rad}52 \Delta \) is epistatic to \( \textit{mms}1 \Delta \) for MMS sensitivity
100

3-10 The effect of \( \textit{mms}1 \Delta \) mutation on the \( \textit{lacZ} \) fusion gene expression
106

3-11 Synthetic growth phenotype of the \( \textit{mms}1 \Delta \textit{apn}1 \Delta \textit{apn}2 \Delta \) mutant strain
108

3-12 Camptothecin-sensitivity of various DNA repair mutants
110

3-13 Hydroxyurea sensitivity of \( \textit{mms}1 \Delta \) mutants
115

3-14 MMS-sensitivity of \( \textit{mms}1 \Delta \) cells is partly alleviated by deleting the non-
homologous end-joining gene \( \textit{YKU}80 \)
119

3-15 \( \textit{mms}1 \Delta \) cells are not deficient in the repair of MMS-induced DSBs
121

3-16 MMS-induced genomic DSBs in wild type and \( \textit{mms}1 \Delta \) cells
123

3-17 DSB formation by MMS requires cellular metabolism
125

3-18 MMS-induced DSBs in DNA repair mutants
127

3-19 The effect of \( \textit{mms}1 \Delta \) on expression of (A) and (C) \( \textit{MAG}1\textit{-lacZ} \)
(YEpMAG1-lacZ), (B) \( \textit{DDI}1\textit{-lacZ} \) (pWX1812), (D) \( \textit{RNR}3\textit{-lacZ} \)
(pZZ13), and (E) \( \textit{RAD}51\textit{-lacZ} \) (YCpRAD51-lacZ)
130

3-20 \( \textit{mms}1 \Delta \) is epistatic to \( \textit{dun}1 \Delta \) for MMS sensitivity
133

3-21 The \( \textit{dun}1 \Delta \) mutant is not camptothecin-sensitive
134

4-1 Proposed model for the requirement of \( \textit{MMS}1 \) in the DNA damage
transcriptional response of \( \textit{MAG}1 \)
147
LIST OF ABBREVIATIONS

Ade  adenine
AP   apurinic/apyrimidinic (abasic)
β-gal β-galactosidase
bp   base pairs
CHEF contour-clamped homogeneous electric field
CPT  camptothecin
Da   Dalton
DEB  1,2;3,4-diepoxybutane
DMSO dimethyl sulphoxide
DNA-PK DNA-dependent protein kinase
DR   direct repeat
DSB  double-strand break
FMS  ethyl methanesulfonate
ENNG 1-ethyl-3-nitro-1-nitrosoguanidine
Fap  2,6-diamino-4-hydroxy-5-amidopyrimidine
His  histidine
HU   hydroxyurea
IPTG isopropylthiogalactoside
MAG  3-MeA DNA glycosylase
3MeA 3-methyladenine
7MeG 7-methylguanine
MMS  methyl methanesulfonate
MNNG 1-methyl-3-nitro-1-nitrosoguanidine
MW   molecular weight
nt   nucleotide
4NQO 4-nitroquinoline-N-oxide
OD   optical density
O\#MeT O\#-methylthymine
O\#MeG O\#-methylguanine
ORF  open reading frame
PCNA proliferating cell nuclear antigen
PFGE pulsed field gel electrophoresis
RPA  replication protein A
SAM  S-adenosylmethionine
SD   synthetic dextrose
SDS  sodium dodecyl sulfate
ss   single strand
SSB  single-strand break
Trp  tryptophan
ts  temperature-sensitive mutation
UAS  upstream activating sequence
Ubc  ubiquitin conjugating enzyme
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>URS</td>
<td>upstream repressing sequence</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>XP</td>
<td>xeroderma pigmentosum</td>
</tr>
</tbody>
</table>
CHAPTER ONE - INTRODUCTION

One of the consequences of having a DNA-based genome is that all cells suffer from DNA damage, be it from endogenously or exogenously derived sources. While some lesions are of little biological significance in that they do not interfere with the processes of transcription, replication, or mitosis, others must be repaired to avoid the consequences of mutation or death. For a unicellular organism, the accumulation of mutations affects only that one cell, and indeed may be beneficial in that it might facilitate evolutionary change allowing a population to adapt to changes in its environment. For a multicellular organism, the accumulation of mutations in a single cell can be detrimental to the survival of the organism as a whole. The consequence of defective DNA repair on human health was cast into the limelight through the linking of two genetic diseases causing a predisposition to cancer with mutations in DNA repair genes; xeroderma pigmentosum with defective nucleotide excision repair, and hereditary nonpolyposis colorectal cancer with defective DNA mismatch repair. DNA repair defects are actually responsible for a number of diseases, with diverse symptoms, including a predisposition to cancer, mental retardation, growth defects, premature ageing, brittle hair, failure of the bone marrow to produce blood cells, and immunodeficiency (see Table 1-1).

1.1 DNA damage

An understanding of the chemistry of DNA damaging agents and the lesions they cause is necessary before one can seek out and study specific repair pathways. Among
<table>
<thead>
<tr>
<th>Disease</th>
<th>Defect</th>
<th>Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xeroderma pigmentosum</td>
<td>Nucleotide excision repair</td>
<td>XPA-XPG</td>
</tr>
<tr>
<td></td>
<td>Post-replication repair</td>
<td>XPV</td>
</tr>
<tr>
<td>Cockayne’s syndrome</td>
<td>Transcription-coupled repair</td>
<td>CSA, CSB</td>
</tr>
<tr>
<td>Trichothiodystrophy</td>
<td>Transcription</td>
<td>XPD</td>
</tr>
<tr>
<td>Fanconi’s anemia</td>
<td>???</td>
<td>FAA-FAH</td>
</tr>
<tr>
<td>Bloom syndrome</td>
<td>recombination?</td>
<td>BLM</td>
</tr>
<tr>
<td>Werner syndrome</td>
<td>recombination?</td>
<td>WRN</td>
</tr>
<tr>
<td>Rothmund-Thomson syndrome</td>
<td>recombination?</td>
<td>RECQL4</td>
</tr>
<tr>
<td>HNPCC</td>
<td>mismatch repair</td>
<td>hMSH2, hMLH1, hPMS1, hPMS2</td>
</tr>
<tr>
<td>Nijmegen breakage syndrome</td>
<td>double-strand break repair</td>
<td>NBS1</td>
</tr>
</tbody>
</table>
the best understood DNA damaging agents (and therefore with the most well-characterized corresponding DNA repair pathways) are UV and ionizing radiation, and alkylating agents.

1.1.1 Exogenous DNA damaging agents and DNA damage

1.1.1.1 Alkylating agents

Alkylating agents are electrophilic compounds with an affinity for nucleophilic centers in organic macromolecules (Friedberg et al., 1995). They will react not only with DNA, but also with proteins (Boffa and Bolognesi, 1985a,b), raising the need not only for DNA repair, but possibly protein repair as well. The ring nitrogens on the DNA bases are more nucleophilic and therefore more susceptible to alkylation than are the oxygens, with $N'\text{ of guanine and } N^3\text{ of adenine being the most reactive (Fig. 1-1).}$

Alkylating agents can be assigned a number $s$ termed the Swain-Scott constant, which correlates with electrophilicity (reviewed in Beranek, 1990). Generally, the closer the $s$ value gets to 1, the greater the preference for reaction with $N'$ versus $O^6$ of guanine. Methyl methanesulfonate (MMS) has an $s$ constant of $>0.83$ and its methylation pattern is skewed towards 7-methylguanine (7MeG) and 3-methyladenine (3MeA), whereas N-methyl-N’-nitro-N-nitrosoguanidine (MNNG) has an $s$ constant of 0.42 and a methylation pattern that includes not only 7MeG and 3MeA, but also a relatively large amount of $O^6$-methylguanine (O$^6$MeG) (Beranek, 1990; Lee et al., 1992).

Base alkylation of DNA can either be innocuous, mutagenic, or lethal to the cell. The most common lesion after treatment of cells with MMS, 7MeG, has no biological consequence per se. It is neither lethal to the cell nor mutagenic. It can be recognized by the base excision repair pathway, however, and processed into a lethal abasic (AP)
Figure 1-1: Potential sites of alkylation by DNA methylating agents.
site (see section 1.2.1). The 3MeA lesion appears innocuous in that it does not distort the double helical structure of the DNA backbone. The N of adenine lies in the minor groove of the DNA molecule, and alkylation here has the effect of blocking replicative DNA polymerases (Larson et al., 1985; Engelward et al., 1998). Hence, 3MeA is a lethal lesion and must be repaired.

Methylation of DNA at O of guanine (O-MeG) (Loechler et al., 1984) and O of thymine (O-MeT) (Preston et al., 1986) is mutagenic due to mispairing of these modified bases with thymine and guanine, respectively. Thus alkylation agents which methylate these sites cause GC→AT and TA→CG transitions if the damage is unrepaired. Mutagenic agents also have the property of being carcinogenic.

1.1.1.2 Ultraviolet radiation

Ultraviolet (UV) radiation encompasses the radiation spectrum from 100-400 nm, and is divided into UV-A (320-400 nm), UV-B (290-320 nm), and UV-C (100-290 nm). UV exposure on the earth’s surface is primarily in the UV-A and B range, although the wavelength most frequently used in DNA repair studies is around 260 nm (UV-C). Two of the most frequently induced DNA lesions are the pyrimidine dimer and the (6-4) photoproduct (Friedberg et al., 1995). Absorption of UV radiation results in adjacent pyrimidines becoming covalently linked and a four membered ring structure is created by the saturation of the pyrimidine 5,6 double bonds. T-T dimers are most common, followed by T-C and C-T, with C-C being relatively rare. Pyrimidine dimers can cause severe distortion of the helical conformation of DNA (Friedberg et al., 1995). The pyrimidine-pyrimidone (6-4) photoproduct also distorts the DNA structure (Taylor et al., 1988). This lesion forms between adjacent TC, CC, and TT, but not between CT.
UV-induced DNA damage is both mutagenic and lethal. The lesions induced block DNA replication, but can be bypassed by a number of mutagenic polymerases (reviewed in Baynton and Fuchs, 2000; Goodman and Tippin, 2000). Outside of S phase, these lesions are efficiently targeted by the nucleotide excision repair pathway (see section 1.2.2) and replaced with pristine nucleotide sequence. In humans, mutations in the nucleotide excision repair group of genes can result in severe sensitivity to sunlight and a predisposition to skin cancer, as observed with the genetic disease xeroderma pigmentosum. Pyrimidine dimers also block the transcription of genes. In fact, one of the signals that triggers the repair of UV-induced DNA damage is a blocked RNA polymerase.

1.1.1.3 Ionizing radiation

DNA damage from ionizing radiation can occur from the direct deposition of energy to DNA, as well as indirectly through the interaction of reactive oxygen species, derived mainly from water, with DNA. Over 80% of the energy deposited by ionizing radiation in cells results in the abstraction of electrons from water creating H₂O⁻. Subsequent reactions result in the accumulation of many reactive oxygen species, including •OH, O₂⁻, and H₂O₂. The majority of DNA damage caused by ionizing radiation appears to be from •OH (Ward, 1988), hence ionizing radiation causes a large amount of oxidative DNA damage.

Hydroxyl radicals attack the C5=C6 double bond of thymine, leading to thymine glycol (5,6-dihydroxy-5,6-dihydrothymine) formation (Friedberg et al., 1995). Other altered bases can also arise, such as 2,6-diamino-4-hydroxy-5-amidopyrimidine (FaPy). These lesions are recognized by DNA glycosylases and repaired by the base excision
repair pathway (see section 1.2.1). Protein-DNA crosslinks might be an important lesion after ionizing radiation treatment (Simic and Dizdaroglu, 1985), as γ-irradiation has been found to cause thymidine-tyrosine crosslinks in the presence of oxygen (Dizdaroglu, 1992).

Ionizing radiation causes less damage to the DNA backbone than it does to bases but nevertheless this sugar damage is of a huge biological significance. 100 Rads of ionizing radiation can induce 600-1000 single strand breaks (SSBs) and 16-40 double strand breaks (DSBs) in mammalian cells (Ward, 1988). The majority of these strand breaks bear damaged termini, so a simple religation is not possible. At the 3’ terminus, the OH group is usually lost, and phosphate or phosphoglycolate groups are present instead (Henner et al., 1982; Henner et al., 1983). Additionally, a terminal base residue is often missing, and many SSBs are therefore single nucleotide gaps. SSBs, if unrepaired, can be converted into lethal DSBs during DNA replication. If these DSBs persist, mitosis will produce daughter cells with an incomplete genome, and eventually cell death will ensue. This type of DNA damage must be repaired by recombinogenic means, either through homologous recombination or non-homologous end joining (see section 1.2.4).

1.1.2 Spontaneous or endogenous DNA damage

DNA damage occurs not only due to exposure to environmental factors, but also as a consequence of cellular metabolism, which is itself intimately linked to the environment in which the cell lives. For example, a cell living in an aerobic environment undergoes aerobic respiration, and many byproducts of this metabolic process are DNA damaging agents. Thus, DNA repair proteins have to deal with a wide
variety of exogenously and endogenously derived DNA lesions. As well, the DNA molecule itself is inherently unstable, suffering from spontaneous base loss and even occasional strand breakages.

Alkylation of DNA from endogenous sources occurs in eukaryotic cells (Xiao and Samson, 1993). Deletion of the MGT1 gene in Saccharomyces cerevisiae, which encodes the methyltransferase that demethylates O6MeG and O4MeT, results in a spontaneous mutator phenotype. Also, in cells deficient in the Apg1 AP endonuclease (see section 1.2.1), overexpression of the MAGI 3-MeA DNA glycosylase, which removes N-alkylated purines (producing more AP sites), was mutagenic, whereas inhibiting MAGI expression (producing fewer AP sites) decreased spontaneous mutagenesis (Xiao and Samson, 1993). Thus, endogenous byproducts of metabolism alkylate DNA on both O and N of bases. One possible source of this damage is S-adenosylmethionine (SAM). SAM is a weak methylating agent often used in enzymatic methylation reactions, but it has been shown to also methylate DNA and proteins non-enzymatically (Rydberg and Lindahl, 1982). In addition, methylating agents are postulated to arise from lipid peroxidation reactions (Vaca et al., 1988) and the nitrosation of amines (Calmels et al., 1987; Tsimis and Yarosh, 1990).

Spontaneous oxidative DNA damage due to aerobic metabolism is a major obstacle to cell survival. A major source of reactive oxygen species is leakage of singlet oxygen, peroxide and hydroxyl radicals, and hydrogen peroxide from the mitochondria. The lesions induced by these reactive oxygen species overlap with those induced by ionizing radiation, since much of ionizing radiation-induced DNA damage is caused by reactive oxygen species.
Abasic (AP) sites are the most common so-called spontaneous DNA lesion. They are formed not only by the first step of base excision repair, wherein a DNA glycosylase removes a damaged base leaving an AP site (see section 1.2.1), but also by spontaneous depurination of DNA. It has been shown that in mammalian cells AP sites persist at a frequency of 50 000-200 000 lesions per cell under normal physiological conditions (Nakamura and Swenberg, 1999), with the most susceptible organ being the brain, presumably due to its high rate of oxygen consumption and therefore oxidative DNA damage. The hydrolytic depurination of DNA at neutral pH occurs 1.5 times faster for guanine than for adenine, and 1/20 as fast for pyrimidines (Friedberg et al., 1995). Spontaneous depurination has been estimated at 1 purine/Escherichia coli genome per 1 hour generation (Lindahl and Nyberg, 1974) or 10 000/mammalian cell/generation (Lindahl, 1979). AP sites are lethal lesions, in that they block replicative DNA polymerases (Johnson et al., 1998; Sagher and Strauss, 1983). They are also mutagenic, as they can be bypassed in S. cerevisiae by the Rev3 mutagenic DNA polymerase (Johnson et al. 1998). DNA-protein crosslinks can potentially occur at AP sites due to reaction of the aldehyde group at the 1' position of the deoxyribose with amino groups of proteins to form Schiff bases (Welsh and Cantor, 1984). Interstrand DNA crosslinks might also form at AP sites (Prakash and Gibson, 1992). Abasic sites can also interfere with the activities of both type I and type II DNA topoisomerases (to be discussed later).

1.2 DNA repair
Many different DNA repair pathways exist, and while each appears to specialize in the correction of a particular type of DNA damage, there is a large degree of substrate overlap amongst them. Thus for alkylation damage induced by the simple methylating agent MMS, the alkylation-specific base excision repair pathway initiated by 3MeA DNA glycosylase (MAG) shares repair duties with the nucleotide excision repair, postreplication repair, and recombination repair pathways. Since MMS induces many different lesions, the different repair pathways might repair different lesions. Sometimes the repair pathways compete for the same lesion, and thus ensure that potentially dangerous damage (such as an AP site) is repaired, even if one repair pathway is compromised. In many cases it is not known how it is decided which pathway will repair which lesion. There might be cell cycle, subnuclear locale, or damage threshold constraints to consider for in vivo DNA repair. Or does repair happen on a "first come, first served" basis, with members of each pathway continually scanning the DNA, competing in seeking out lesions? These questions have yet to be answered.

1.2.1 Base excision repair and alkylation damage

Base excision repair, as the name implies, is a process whereby single damaged bases are removed from the DNA and replaced with pristine sequence. The base excision repair pathway repairs DNA damage caused by both endogenous and exogenous sources, typically modifications by alkylating and oxidative agents, and spontaneous decomposition products. Base excision repair begins with the removal of a damaged base from the DNA by DNA glycosylase-mediated cleavage of the N-glycosidic bond resulting in an AP site (Friedberg et al., 1995). AP sites are processed either by an AP endonuclease which cleaves phosphodiester bonds 5' to the AP site and
<table>
<thead>
<tr>
<th>Nucleotide excision repair (RAD3 group)</th>
<th>Postreplication repair (RAD6 group)</th>
<th>Recombination repair (RAD52 group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD1</td>
<td>RAD5</td>
<td>RAD50</td>
</tr>
<tr>
<td>RAD2</td>
<td>RAD6</td>
<td>RAD51</td>
</tr>
<tr>
<td>RAD3</td>
<td>RAD18</td>
<td>RAD52</td>
</tr>
<tr>
<td>RAD4</td>
<td>MMS2</td>
<td>RAD54</td>
</tr>
<tr>
<td>RAD7</td>
<td>UBC13</td>
<td>RAD55</td>
</tr>
<tr>
<td>RAD10</td>
<td>REV1</td>
<td>RAD56</td>
</tr>
<tr>
<td>RAD14</td>
<td>REV3</td>
<td>RAD57</td>
</tr>
<tr>
<td>RAD16</td>
<td>REV7</td>
<td>RAD59</td>
</tr>
<tr>
<td>RAD23</td>
<td>RAD30</td>
<td>MRE11</td>
</tr>
<tr>
<td>RAD25</td>
<td>POL30</td>
<td>XRS2</td>
</tr>
<tr>
<td>SSL1</td>
<td>POL3</td>
<td></td>
</tr>
<tr>
<td>TFB1</td>
<td>SRS2</td>
<td></td>
</tr>
</tbody>
</table>
leaves a 3'-OH and a 5'-deoxyribose phosphate, or by an AP lyase which cleaves 3' to the AP site, leaving a 5'-phosphate and a 3'-unsaturated sugar derivative incapable of priming DNA synthesis (Friedberg et al., 1995). It is possible that this abnormal 3' moiety is removed by an AP endonuclease. The one base gap is subsequently filled in by a DNA polymerase, the abnormal 5' group left by the AP endonuclease is removed by flap endonuclease I (FEN-1) (Rad27 in S. cerevisiae), and the DNA ends are ligated.

The initiating DNA glycosylase sets the stage for subsequent base excision repair reactions. Alkylated bases and uracil are removed by monofunctional DNA glycosylases, while oxidative base damage is typically repaired by DNA glycosylases with an associated AP lyase activity (Doetsch and Cunningham, 1990; Friedberg et al., 1995). MAG initiates the base excision repair of DNA alkylation damage. MAG actually has a relatively broad substrate range, and depending upon the species from which it was isolated, can remove the methylated bases 3MeA, 3-methylguanine, 7-MeG, O6-methylthymidine, and O6-methylcytosine, the more complex alkylated bases 7-(2-chloroethyl)guanine, 7-(2-hydroxyethyl)guanine, and 7-(2-ethoxyethyl)guanine, the bridged alkyl adducts 1,N6-ethenoadenine and 3,N2-ethenoguanine, hypoxanthine (deaminated adenine), the oxidized bases 8-oxoguanine, 5-hydroxymethyluracil, and 5-formyluracil, as well as all four normal DNA bases (reviewed in Wyatt et al., 1999). *E. coli* has two 3MeA DNA glycosylases, encoded by *tag*+ and *alkA*−, the former of which is constitutively expressed, and the latter involved in the adaptive response to alkylation damage (Friedberg et al., 1995). The yeast *S. cerevisiae* has only one 3MeA DNA glycosylase, encoded by *MAG1*, and it shows homology to the *alkA*+ gene product (Chen et al., 1990). *MAG1* expression is induced by DNA damage, including but not limited
to, alkylation damage (Chen et al., 1990; Chen and Samson, 1991; Liu and Xiao, 1997). Deletion of \( MAG1 \) renders yeast cells hypersensitive to killing by the alkylation agent MMS (Chen et al., 1990), but has no effect on spontaneous mutagenesis (Xiao and Samson, 1993).

Mag1 catalyzes the first step in the base excision repair of alkylation damage in \( S. \) cerevisiae. The AP site created is next recognized by an AP endonuclease, of which there are two in \( S. \) cerevisiae. \( APN1 \) is a yeast homolog of the \( E. \) coli endonuclease IV (Popoff et al., 1990). Apn1 hydrolyses DNA 5' to the abasic site, generating 3'-OH and 5'-deoxyribosephosphate termini (Johnson and Demple, 1988). Yeast \( apn1\Delta \) cells are slightly sensitive to killing by MMS, and have an increased spontaneous mutation rate (Ramotar et al., 1991). Overexpression of \( MAG1 \), increasing the number of genomic AP sites, increases the mutation rate of \( apn1\Delta \) cells, whereas decreasing \( MAG1 \) expression reduces this rate (Xiao and Samson, 1993). \( APN2 \) is homologous to \( E. \) coli exonuclease III (Johnson et al., 1998). \( apn2\Delta \) mutant cells show no MMS-sensitivity, suggesting that Apn1 is the major yeast AP endonuclease (Johnson et al., 1998). However, \( apn1\Delta \ apn2\Delta \) double mutants are extremely sensitive to killing by MMS (a synergistic effect), indicating that in the absence of Apn1, Apn2 AP endonuclease activity can compensate (Johnson et al., 1998). MMS-induced mutagenesis is also synergistically increased in the \( apn1\Delta \ apn2\Delta \) double mutant, an effect requiring the Rev3 mutagenic DNA polymerase. As well, \( apn1\Delta \ apn2\Delta \) cells were almost totally defective in the repair of MMS-induced AP sites, compared to only a partial defect in \( apn1\Delta \) cells and no defect in \( apn2\Delta \) cells (Johnson et al., 1998).
Cleavage by an AP endonuclease leaves a gapped DNA strand with an abnormal 5'-terminus. The 5'-deoxyribosephosphate can be removed either by a deoxyribophosphodiesterase activity (or 5'→3' exonuclease activity), or by repair-synthesis-mediated strand displacement followed by removal of the displaced strand by a flap endonuclease. The \textit{S. cerevisiae} Rad27 protein can accomplish both tasks (Harrington and Lieber, 1994; Zhu \textit{et al}., 1997). Rad27 was first identified as being a Rad2 nuclease family member (Reagan \textit{et al}., 1995; Johnson \textit{et al}., 1995; Harrington and Lieber, 1994). It possesses both 5'→3' exonuclease and flap endonuclease activities (Harrington and Lieber, 1994; Zhu \textit{et al}., 1997). Apart from its role in base excision repair, Rad27 is important for the processing of Okazaki fragments in lagging strand DNA synthesis (Ishimi \textit{et al}., 1988; Waga \textit{et al}., 1994; Murante \textit{et al}., 1998). Inactivation of Rad27 leads to temperature-sensitive growth with a G2 arrest characteristic of replication mutants, a mutator phenotype, a hyperrecombinant phenotype, and MMS hypersensitivity (Reagan \textit{et al}., 1995; Johnson \textit{et al}., 1995; Sommers \textit{et al}., 1995). Wu and Wang (1999) showed that the 5'-deoxyribosephosphate moiety left by AP endonucleases is removed in yeast by hydrolysis of 1-5 nt 3' to the baseless sugar phosphate. This was likely due to the exonuclease activity of Rad27, as no strand displacement synthesis occurred during the assay (Wu and Wang, 1999). However, a role for the flap endonuclease activity of Rad27 \textit{in vivo} was not ruled out.

Base excision repair is completed by gap-filling DNA synthesis and ligation of the broken DNA sugar-phosphate backbone. The 3'-OH terminus left by AP endonuclease serves as an appropriate primer for DNA synthesis. Evidence in the yeast
S. cerevisiae implicates DNA polymerase δ in repair synthesis during base excision repair (Blank et al., 1994).

3MeA-DNA glycosylases protect cells against the toxic effects of numerous DNA damaging agents. For MMS-induced DNA damage, the major lethal lesion is 3MeA, which has been shown to inhibit both in vitro DNA synthesis (Larson et al., 1985) and in vivo DNA replication (Engelward et al., 1998). 3MeA is a relatively minor DNA modification, situated in the minor groove of the DNA molecule (Friedberg et al., 1995), and it was a puzzle as to how this extra methyl group interfered so dramatically with DNA replication. Recently, the crystal structures of four DNA polymerases (rat Pol β, Thermus aquaticus Taq polymerase, T7 DNA polymerase, and E. coli Pol I) have shown that contact is made between the polymerase and the N3 atom of purines in the template strand (Pelletier et al., 1994; Eom et al., 1996; Spratt, 1997; Doublié et al., 1998). In the case of DNA pol β and T7 DNA polymerase, this contact has been shown to be required for catalytic activity of the enzymes (Beard et al., 1996; Doublié et al., 1998).

In addition to protecting against cell death, the murine Aag 3MeA-DNA glycosylase protects against sister chromatid exchange events, as well as the formation of chromatid breaks and gaps, S phase arrest, and apoptosis (Engelward et al., 1996; Engelward et al., 1998). The lesion responsible for triggering these events in Aag-deficient cells is 3MeA (Engelward et al., 1998). Possible mechanisms for the clastogenicity of MMS in the context of DNA replication blocks will be discussed later (Section 1.4: DNA replication, DSB production, and recombination).

1.2.2 Nucleotide excision repair and alkylation damage
In nucleotide excision repair in eukaryotes, the DNA lesion is removed as part of a 24-32 base oligonucleotide, and the resulting ss gap is filled by a DNA polymerase and ligated (Friedberg et al., 1995). The nucleotide excision repair proteins recognize and excise lesions that typically cause a great deal of helical distortion in the DNA molecule, such as (6-4) photoproducts and cyclobutane pyrimidine dimers induced by UV light. Various nucleotide excision repair genes are mutated in patients with the disease xeroderma pigmentosum (XP); phenotypes associated with this disease include extreme photosensitivity and a susceptibility to developing skin cancers (Friedberg et al., 1995), highlighting the role that nucleotide excision repair plays in repairing UV-induced DNA damage.

Nucleotide excision repair begins with recognition of the DNA lesion. This can occur by two methods. In the so-called "global genome" nucleotide excision repair, a complex of XPC-hHR23B in humans (Rad4-Rad23 in S. cerevisiae) detects the presence of a lesion in DNA and recruits the rest of the repair machinery, including the general transcription factor TFIIH, to the site of the damage (Sugasawa et al., 1998). In "transcription coupled" repair, the damage signal is a blocked RNA Pol II elongation complex. The RNA Pol II complex is removed by the CSA and CSB proteins (Rad26 and Rad28) (reviewed in van Gool et al., 1997) allowing for the nucleotide excision repair of transcription-blocking DNA lesions on the template strand of actively transcribed genes. In both cases, TFIIH is required for melting the DNA around the lesion, thereby increasing accessibility for subsequent repair proteins. TFIIH is a multisubunit protein containing two helicases, XPB (Rad25) (3'→5') and XPD (Rad3) (5'→3'), which can open the bubble around the lesion to a 10-20 nucleotide (nt) size.
The fully open bubble is stabilized by the DNA damage-binding protein XPA (Rad14) (Robins et al., 1991; Jones and Wood, 1993; Asahina et al., 1994), the ssDNA binding protein replication protein A (RPA) (de Laat et al., 1998b), and the endonuclease XPG (Rad2). XPG cleaves at the 3' border of the open nucleotide excision repair DNA intermediate (O'Donovan et al., 1994; Evans et al., 1997). A complex of ERCC1-XPF (Rad10-Rad1) forms the corresponding 5' endonuclease (Matsunaga et al., 1995; Sijbers et al., 1996; de Laat et al., 1998a). The endonucleases are correctly positioned by virtue of interactions with RPA. Repair synthesis uses the newly exposed 3'-OH as a primer for DNA replication across the gap. Both DNA Polδ and ε are required in humans and in yeast (Dresler and Frattini, 1986; Nishida et al., 1988; Hunting et al., 1991; Coverley et al., 1992; Budd and Campbell, 1995). The replication cofactors RF-C, RPA, and PCNA are also required. Repair synthesis does not result in strand displacement downstream of the gap, as can occur with base excision repair, but stops at the 3' cleavage site, where strand ligation occurs to complete repair of the damaged DNA.

As previously mentioned, nucleotide excision repair typically functions to repair DNA damaged by helix-distorting lesions. However, nucleotide excision repair also plays a role in the repair of the structurally innocuous 3MeA and AP lesions (Xiao and Chow, 1998; Torres-Ramos et al., 2000). Xiao and Chow (1998) showed that while rad2Δ and rad4Δ yeast cells were only slightly MMS-sensitive, and rad10Δ cells displayed no MMS sensitivity, double mutants combining nucleotide excision repair mutations and either mag1Δ or apn1Δ displayed a synergistic increase in MMS sensitivity. They further showed that deleting RAD1 led to a synergistic increase in the MMS-induced mutagenesis frequency of apn1Δ cells. Torres-Ramos et al. (2000)
repeated the results of Xiao and Chow (1998), using rad2Δ, rad4Δ, and rad14Δ as their nucleotide excision repair mutations, and a combined apn1Δ apn2Δ double mutant as their base excision repair mutation. They extended their results by showing that the deletion of RAD14 in both the apn1Δ single and apn1Δ apn2Δ double mutant backgrounds decreased the rate of removal of abasic DNA lesions after MMS treatment. Together, these two studies implicate nucleotide excision repair in the removal of both 3MeA and abasic lesions, albeit in otherwise wild type cells the effect is negligible. Perhaps the affinity of the damage recognition protein for these lesions is so low that unless base excision repair is nonfunctional, the rate of repair by nucleotide excision repair proteins is too slow to be observed.

1.2.3 Postreplication repair and alkylation damage

The postreplication repair pathway of yeast exists to enable the completion of DNA replication in the presence of polymerase-blocking lesions; these lesions are not removed, merely bypassed, so the term “repair” is a misnomer. Postreplication repair is actually a damage tolerance pathway. Traditionally, there are two arms to postreplication repair, namely mutagenic and error-free (Friedberg et al., 1995). Both are under the control of the Rad6 ubiquitin-conjugating enzyme (Ubc) and the Rad18 ssDNA-binding protein.

Mutagenic translesion bypass has been in the limelight recently due to the discovery of multiple mutagenic DNA polymerases in many species. The list includes in yeast Polζ (Rev3-Rev7), Rev1, and Polη (Rad30), in humans hRev3, XPV (hRAD30A, Polη), and hRAD30B (Polτ), and in E. coli UmuC and DinB (reviewed in Baynton and Fuchs, 2000; Goodman and Tippin, 2000; Johnson et al., 2000).
Mutagenic DNA polymerases have the ability to replicate over DNA lesions that block replicative DNA polymerases, at the expense of an increased mutation rate. In the yeast *S. cerevisiae*, Rad6 and Rad18 somehow control the Rev1 and Polζ proteins, although how polymerase switching occurs remains unknown. Mutants of the mutagenic arm of postreplication repair display little sensitivity to DNA damaging agents such as UV or MMS as compared to mutants of the error-free arm, so the relative contribution of the mutagenic response to cell survival is minimal. However, when mutagenic (i.e. rev3) and error-free (i.e. mms2) double mutants are exposed to DNA damaging agents, a synergistic sensitivity approaching that of rad6 or rad18 is observed (Broomfield *et al.*, 1998; Brusky *et al.*, 2000). Hence the mutagenic response could be seen as a vital backup for error-free postreplication repair.

Error-free postreplication repair in yeast is currently known to require Rad6, Rad18, Mms2, Ubc13, Rad5, Pol30 (PCNA), and Pol3 (Polδ) (Broomfield *et al.*, 1998; Brusky *et al.*, 2000; Hofmann and Pickart, 1999; Johnson *et al.*, 1992; Prakash, 1981; Torres-Ramos *et al.*, 1996; Torres-Ramos *et al.*, 1997). Genetic studies have further subdivided error-free postreplication repair into two arms, defined by *RAD5* on one side and *POL30* on the other side (Xiao *et al.*, 2000). Both of these arms are thought to be under the control of Mms2 and Ubc13, and these in turn under Rad6 and Rad18. How all of this is coordinated biochemically is not well characterized.

Rad6 and Ubc13 are both Ubcs (Jentsch *et al.*, 1987; Hofmann and Pickart, 1999), while Mms2 is a Ubc-like protein (Broomfield *et al.*, 1998). Rad6 Ubc function is required for all of its activities, including DNA repair, sporulation, and N-end rule protein degradation (Sung *et al.*, 1990). The target(s) of Rad6 Ubc activity in
postreplication repair are unknown. Ubc13 complexes with Mms2 (Hofmann and Pickart, 1999), and catalyzes the assembly of polyubiquitin chains through lysine (K) 63 rather than the usual K48. This suggests that Mms2-Ubc13 mediated ubiquitination might not target proteins for degradation, but rather be a form of signal transduction, possibly to trigger the nucleation of postreplication repair proteins at a polymerase-blocking lesion (Hofmann and Pickart, 1999). Interestingly, yeast strains which produce K63R ubiquitin (where arginine replaces lysine at position 63) display sensitivity to killing by UV and MMS, and a defect in UV-induced mutagenesis (Spence et al., 1995). That these ubiquitin mutants are deficient in damage-induced mutagenesis suggests that the Rad6 Ubc protein also utilizes the alternative K63 linkage.

Rad5 and Rad18 both bear the RING finger motif (Ulrich and Jentsch, 2000). Many RING finger proteins are found to be associated with ubiquitin conjugating protein complexes (reviewed in Joazeiro and Weissman, 2000; Tyers and Jorgensen, 2000). Ulrich and Jentsch (2000) found that Rad5, through its RING finger motif, binds Ubc13-Mms2. Rad5 can also interact with Rad18, and it is envisioned that upon encountering a polymerase-blocking lesion, Ubc13-Mms2, through Rad5, is brought into association with Rad6-Rad18. Hence two Ubcs would be brought into close proximity at the site of DNA damage. It is not known what the targets of ubiquitination are, if the ubiquitinated proteins are degraded, or how ubiquitination allows for error-free bypass of the replication-blocking lesion.

In cycling cells, exposure to UV light leads to the synthesis of DNA fragments of a size equal to the average distance between pyrimidine dimers (Rupp and Howard-Flanders, 1968; Lehmann, 1972; Buhl et al., 1972; Sarasin and Hanawalt, 1980).
Incubating cells after UV irradiation allows for the conversion of this low MW DNA into high MW, chromosomal length DNA, as seen in unirradiated cells. This phenomenon is referred to as “postreplication repair”. Postreplication repair in *E. coli* is mainly recombinational (Rupp *et al.*, 1971), whereas in mammalian cells it is possibly non-recombinational and requires *de novo* DNA synthesis (Lehmann, 1972; Buhl *et al.*, 1972). Attempts to delineate error-free postreplication repair in the yeast *S. cerevisiae* have led to ambiguous results, and it appears that recombinational mechanisms may be involved in some, but not all, cases. Louise Prakash (1981) designed a yeast postreplication repair assay which shows that, following the definition of postreplication repair set out at the beginning of this paragraph, *rad6* cells are completely defective in postreplication repair. She also showed that *rad18* and *rad52* (recombination-defective) cells were severely but not completely impaired, and the *rev3* mutation had no effect on the outcome of the postreplication repair assay (Prakash, 1981). Subsequent experiments showed that the *pol30-46* allele of PCNA renders cells postreplication repair-defective (Torres-Ramos *et al.*, 1996). The effects of other *RAD6* group mutants on the postreplication repair assay are unknown.

The postreplication repair assay measures the post-UV replication of DNA. Immediately after irradiation, cells are labeled with [*H]-uracil for 15 minutes, and then incubated for various times in a high-uracil medium before cell lysis and DNA separation through an alkaline sucrose gradient (Prakash, 1981). Over time in wild type cells the ssDNA fragment size makes a transition from low MW to high MW, as postreplication repair completes replication over or around the UV-induced DNA lesions.
There is good evidence for a recombination mechanism for the completion of postreplication repair in yeast. When yeast cells attempt to replicate a UV-damaged DNA template, sister chromatid exchange is induced (Kadyk and Hartwell, 1993). The UV-irradiation of diploid rad1/rad1 cells, defective in nucleotide excision repair, stimulated sister chromatid exchange in a replication-dependent manner (Kadyk and Hartwell, 1993). It is suggested that sister chromatid exchange is a mechanism used by cells to bypass UV-induced damage (and presumably other polymerase-blocking lesions) during S phase. Interestingly, not only is RAD52 required for UV-induced sister chromatid exchange (as expected; see section 1.2.4.1) (Kadyk and Hartwell, 1993), the DNA damage cell cycle checkpoint genes RAD9 and RAD17 (see section 1.3.1) are also required for maximal induction of sister chromatid recombination (Paulovich et al., 1998). Either a slowing of S phase progression is required to attain full UV-induction of sister chromatid exchange, or the Rad9 and Rad17 proteins play a direct role in the recombination events (Paulovich et al., 1998).

Two models have been proposed to account for UV-induced sister chromatid exchange (reviewed in Naegeli, 1994; Friedberg et al., 1995). In one, the free 3’ end of the daughter strand gap invades the sister duplex and a Holliday junction is formed. Branch migration across the damaged portion of the template bypasses the lesion (Fig. 1-2). In the second model, the 3’ end of the daughter strand gap invades the sister duplex and uses the nascent strand as a template for replicating beyond the lesion on the leading strand template. A second template switch downstream of the lesion restores the normal replication fork. This is termed a copy choice mechanism of DNA synthesis. Evidence for something remarkably similar has been found in mammalian cells.
Figure 1-2: UV-induced sister chromatid exchange: daughter strand gap repair without Holliday junction cleavage. In this model, the Holliday junction is resolved by reverse branch migration.
Experiments by Higgins et al. (1976) pointed to the possibility of strand displacement and branch migration creating a template for replicative bypass of a DNA lesion (Fig. 1-3). It was proposed that after the leading strand was blocked, lagging strand synthesis continues for short distance, replicating beyond the lesion on the leading strand template. The newly synthesized strands are displaced, the parental strands anneal (fork backs up), and the daughter strands anneal. This creates an alternative template for leading strand DNA synthesis. After synthesis the structure needs to be resolved to restore a normal replication fork (Higgins et al. 1976). The authors found evidence to support their model. After treatment of cells with MMS and incubation in the presence of BrdU to label newly synthesized DNA, genomic DNA was isolated, sheared, and applied to a neutral CsCl density gradient. Unreplicated parental DNA appears as a light peak, while newly synthesized DNA appears as a peak of intermediate density. There was a third heavy peak of DNA, indicative of conservative DNA synthesis or daughter-daughter annealing. The average length of the DNA molecules in the heavy peak coincide with the length of the short arm of a four-armed structure seen in electron micrographs of the same genomic DNA from MMS-treated cells (short arm = 440-4800 nt). Thus error-free postreplication repair in mammalian cells might proceed by a copy-choice DNA synthesis mechanism.

1.2.4 Recombination and alkylation damage

1.2.4.1 Gene conversion

Genetic recombination is the exchange of information between DNA molecules. Recombinational DNA repair usually occurs by gene conversion, where new DNA is copied from a donor template, rather than a reciprocal exchange of information. We
Figure 1-3: UV-induced sister chromatid exchange: strand displacement, branch migration, and template switching to allow DNA replication beyond the lesion. No daughter strand gaps are produced.
typically think of the substrate for recombinational repair being a DNA DSB, however a ss gap or SSB may also suffice.

In *S. cerevisiae*, recombination-defective mutants were isolated by their enhanced sensitivity to ionizing radiation and chemical agents which induce DNA strand breaks, but relatively normal sensitivity to UV (Friedberg *et al.*, 1995). As well, some recombination-defective strains are defective in meiosis (due to aberrant meiotic recombination) and cannot produce viable spores. Most of the genes responsible for recombination in *S. cerevisiae* fall into the RAD52 group.

Recombination is the most effective way to repair a radiation-induced strand break. As mentioned earlier, ionizing radiation does not produce DNA breaks with ligatable ends; the ends are usually modified. Recombination mutants are also extremely sensitive to DNA alkylating agents. Because of this phenomenon, alkylating agents such as MMS have been called X-ray mimetic. Alkaline elution of DNA from lung and liver tissue of mice that had been administered MMS showed that the alkylating agent induces DNA SSBs *in vivo* (Eastman and Bresnick, 1978). Similar results were found by use of an alkaline sucrose gradient for separating DNA from human or mouse cells cultured in the presence of MMS (Pavlis *et al.*, 1978). Later studies showed that MMS is an S-phase-dependent clastogenic agent; chromosomal abnormalities were not observed unless the cells had undergone DNA replication (Schwartz, 1989). All of these studies showed that DNA alkylation leads to SSBs. None were designed to show the appearance of DSBs after alkylation. Another problem with these studies is that in order to show SSBs, the DNA strands are separated under alkaline conditions, and high pH causes strand breakage at AP sites (Tamm *et al.*, 1953).
Since DNA alkylating agents induce a high number of AP sites due to base excision repair, this technique overestimates the number of frank SSBs induced by the DNA damaging treatment. Nonetheless, it is assumed that MMS induces DSBs, probably through the replication-blocking action of 3MeA (see section 1.4 below).

There are a few models to explain the process of gene conversion (reviewed in Pâques and Haber, 1999). The Szostak model (Szostak et al., 1983) begins with the 5'→3' resection (chewing back) of the DSB ends (Fig. 1-4). The 3' ends then invade the homologous sequence, and act as primers for de novo DNA synthesis. Two Holliday junctions are formed; if they are resolved in the same manner, gene conversion without crossing over of flanking markers results. If the two junctions are resolved differently, some crossing over (exchange of flanking DNA) occurs.

The synthesis-dependent strand annealing model was designed to account for the lack of crossing over associated with many gene conversion events. It begins similar to the Szostak model, except that the newly synthesized strands are displaced from their respective templates and anneal to one another, allowing for the completion of DNA synthesis and ligation of the broken molecule (Fig. 1-5). Thus, in this model, DNA synthesis is conservative, not semi-conservative. The strands could be displaced by dismantling of the replication structure by a topoisomerase or helicase (McGill et al., 1989; Thaler et al., 1987), or by keeping the replication bubble small and continually unwinding the newly synthesized strand from its template (Formosa and Alberts, 1986).

The above two models require only leading strand DNA synthesis, primed by the 3' end of the invading strand, during recombination. Holmes and Haber (1999) showed that both leading and lagging strand DNA synthesis is involved in DSB-induced gene
Figure 1-4: Szostak model of DSB-induced gene conversion. Adapted from Szostak et al. (1983).
Figure 1-5: Synthesis-dependent strand annealing model of DSB-induced gene conversion. Note that gap-filling DNA synthesis is conservative. Adapted from Pâques and Haber (1999).
conversion in *S. cerevisiae* (at least at the MAT locus). Since a lagging strand synthesis defect was able to inhibit gene conversion in G1 cells, the observed effect was not an indirect one due to an inhibition of S-phase DNA replication (Holmes and Haber, 1999). In addition, mating type switching, the outcome of which was followed in the authors' assay, was not affected by mutations in the genes encoding the origin-recognition complex, showing that the initiation of DNA replication by a DSB differs from origin-dependent initiation. A model was proposed whereby strand invasion leads to the creation of a modified DNA replication fork, requiring both leading and lagging strand synthesis using the donor template (Fig. 1-6). Replication is terminated when the fork reaches the second end of the DSB.

Recombinational repair requires many proteins. There are currently ten members of the *RAD52* epistasis group: *RAD50, RAD51, RAD52, RAD53, RAD54, RAD55, RAD56, RAD57, MRE11*, and *XRS2* (Pâques and Haber, 1999). *RAD52* is required for all homologous recombination events (Friedberg *et al.*, 1995). *RAD50, MRE11*, and *XRS2* encode a group of interacting proteins with roles in homologous recombination, non-homologous end joining, and telomere maintenance (reviewed in Haber, 1998). *RAD51, RAD54, RAD55*, and *RAD57* are required for certain recombination events, but not others. The Rad53 protein is actually involved in controlling DNA damage cell cycle checkpoints (Allen *et al.*, 1995; Weinert *et al.*, 1994), while the *RAD56* gene has not yet been cloned. In addition to these genes there exists a *RAD52* homolog, *RAD59* (Bai and Symington, 1996), and a *RAD54* homolog, *RDH54* (Klein, 1997; Shinohara *et al.*, 1997).
Figure 1-6: DSB-induced recombination requiring both leading and lagging strand DNA synthesis. Adapted from Holmes and Haber (1999).
The Rad50-Mre11-Xrs2 complex acts at DSBs and affects the 5'→3' resection of the two ends (Sugawara and Haber, 1992; Ivanov et al., 1994; Johzuka and Ogawa, 1995). The actual exonuclease activity is encoded by MRE11. Strangely, although in vivo, a 5'→3' exonuclease activity is needed, in vitro Mre11 acts as a dsDNA 3'→5' exonuclease and a ssDNA endonuclease (Furuse et al., 1998; Paull and Gellert, 1998; Usui et al., 1998; Moreau et al., 1999). Mutations in amino-terminal residues of Mre11 eliminate this in vitro activity and eliminate or reduce 5'→3' resection of DSBs in vivo (Paull and Gellert, 1998; Usui et al., 1998; Moreau et al., 1999). Rad52 appears to control the rate of resection (Sugawara and Haber, 1992), possibly through its ability to bind DNA ends (van Dyck et al., 1999). Thus DNA ends at DSBs are protected by either Rad52, promoting homologous recombination, or by the Ku proteins, promoting non-homologous end-joining (see section 1.2.4.3). RAD51 encodes the yeast homolog of E. coli recA (Aboussekhra et al., 1992; Shinozawa et al., 1992). Like RecA, Rad51 forms protein filaments on ssDNA (Baumann et al., 1996; Ogawa et al., 1993) and catalyzes an ATP-dependent strand exchange between a ss circular DNA molecule and a homologous linear duplex in vitro (Namsaraev and Berg, 1997; Sung, 1994; Sung and Robberson, 1995; Sung and Stratton, 1996). The ssDNA binding protein RPA (replication protein A) is required for efficient nucleoprotein filament formation by Rad51, presumably because it removes secondary structures from the ssDNA, but RPA also competes with Rad51 for ssDNA binding (Sung, 1997). It was shown that Rad52, which can bind to both Rad51 and RPA (Sung, 1997) and to ssDNA (Benson et al., 1998), allows for Rad51 ssDNA binding in the presence of RPA (Sung, 1997; Benson et al., 1998; New et al., 1998; Shinozawa and Ogawa, 1998). Another study showed that at
least for meiotic recombination, Rad55 and Rad57 are also involved in promoting the formation of the strand exchange complex (Gasior et al., 1998). D-loop formation, the heteroduplex joint formed when an invading ssDNA pairs with its ds homolog, requires factors beyond Rad51. Rad54 can promote D-loop formation by Rad51 and RPA (Petukhova et al., 1998), probably due to its ability to modify DNA topology (Petukhova et al., 1999). The Rad54 homolog, Rdh54, can also promote D-loop formation (Petukhova et al., 2000). Rdh54 can generate positive and negative supercoils in an ATP-dependent manner, and negative supercoils stimulate D-loop formation, so it is likely that Rdh54 promotes D-loop formation by virtue of its ability to generate negative supercoils in the DNA template (Petukhova et al., 2000).

For all the years of studying recombination, and for all the models used to explain such events, very little is known about the in vivo reactions themselves, and the actions of the various cellular components involved. In vitro biochemical studies, such as those described above, are helping to determine the roles of the various proteins involved; however, it is possible that there remain recombination factors yet to be discovered. Specific recombination events in the cell might require accessory factors to facilitate recombination in vivo that are not required for in vitro recombination assays.

1.2.4.2 Single-strand annealing

An alternative DSB repair pathway exists, which utilizes direct repeat sequences flanking the DSB, and leads to repair with deletion of one of the two repeats (Lin et al., 1984; Lin et al., 1985; Fishman-Lobell et al., 1992). This single-strand annealing pathway requires resection of DSB ends, exposing long tails each bearing the repeated sequence, which anneal. The non-complementary ss flaps are cleaved by an
endonuclease, and any gaps remaining are filled in by a polymerase and ligated (Fig. 1-7). In the yeast *S. cerevisiae*, single-strand annealing is Rad52-independent to a certain extent (Jackson and Fink, 1981; Klein, 1988; Dornfeld and Livingston, 1992; Prado and Aguilera, 1995); if the repeats are 1-2 kb in length, Rad52 is required (Fishman-Lobell et al., 1992; Sugawara and Haber, 1992). However, in longer repeats the level of homology seems to compensate for a lack of Rad52. The Rad52 homolog Rad59 also plays a role in single-strand annealing, especially if the repeat length is short. As the region of homology increases to 1 kb, the dependence of single-strand annealing on Rad59 decreases, although *rad59* mutants still display a 4-fold reduction in single-strand annealing with 1.17 kb repeats (Sugawara et al., 2000). The Rad1-Rad10 endonuclease cleaves the unpaired ss tails regardless of homology length (Fishman-Lobell and Haber, 1992). Msh2 and Msh3 mismatch repair proteins are also involved in removing the nonhomologous ss tails, but like Rad52, their necessity varies with the length of the direct repeats, decreasing as the homologous sequence increases in length (Sugawara et al., 1997). It is thought that the mismatch repair proteins stabilize short annealed sequences, allowing Rad1-Rad10 to cleave the unpaired ss tails. The protein(s) responsible for the resection activity is not known, although *rad50Δ* and *xrs2Δ* alleles slow down this process (Ivanov et al., 1994). As it leads to deletions, the single-strand annealing pathway is a source of genetic instability in areas of DNA repeats. Interestingly, although gene conversion is a non-mutagenic process, single-strand annealing can outcompete gene conversion for the repair of DSBs in mitotic *S. cerevisiae* cells (Fishman-Lobell et al., 1992; Wu et al., 1997).
Figure 1-7: Single-strand annealing. Box represents direct repeated DNA sequence.
1.2.4.3 Non-homologous end joining

In mammalian cells, most DSB repair occurs through an end-joining mechanism rather than through homologous recombination. This non-homologous end-joining process is dependent upon the Ku protein, a heterodimer of ~70 and ~80 kDa subunits (reviewed in Dynan and Yoo, 1998; Featherstone and Jackson, 1999). The Ku protein binds to DNA ends, stabilizing them for processing and religation. DNA ligase IV is responsible for the ligation activity (Frank et al., 1998; Grawunder et al., 1998). In addition to its role in DNA repair, Ku end-binding allows for the formation of the active DNA-dependent protein kinase (DNA-PK), by forming a nucleation site for the recruitment of the catalytic subunit of the enzyme (DNA-PKcs) (Dvir et al., 1992; Gottlieb et al., 1993). DNA-PK can then phosphorylate downstream effectors, possibly recruiting other repair proteins to the damage site, or even triggering a checkpoint response or apoptosis (Featherstone and Jackson, 1999). Consistent with this, DNA-PK phosphorylates p53 (Shieh et al., 1997) and is involved in the induction of p53 DNA binding after DNA damage (Woo et al., 1998).

*S. cerevisiae* has Ku70 and Ku80 homologs, originally termed Hdf1 (high affinity DNA-binding factor) (Feldmann and Winnacker, 1993) and Hdf2 (Feldmann and Winnacker, 1993; Milne et al., 1996), respectively (hereafter referred to as yKu70 and yKu80). Like their mammalian counterparts, yKu70 and yKu80 form a stable heterodimer which binds DNA ends (Feldmann and Winnacker, 1993). No DNA-PKcs homolog exists in *S. cerevisiae*, which possibly reflects a need for greater fidelity in the DNA damage response in multicellular organisms.
Both yKu70 and yKu80 are required for efficient rejoining of the DNA ends of a restriction endonuclease-digested plasmid (Boulton and Jackson, 1996; Milne et al., 1996). Interestingly, yku70Δ and yku80Δ mutants display little (Milne et al., 1996) to no (Boulton and Jackson, 1996; Siede et al., 1996) MMS sensitivity and no γ sensitivity, indicating that under normal circumstances, non-homologous end-joining is a minor DNA repair pathway in yeast. When homologous recombination is defective, however, loss of non-homologous end-joining increases the cell’s sensitivity to strand breaking agents such as MMS, γ, or X-rays (Boulton and Jackson, 1996; Milne et al., 1996; Siede et al., 1996). Thus, the non-homologous end-joining pathway offers an alternative mode of DSB repair if the homologous recombination pathway is rendered inoperable.

As previously stated, strand breaks induced by ionizing radiation (and probably other agents as well) cannot simply be religated, as they bear modified termini. Hence in many cases, Ku end-binding does not act simply by stabilizing the DNA ends until ligation can occur; Ku binding must facilitate processing of the ends prior to their ligation. The *S. cerevisiae* Rad50-Mre11-Xrs2 complex is required for non-homologous end-joining (Moore and Haber, 1996; Tsukamoto et al., 1996), and is a possible candidate for end processing. The yeast homolog of mammalian DNA ligase IV has been found, and ligIV mutants show γ-sensitivity in a rad52 background and defective non-homologous end-joining as expected (Schar et al., 1997; Teo and Jackson, 1997; Wilson et al., 1997). DNA polymerase β (DNA Polβ) is needed for end-joining reactions that require the removal of a 5’ or 3’ terminal mismatch, although DNA Polβ does not possess the nuclease activity required for the mismatch removal (Wilson and Lieber, 1999). Deletion of *RAD27* (FEN-1) leads to a reduction in non-homologous
end-joining events that proceed through a 5' flap intermediate, suggesting that Rad27 is one of the nucleases involved in end processing for non-homologous end-joining (Wu et al., 1999). It is possible that Mre11 possesses the other (3') nuclease activity.

There appears to be multiple pathways of non-homologous end-joining. One of the methods used to assay non-homologous end-joining in S. cerevisiae is to cleave a plasmid with a restriction endonuclease in a region with no homology to yeast DNA sequence, transform various yeast strains, and compare transformation efficiency to that obtained with the undigested plasmid. Boulton and Jackson (1996) found that yku70Δ rad52Δ strains had >100 fold decrease in transformation efficiency with digested plasmids versus rad52Δ single mutants. The yku70Δ single mutant reduces transformation efficiency of the linearized plasmid by a factor of 10 fold versus the wild type. The authors found that plasmids recovered from yKu70+ cells were able to be reclaved by the appropriate restriction enzyme, indicating an error-free repair. Plasmids recovered from yku70 cells, on the other hand, could not be reclaved, and were thus repaired by an error-prone means (Boulton and Jackson, 1996). Sequencing of these plasmids revealed that deletions of various sizes had occurred, and religation was through short direct repeat sequences of usually 4-8 bp. Thus in the absence of yKu70, some mechanism of end-joining still operates, but its fidelity is reduced.

Another method of assaying non-homologous end-joining in S. cerevisiae is by examining the repair of an HO endonuclease-induced DSB at the yeast mating type locus. Moore and Haber (1996) found that the HO cut was repaired by two pathways, one producing deletions whose ends have 1 to 6 bp of homology, and the other producing 2 or 3 bp insertions. Deleting RAD50, MRE11, or XRS2 dramatically
decreased the proportion of insertional mutations. Interestingly, when HO is expressed in S and G2 phases, the \textit{RAD50/MRE11/XRS2} insertion-producing pathway is more important, and when HO is expressed only in G1, the deletion pathway predominates (Moore and Haber, 1996).

1.2.5 \textbf{Substrate overlap for DNA repair pathways}

There is some degree of overlap in substrate specificity between the known DNA repair pathways. It has been shown that nucleotide excision repair and base excision repair both can repair damage arising from DNA alkylating agents. \textit{S. cerevisiae} double mutants of \textit{apn1} or \textit{mag1}, and \textit{rad2}, \textit{rad4}, or \textit{rad10} all show synergism in their survival curves after treatment with the alkylating agent MMS (Xiao and Chow, 1998). It is interesting that a synergistic response is seen, as nucleotide excision repair typically targets helix-distorting lesions, whereas 3MeA and therefore the AP lesions arising from 3MeA are present in the minor groove of the DNA, and are non-distorting lesions (Friedberg \textit{et al}, 1995). The \textit{apn1 rad1} and \textit{apn1 rad10} double mutants show a growth defect, while \textit{apn1 rad2} grows at a normal rate (Xiao and Chow, 1998). Additionally, deleting \textit{rad1} in the \textit{apn1} background further increases the spontaneous mutation rate of the \textit{apn1} mutant. These data indicate that while nucleotide excision repair does play a role in the repair of alkyl or abasic lesions, the single-strand annealing pathway of Rad1 and Rad10 plays an even more important role in tolerating this damage (Xiao and Chow, 1998).

This overlap was also observed for the cellular response to oxidative DNA damage (Swanson \textit{et al}., 1999). The Ntg1 and Ntg2 proteins are N-glycosylase-associated AP lyases that recognize a variety of damaged pyrimidines (Senturker \textit{et al}.,
The $ntg1 \ ntg2 \ apnl$ triple mutant is hyperrecombinant and has a mutator phenotype. It is not, however, sensitive to killing by oxidizing agents (Swanson et al., 1999). The mutator phenotype is lost and the recombination phenotype enhanced if $REV3$ is deleted, indicating that Pol$\zeta$ is required for the translesional bypass of AP lesions acquired in this genetic background (Swanson et al., 1999). If $RAD52$ is deleted in the $ntg1 \ ntg2 \ apnl$ triple mutant, the cells become sensitive to killing by oxidative damage. As well, simultaneous deletion of $NTG1$, $NTG2$, $APNL$, and either $RAD1$ or $RAD52$, resulted in a poor growth phenotype for the cells. The hyperrec and mutator phenotypes of the $ntg1 \ ntg2 \ apnl$ triple mutant were further enhanced by deletion of the $RAD1$ gene. These results indicate that the base excision repair, nucleotide excision repair, recombination repair, and translesional DNA synthesis pathways overlap for the repair or tolerance of oxidative DNA damage, probably specifically at the AP site (Swanson et al., 1999).

### 1.3 Cell cycle control, transcriptional upregulation, and DNA damage

#### 1.3.1 Cell cycle arrest and DNA damage

For eukaryotic cells, controlling cell cycle progression is part of the DNA damage response. DNA damage cell cycle checkpoints (Weinert and Hartwell, 1988) exist to allow the cell time to repair DNA damage during the cell cycle phase in which it was incurred, thereby preventing an easily repairable lesion being converted into a mutation, or even into an irreparable lethal lesion. They are also responsible for controlling much of the DNA damage-inducible gene expression. There are three cell cycle phases during which checkpoints can be triggered by DNA lesions: within G1, S
phase, or G2. The G1 checkpoint allows the repair of lesions incurred during G1 before entry into S phase, and can be triggered by UV, 4NQO, MMS, and ionizing radiation (Siede et al., 1993; Sidorova and Breeden, 1997). This checkpoint is probably sensitive to the gaps produced during repair of these lesions, as unexcised pyrimidine dimers do not trigger the checkpoint (Nelson and Kastan, 1994; Siede et al., 1994), and neither do DSBs (Raghuraman et al., 1994). The S phase checkpoint slows down the rate of DNA replication, allowing the cell time to repair potential mutagenic or polymerase-blocking lesions before the replication apparatus encounters them. This checkpoint is important for the cellular response to DNA alkylation damage (Paulovich and Hartwell, 1995; Paulovich et al, 1997). The G2 checkpoint exists mainly to allow for the repair of DSBs before mitosis, and is triggered by ionizing radiation and replication-dependent DNA damage (Weinert and Hartwell, 1988; Weinert et al., 1994).

Both the G1 and S phase checkpoints serve to prevent DNA replication in the presence of DNA damage or incomplete DNA repair, thereby inhibiting the clastogenic and mutagenic effects of some DNA damaging agents. Certain lesions, such as pyrimidine dimers or 3MeA, block the replication fork if encountered on the template strand. Blocked replication forks are unstable, and may lead to breakage and rescue by recombination proteins or by postreplication repair (if indeed these are two are separate pathways). In some cases, this problem is overcome through the use of mutagenic DNA polymerases which are able to replicate over these lesions, but at the cost of incorporating mutations into the newly replicated DNA. A SSB or gapped template, either directly due to template damage or to incomplete nucleotide excision repair or base excision repair, can be converted into a DSB by the replication process.
The G1 checkpoint is defective in p53-mutant mammalian cells (reviewed in Levine, 1997). In response to a DNA damage signal, p53 activates transcription of p21, a cyclin-dependent kinase inhibitor. p21 in turn inhibits Cdk4-cyclin D1, preventing it from phosphorylating Rb. Rb protein binds to and inhibits the E2F transcription factors, which regulate the expression of many genes required for entry into and progression through S phase. Phosphorylated Rb releases E2F complexes thereby allowing entry into S phase. Atm is upstream of p53 in the DNA damage response pathway, and the ATM gene is mutated in the cancer-prone disease ataxia telangiectasia (reviewed in Meyn, 1995). atm mutant cells are defective for all DNA damage checkpoints, and it was in these cells that an S phase checkpoint defect was first noticed, as atm mutants fail to inhibit DNA replication in response to X-irradiation (Painter and Young, 1980). This observation predated Weinert and Hartwell's coining of the term "cell cycle checkpoint" (Hartwell and Weinert, 1989).

The G2 checkpoint serves to ensure that the cell has enough time to utilize recombinational repair, if required. The reason for this is twofold. First, sister chromatids are the preferred template for recombinational repair in S. cerevisiae (Kadyk and Hartwell, 1992), and sister chromatid exchange is important for the repair of not only DSBs, but for replication-blocking lesions as well. After mitosis, the opportunity to use the sister chromatid for a recombinational substrate is lost. Second, if mitosis were to occur before a broken chromosome could be repaired, then the centromeric and distal portions of the chromosome would be partitioned into different cells, and some genetic information permanently lost to one of the resulting daughters. This may lead to the extinction of that daughter cell line.
DNA damage cell cycle checkpoint proteins can be loosely divided into three categories: damage sensors, signal transducers, and targets (Weinert, 1998). In *S. cerevisiae* there are two groups of DNA damage sensors; as determined through the use of a UV-induced DNA damage signal, Rad9, Rad24, and Mec3 are required to activate the checkpoint in G1 or G2, while Pole is required to activate it during S phase (Navas *et al.*, 1996). Contrasting this find, it was shown that Rad9, Rad17, and Rad24 are also required for the full inhibition of DNA replication in response to MMS-induced DNA damage (Paulovich *et al.*, 1997). Both sensor groups activate the checkpoint kinase Mec1, which in turn is responsible for controlling the activation of the Rad53 kinase (reviewed in Weinert, 1998). To activate the G1 checkpoint, Rad53 phosphorylates Swi6. Swi6 forms a complex with the transcriptional activator Swi4, and together they control the expression of the G1 cyclin genes (*CLNs*) at the G1/S interface.

Phosphorylation of Swi6 inhibits the Swi6/Swi4 complex, thereby arresting the cell in G1 (Sidorova and Breeden, 1997). During S phase, it is possible to inhibit replication both at the levels of initiation and elongation. DNA primase (Pri1) is required for both initiation of replication and elongation of the lagging strand. A primase mutant (*pri1-M4*) was isolated which fails to inhibit DNA replication when the cells are treated with MMS, suggesting that primase might be a target of Mec1 and Rad53 (Marini *et al.*, 1997). As well, Rad53 and Mec1 are required for the inhibition of late origin of replication firing in response to hydroxyurea (Santocanale and Diffley, 1998) and MMS (Shirahige *et al.*, 1998) treatment of cells, implicating these two proteins in controlling both initiation and elongation phases of DNA replication in response to a DNA damage signal. G2 arrest is mediated by two different pathways, one Pds1-dependent and one
Rad53- and Dun1-dependent (Cohen-Fix and Koshland, 1997). While Pds1 inhibits the anaphase promoting complex, thereby inhibiting the onset of anaphase, the downstream targets of the Rad53 and Dun1 kinases are unknown.

It is thought that the purpose of DNA damage cell cycle checkpoints is to ensure the cell enough time to repair DNA damage before entry into the next phase of the cell cycle. Recently, a direct coupling of checkpoints and DNA repair was found, as Rad55 was determined to be a target of the DNA damage checkpoint (Bashkirov et al., 2000). The authors showed that Rad55 was phosphorylated in a Mec1-, Rad53-, and Dun1-dependent manner in response to MMS, UV, and gamma irradiation. Most interestingly, mec1 cells were found to be deficient in DNA damage-induced homologous recombination. The assay utilized stationary phase (G1 DNA content) diploid cells treated with MMS and measured recombination between alleles on homologous chromosomes (Bashkirov et al., 2000). The authors suggest that phosphorylation of Rad55 might be required for upregulation of Rad55 activity and hence an inducible recombinogenic response to DNA DSBs, although they admit that there is no hard evidence to support this assertion.

1.3.2 Transcriptional response to DNA damage

Induction of a specific set of genes in response to DNA damage is a ubiquitous phenomenon in living cells. In E. coli, a group of genes (regulon) may respond to a specific type of DNA damage and serve a common purpose, as in the responses to oxidation or alkylation damage, or there may be a general effect on cell physiology, as in the SOS response to UV damage (Friedberg et al., 1995). In the adaptive response to DNA alkylation damage, Ada protein transfers the methyl group from
methylphosphotriesters to a specific cysteine residue, resulting in a conformational change allowing the protein to act as a transcriptional activator for aidB, ada, alkA, and alkB (Lindahl et al., 1988). In response to oxidative damage, high •O2 levels induce a conformational change in the SoxR protein, causing it to activate transcription of soxS, which in turn activates the genes for superoxide dismutase, endonuclease IV, and others (Demple, 1991). Similarly H2O2 activates the oxyR regulon, inducing the expression of other protective proteins (Farr and Kogoma, 1991).

The archetype of a coordinated transcriptional response to DNA damage is the SOS response in E. coli. DNA damage induces the expression of approximately 30 genes with wide ranging effects on the physiology of the organism, from DNA repair and mutagenesis to prophage activation (Walker, 1984). LexA protein acts as a repressor, binding to operator sites in the promoters of SOS genes. When the cell suffers DNA damage such as from UV radiation, RecA is activated by binding to the resulting ss DNA at the damaged sites, and activates an autoproteolytic activity in LexA. LexA then cleaves itself, inactivating its repressor activity and thereby inducing the SOS genes (Little, 1993).

While there is no evidence as of yet to suggest that eukaryotic cells have an analogous system for the coordinate induction of a set of genes in response to specific types of DNA damage, there appears to be a transcriptional response to several types of DNA damage in these cells. Mammalian cells respond to UV by inducing many genes, including those for signal transduction (Devar et al., 1992), growth control (Sachsenmaier et al., 1994), and DNA repair (Kastan et al., 1992). A key regulator of the mammalian DNA damage response is p53. In response to a DNA damage signal,
p53 protein levels increase, and p53 binds specific cis-acting regulatory sequences of certain genes, inducing their expression (Levine, 1997). Among the genes activated are WAF1/CIP1, a cyclin-dependent kinase inhibitor (El-Deiry et al., 1993; Harper et al., 1993), and GADD45, whose product is involved in inhibiting DNA replication (Fornace et al., 1989). However, not all DNA damage signals flow through p53; while the induction of GADD45 in response to ionizing radiation requires p53, its induction in response to MMS treatment can occur in p53 mutants (Fornace et al., 1989), suggesting the existence of multiple regulatory pathways in response to DNA damage in mammalian cells.

There are at least four overlapping DNA damage-responsive transcriptional induction pathways in Saccharomyces cerevisiae (Kiser and Weinert, 1996). This results in the induction of genes from the three major DNA repair epistasis groups (RAD3, RAD6, and RAD52 groups) as well as some genes involved in DNA synthesis, such as RNR1, RNR2, RNR3, and POL1 (Friedberg et al., 1995). That three of these pathways rely on cell cycle checkpoint proteins demonstrates the interconnected nature of the DNA damage responses. The transcriptional response to UV-induced damage utilizes Pole as a sensor during S phase, and Rad9 during G1 and G2 (Navas et al., 1996). The damage signal flows through Rad53 and Dun1, resulting in their phosphorylation (Allen et al., 1994; Sanchez et al., 1996; Sun et al., 1996). Not all DNA damage signals for transcriptional induction flow through this pathway though, as UB14 and DDR48 are induced in both dun1 (Zhou and Elledge, 1993) and cell cycle checkpoint mutant strains (Kiser and Weinert, 1996).
In many cases, transcriptional induction is actually transcriptional derepression. Like the SOS response in *E. coli*, where the LexA repressor is removed from the promoters of genes in the SOS regulon in response to DNA damage, the *RNR2*, *RNR3*, and *RNR4* genes in *S. cerevisiae* are normally repressed by Crt1 (Huang *et al.*, 1998). Crt1 binds to a 13 bp sequence called the X-box, which has been found in the promoter region of *RNR2*, *RNR3*, *RNR4*, and *CRT1* (Huang *et al.*, 1998). The Crt1 protein also binds a heterodimeric complex of Ssn6-Tup1 (Huang *et al.*, 1998), which is involved in the transcriptional repression of many genes through interactions with different DNA-binding proteins (Tzamarias and Struhl, 1995). In response to DNA damage, Crt1 is phosphorylated and no longer binds to the X-box sequence (Huang *et al.*, 1998). Crt1 phosphorylation after MMS treatment is completely dependent upon *MEC1* and *RAD53*, and partially dependent upon *DUN1*. Since Crt1 regulates its own transcription, it is envisioned that this provides a degree of feedback control to the system; the DNA damage signal not only “induces” the expression of needed DNA synthesis genes such as the *RNR* genes, and possibly other DNA repair genes, but it also replenishes the pool of unphosphorylated Crt1, allowing for termination of transcriptional upregulation of this regulon (Huang *et al.*, 1998).

One would assume that transcriptional induction of DNA repair genes in response to DNA damage would increase cell survival. For certain genes, this is not the case. While non-inducible *MAG1* strains show a decrease in MMS-resistance versus the inducible wild type (Liu and Xiao, 1997), no effect was found for non-inducible *RAD54* mutant cells (Cole and Mortimer, 1989). It is possible that the induction of *RAD54* might affect the ability of the cell to repair or tolerate DNA damage, but that the effect is
not strong enough to be noticed in the standard killing assay. Induction of repair genes might be part of a program to increase general cellular fitness after stress. Indeed, many classes of genes are induced after treatment of cells with MMS, including repair genes, DNA synthesis genes, general stress response genes, structural genes, metabolism genes, and mRNA processing genes (Jelinsky and Samson, 1999). In many cases this induction might be to replace vital proteins potentially damaged by the alkylation treatment, a probable hypothesis as many protein degradation genes are induced in response to MMS treatment (Jelinsky and Samson, 1999).

1.4 DNA replication, DSB production, and recombination

It is becoming increasingly clear that the processes of genetic recombination and DNA replication are very much linked. In fact, it is suggested that the recombination-driven shuffling of the genome is a consequence of the rescue of many “derelict” replication forks during each round of DNA replication, with more rapid evolution a side effect of this process (von Hippel, 2000).

1.4.1 Replication fork blocks and recombination in E. coli

In E. coli, blockage of the replication apparatus by inhibiting the replicative helicase leads to DNA rearrangements. The replication terminator (ter) sites have been shown to be deletion hotspots. ter sites are recognized and bound by the Tus protein, which blocks replicative helicases, thus inhibiting DNA replication (Lee et al., 1989). Through the use of a plasmid-based assay it was shown that ter sequences induce deletions between microhomologies. Deletion formation involves a linear intermediate, and hence a DSB (Bierne et al., 1991; 1997). Cells in which a ter sequence is inserted
into the lacZ locus are dependent upon recA, recB, and recC for growth (Horiuchi and Fujimura, 1995). It was suggested that at the blocked replication fork, a DSB occurs. This acts as a substrate for RecBCD, which creates a 3’ ss tail for strand invasion. Resolution of the resulting Holliday structure would lead to the repair of the replication fork (Horiuchi and Fujimura, 1995).

recB and recC mutant strains grow poorly, and it is proposed that this phenotype is the result of spontaneous replication arrest and DSB formation (Capaldo-Kimball and Barbour, 1971). Since this spontaneous arrest might not mimic that observed at ter sites, a series of studies using DNA helicase mutants was carried out. It had been shown that defects in the essential replicative helicase DnaB lead to RecB-dependent chromosomal DNA degradation (Buttin and Wright, 1968; Fangman and Russel, 1971; Wescbler and Gross, 1971), and that ter sequences are hotspots for RecBCD-dependent recombination, as detailed above. There is a second replicative helicase in E. coli called Rep. It was proposed that the function of this helicase is to remove DNA-bound proteins and thus facilitate replication (Yancey-Wrona and Matson, 1992; Matson et al., 1994). rep recB and rep recC double mutants are inviable, suggesting that in rep strains, like dnaB strains, DSBs occur at an elevated rate (Uzest et al., 1995). Using pulsed field gel electrophoresis (PFGE), Michel et al. (1997) showed that upon arrest of DNA replication due to defects in replicative DNA helicases, DSBs form. In recB, recC, and recA recD mutants, some linear DNA was detected, indicating that even in cells with a normal replication apparatus DSBs occurred. Introduction of a rep mutation into these strains greatly increased the percentage of linear DNA detected. Similar results were seen using a dnaB temperature sensitive mutation in combination with recB, confirming
the hypothesis that defects in replicative helicases can lead to DSB formation, at least in the absence of effective DSB repair (Michel et al., 1997).

Seigneur et al. (1998) screened for mutations that restore the viability of *rep recBts recCts* mutants at the restrictive temperature and isolated *ruvAB* alleles. These mutations also suppressed the formation of linear DNA in the *rep recBts recCts* strain. DSB formation in the *dnaBts recB* strain is dependent upon RuvA, RuvB, and RuvC, as are roughly half of the spontaneous DSBs formed in *recB* cells wild type for the replicative helicases (Seigneur et al., 1998). A model was proposed whereby a Holliday junction forms at blocked replication forks through the annealing of the newly synthesized strands of DNA. RuvAB binds to this structure and catalyzes branch migration, creating a double stranded tail recognized by RecBCD. RecBCD initiates homologous recombination, creating a structure recognized by the primosome, which reinitiates DNA replication. Thus, in wild type cells, it is possible that RuvAB and RecBCD catalyze replication fork repair without actual breakage of the replication fork (without replication fork “collapse”). However, if RuvC recognizes and cleaves the Holliday structure, a DSB is formed; this event is lethal in *recBCD* mutants (Seigneur et al., 1998).

1.4.2 Mutations in DNA replication genes are recombinogenic in *S. cerevisiae*

Replication and recombination are also linked in eukaryotic cells. In *S. cerevisiae*, many replication-defective mutants cause genetic instability which manifests as a hyper-rec phenotype. For example, certain alleles of *pol3* (Polδ), *rad27* (FEN1), *pol30* (PCNA), and *rfa1-3* (RFA/RPA) all accumulate double strand breaks (DSBs) during S phase (Chen et al., 1998; Chen and Kolodner, 1999; Merrill and Holm, 1998;
Tishkoff et al., 1997; Zou and Rothstein, 1997). As well, many mutations affecting PCNA, RFC1, FEN1, DNA ligase, RPA, and Polδ are synthetically lethal with mutants of the RAD52 recombinational repair pathway, indicating that recombination events are required for the repair of this replication-induced DNA damage (Montelone et al., 1981; Giot et al., 1997; Tishkoff et al., 1997; Chen et al., 1998; Merrill and Holm, 1998).

Using 2D gel electrophoresis and a probe specific for a region of the rDNA array in S. cerevisiae, it has been shown that Holliday junctions can be detected only during S phase, suggesting that recombination is required to repair replication-induced DNA damage (Zou and Rothstein, 1997). It was found that Holliday junction formation was stimulated by mutations in subunits of DNA polymerase α and δ (Zou and Rothstein, 1997). Interestingly, it was shown that rad52 mutants are defective for Holliday junction formation, whereas rad51, rad55, and rad57 mutants (all RecA homologs), as well as the rad51 rad55 rad57 triple mutant, are all proficient in this activity, suggesting that Rad52 can catalyze Holliday junction formation and strand exchange without the assistance of a RecA homolog.

Hydroxyurea causes DNA synthesis arrest by inhibiting ribonucleotide reductase, thus depleting the pool of dNTPs available for DNA replication. Hydroxyurea inhibition of DNA replication causes DNA damage, as mutants of the RAD52 pathway are sensitive to killing by hydroxyurea (Allen et al., 1994) and accumulate a large number of DSBs, as shown by sedimentation through a neutral sucrose gradient (Merrill and Holm, 1999). The authors suggested that the requirement for the RAD52 pathway after hydroxyurea treatment of yeast is analogous to the requirement for RecBCD in rep and dnaB cells in E. coli. Although there are no yeast homologs of the E. coli Ruv proteins,
perhaps there is an analogous activity that converts a collapsed replication fork into a Holliday junction for recombination-dependent priming of DNA replication (Merrill and Holm, 1999).

1.4.3 DNA topoisomerase I and DNA damage

DNA topoisomerases catalyze the interconversion of different topological isomers of DNA. Type I topoisomerases make a ss break in the DNA and change the linking number of the DNA molecule in increments of one, whereas type II topoisomerases make a ds break in the DNA and change the linking number in steps of two. Certain cellular processes require topoisomerase activity (for reviews, see Wang, 1991; Duguet, 1997). An advancing replication fork produces positive supercoils in front of the replication apparatus, which can be removed by either type I or type II topoisomerases in eukaryotes. Newly replicated chromosomes are intertwined, and their separation during mitosis requires a type II topoisomerase. Transcription can induce positive supercoiling in front and negative supercoiling behind the RNA polymerase, which are relaxed by DNA gyrase and topoisomerase I, respectively, in E. coli (Liu and Wang, 1987). While the ability to produce transient DNA breaks is vital for cell survival, this ability also poses a danger, as conditions which increase the number or duration of these breaks will have ill effects such as mutations, chromosomal rearrangements, and death (reviewed in Froelich-Ammon and Osheroff, 1995).

S. cerevisiae Top1 is a type I topoisomerase. There are four steps in the catalytic mechanism of Top1 (reviewed in Pommier et al., 1998). First, Top1 binds to DNA, with a preference for bent or supercoiled segments. Second, one strand of the DNA is cleaved. The cleavage recognition site is fairly conserved between eukaryotic species,
and Top1 usually cleaves immediately 3' of (G/C)(A/T)T sequences (Champoux and Bullock, 1988). The cleavage is a transesterification reaction, ending with Top1 linked to a 3'-phosphate through a tyrosine hydroxyl group, and a 5'-OH. Next, the cleaved strand winds around the intact strand, relaxing supercoils. Finally, the DNA ends are religated. The yeast type II topoisomerase Top2 is a homodimer which requires a Mg²⁺ cofactor and ATP hydrolysis to effect the ds cleavage of DNA (reviewed in Kornberg and Baker, 1992). The DSB produced has 4 bp 5' overhanging ends, which are covalently attached to a tyrosine residue in the enzyme, one per subunit. After passage of the double helix through the cut, the ends are religated to reform an intact DNA molecule. TOP2 is essential, and the Top2 enzyme is required specifically at mitosis to effect the separation of entwined chromosomes (Holm et al., 1985). Top1 is not an essential enzyme, and top1Δ cells show no DNA replication defects. However, top1Δ top2-l(ts) double mutants differ from top1Δ single mutants in this respect (Thrash et al., 1985; Goto and Wang, 1985; Brill et al., 1987). While the top2-l(ts) strain completes one round of DNA replication at the restrictive temperature, the top1Δ top2-l(ts) strain ceases DNA replication immediately upon shift to the restrictive temperature. Hence, the presence of either Top1 or Top2 is sufficient for the completion of DNA replication, but when both enzymes are missing, DNA replication is blocked. It is suggested that topoisomerasases are required to form a swivel to facilitate replication fork movement; Top1 might act ahead of the advancing fork, and Top2 behind it (Brill et al., 1987). The double mutant also has a defect in rRNA synthesis, but not mRNA or tRNA synthesis (Brill et al., 1987), and the Top1 and Top2 enzymes have been shown to suppress recombination within the rDNA array (Christman et al., 1988). In S. cerevisiae, the
rDNA locus consists of roughly 200 repeats of the 9.1 kb rDNA cistron on chromosome XII (Schweizer et al., 1969). This region is heavily transcribed, and it is possible that repetitive transcription in this area without the aid of topoisomerases leads to the accumulation of recombinogenic lesions in the DNA (Christman et al., 1988). Kim and Wang (1989) found that top1Δ top2-ts cells accumulate extrachromosomal rDNA circles at the permissive temperature (Kim and Wang, 1989). The accumulation of these rDNA circles has been shown to correlate with an aging phenotype in S. cerevisiae (Sinclair and Guarente, 1997).

The antitumor chemotherapeutic agent camptothecin stabilizes the Top1 cleavage complex, leading to the persistence of Top1-bound ssDNA breaks (Pommier et al., 1998). Camptothecin cytotoxicity is dependent upon ongoing DNA replication. DNA replication forks which collide with a Top1 cleavage complex are blocked (Hsiang et al., 1989; Tsao et al., 1993) and result in DSB formation (Ryan et al., 1991), a lethal event if not repaired. In agreement with this, S. cerevisiae RAD52-group mutants are extremely sensitive to killing by camptothecin (Eng et al., 1988; Nitiss and Wang, 1988).

The persistence of a Top1 cleavage complex is detrimental to the survival of a cycling cell. In S. cerevisiae, Tdp1 is able to cleave the bond between the 3'-phosphate and the Top1 active site tyrosine residue (Yang et al., 1996; Poulion et al., 1999). This tyrosine-DNA phosphodiesterase activity exists not only in yeast, but in Drosophila, rodents, and humans as well (Yang et al., 1996). One would expect that tdp1Δ mutants would display camptothecin sensitivity, but surprisingly they do not (Poulion et al.,
Deletion of TDP1 does, however, increase the camptothecin sensitivity of rad9A DNA damage checkpoint-defective mutants.

The presence of abnormal bases or secondary structure in the vicinity of the Top1 cleavage site affects Top1 activity (Pourquier et al., 1997B; C). Using defined substrates, it was shown that the presence of a ssDNA loop immediately 3' to the cleavage site on the intact strand irreversibly traps the topoisomerase in the cleavage complex, similar to the effect of camptothecin (Pourquier et al., 1997C). An abasic site immediately 3' to the cleavage site on either strand has the same effect. It was proposed that base pairing immediately downstream from the Top1-DNA linkage is needed for the correct alignment of the cleaved strand for religation (Pourquier et al., 1997C). If Top1 cleaves the DNA opposite from a nick or gap in the template, a Top1-linked DSB is created (Pourquier et al., 1997A). This could conceivably occur after ionizing radiation exposure or incomplete base excision repair. Thus replication-blocking DNA lesions can not only act at the level of the DNA polymerase, but also ahead of the polymerase at a topoisomerase. The DNA damage that a cell has to deal with is always in flux; if repair fails or is incomplete, a simple lesion may evolve into a more complex and potentially irreparable structure.

1.5 Rationale for the project

There are multiple pathways capable of repairing DNA damage caused by simple alkylating agents. In S. cerevisiae there exists a base excision repair pathway which repairs DNA alkylation damage, headed by the MAG1-encoded 3MeA DNA glycosylase, and the methyltransferase Mgt1, which removes the methyl group from
O\textsuperscript{6}MeG and O\textsuperscript{4}MeT (Friedberg et al., 1995). Mutations in MAG1, APN1, and RAD27, all in the base excision repair pathway, render cells sensitive to MMS, as does mutating MGT1. Mutants of the three "radiation" epistasis groups, the RAD3 nucleotide excision repair group, RAD6 postreplication repair group, and RAD52 recombination repair group, are also MMS-sensitive (Friedberg et al., 1995). However, the existence of other uncharacterized MMS-sensitive *S. cerevisiae* mutants, such as *mms1, mms2, mms4,* and *mms22* (Prakash and Prakash, 1977) and *ngs1* (Nisson and Lawrence, 1986) suggested that the list of enzymes capable of repairing DNA alkylation damage was far from complete. The Xiao lab obtained these mutant strains and began cloning and characterizing the corresponding wild type genes in an attempt to elucidate novel DNA repair pathways. It is hoped that the characterization of these genes will aid not only in the understanding of DNA repair mechanisms in general, but that the discovery of human homologs will also assist in the understanding of the genesis of cancer and perhaps lead to new therapies for patients. Most DNA repair pathways found in lower eukaryotes such as yeast are conserved in humans, and some are amplified such that a single yeast DNA repair protein has multiple human homologs. Thus studying a novel repair pathway in *S. cerevisiae* is likely to pay dividends in understanding how human cells cope with DNA damage.
CHAPTER TWO - MATERIALS AND METHODS

2.1 Yeast genetics

2.1.1 Yeast strains, culture, transformation, and storage

The *Saccharomyces cerevisiae* strains used in this thesis are listed in Table 2-1. Yeast cells were cultured at 30°C either in a rich YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose), YM-1 medium (0.5% Bacto-yeast extract, 1% Bacto-peptone, 0.67% Bacto-yeast nitrogen base without amino acids, 10 mg/L adenine sulfate, 10 mg/L uracil, 1% succinic acid, 0.6% NaOH, 1% glucose, pH 5.8), or in a synthetic dextrose (SD) medium (0.67% Bacto-yeast nitrogen base without amino acids, 2% glucose) supplemented with amino acids and bases as required (20 mg/L each of adenine sulfate, uracil, L-tryptophan, L-histidine HCl, L-arginine HCl, and L-methionine; 30 mg/L each of L-tyrosine, L-isoleucine, and L-lysine HCl; 50 mg/L L-phenylalanine; 100 mg/L each of L-leucine, L-isoleucine, and L-aspartic acid; 150 mg/L L-valine; 200 mg/L L-threonine; 400 mg/L L-serine). Solid media was made by adding 2% Bacto-agar to the liquid media prior to autoclaving. For canavanine selection, 60 mg/L canavanine was added to arginine-free SD media.

A dimethyl sulfoxide-enhanced method (Hill *et al.*, 1991) was used to transform intact yeast cells. Roughly 3 ml of a log-phase yeast culture is pelleted, washed in 500 μl LiOAc solution (0.1M lithium acetate, 10mM Tris-HCl, pH 8.0), and resuspended in 100 μl of the same. 4 μl of denatured sheared salmon sperm DNA (10 mg/ml stock) and an appropriate amount of transforming DNA are added. After a 5 min incubation at room temperature, 280 μl of 50% PEG4000 is added, and cells incubated for 45 min at
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B635</td>
<td>MATa cyc1-115 his1 lys2 trp2</td>
<td>L. Prakash</td>
</tr>
<tr>
<td>FY86</td>
<td>MATa his3-Δ200 leu2Δ1 ura3-52 GAL⁺</td>
<td>F. Winston</td>
</tr>
<tr>
<td>WXY348</td>
<td>FY86 with mms1Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY172</td>
<td>FY86 with rad52Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>THY173</td>
<td>THY172 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>WX15-1c</td>
<td>MATa his3-Δ200 leu2-Δ1 lys2 ura3-52 mms1-1</td>
<td>Lab stock</td>
</tr>
<tr>
<td>DBY747</td>
<td>MATa his3-Δ1 leu2-3,112 trp1-289 ura3-52</td>
<td>D. Botstein</td>
</tr>
<tr>
<td>WXY344</td>
<td>DBY747 with mms1Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>WXY347</td>
<td>DBY747 with mms1Δ::hisG-URA3-hisG</td>
<td>Lab stock</td>
</tr>
<tr>
<td>JC8901</td>
<td>DBY747 with mag1Δ::hisG-URA3-hisG</td>
<td>L. Samson</td>
</tr>
<tr>
<td>WXY406</td>
<td>DBY747 with mms1Δ::LEU2 mag1Δ::hisG-URA3-hisG</td>
<td>Lab stock</td>
</tr>
<tr>
<td>WXY105</td>
<td>DBY747 with apn1Δ::HIS3</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY162</td>
<td>DBY747 with apn1Δ::HIS3 mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>WXY791</td>
<td>DBY747 with apn2Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY163</td>
<td>WXY791 with mms1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>WXY814</td>
<td>WXY105 with apn2Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY164</td>
<td>WXY814 with mms1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>WXY394</td>
<td>DBY747 with rad4Δ::hisG-URA3-hisG</td>
<td>Lab stock</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>THY170</td>
<td>WXY394 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>WXY376</td>
<td>DBY747 with rad6Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY132</td>
<td>DBY747 with mms1Δ::URA3 rad6Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>WXY561</td>
<td>DBY747 with rad50Δ::hisG</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY113</td>
<td>WXY561 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>WXY387</td>
<td>DBY747 with rad52Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY171</td>
<td>WXY387 with mms1Δ:: hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>THY161</td>
<td>DBY747 with mms1Δ:: hisG-URA3-hisG top1-7::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>THY123</td>
<td>DBY747 with rad9Δ::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>THY124</td>
<td>THY123 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>THY156</td>
<td>DBY747 with yku70Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>THY157</td>
<td>THY156 with mms1Δ::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>THY158</td>
<td>DBY747 with yku80Δ::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>THY159</td>
<td>THY158 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>THY142</td>
<td>DBY747 with dun1Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>THY143</td>
<td>THY142 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>WXY379</td>
<td>DBY747 with rad1Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>WXY555</td>
<td>DBY747 with rad2Δ::TRP1</td>
<td>Lab stock</td>
</tr>
<tr>
<td>WXY537</td>
<td>DBY747 with rad10Δ::URA3</td>
<td>Lab stock</td>
</tr>
<tr>
<td>WXY482</td>
<td>DBY747 with rad18Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>WXY394</td>
<td>DBY747 with rad4Δ::hisG-URA3-hisG</td>
<td>Lab stock</td>
</tr>
<tr>
<td>MCY27</td>
<td>DBY747 with mre11Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>6613-53a</td>
<td>MATa ura3-52 his3-A200 leu2-3,112 hom3 gal1 can1 cdc2-2</td>
<td>L. Hartwell</td>
</tr>
<tr>
<td>WXY433</td>
<td>6613-53a with rad52Δ::LEU2</td>
<td>Lab stock</td>
</tr>
<tr>
<td>THY109</td>
<td>6613-52a with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>B365-14c</td>
<td>MATa his3::ade2-5Δ-TRP1-ade2-n leu2-3,112 trpl-1 ura3-1 ade2-1 can1-100</td>
<td>L. Symington</td>
</tr>
<tr>
<td>WXY687</td>
<td>B365-14c with mms1Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>yMP10381</td>
<td>MATa ade2 ade3-130 leu2 trpl ura3 cyh2 SCR::URA3*</td>
<td>L. Hartwell</td>
</tr>
<tr>
<td>THY151</td>
<td>yMP10381 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>CY184</td>
<td>MATa ura3-1 ade2-1 his3-11,15 trpl-1 leu2-3,112 rDNA::ADE2</td>
<td>M. Christman</td>
</tr>
<tr>
<td>THY153</td>
<td>CY184 with mms1Δ::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>LSY391</td>
<td>MATa trpl-1 his3-11,15 can1-100 ura3-1 leu2-3,112 ade2-1</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY399</td>
<td>LSY391 with xrs2Δ::URA3</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY395</td>
<td>LSY391 with rad50Δ::hisG-URA3-hisG</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY401</td>
<td>LSY391 with rad51Δ::LEU2</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY387</td>
<td>LSY391 with rad52Δ::TRP1</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY404</td>
<td>LSY391 with rad54Δ::LEU2</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY406</td>
<td>LSY391 with rad55Δ::LEU2</td>
<td>L. Symington</td>
</tr>
<tr>
<td>LSY408</td>
<td>LSY391 with rad57Δ::LEU2</td>
<td>L. Symington</td>
</tr>
<tr>
<td>THY174</td>
<td>LSY391 with mms1Δ::LEU2</td>
<td>This study</td>
</tr>
</tbody>
</table>
*SCR::URA3 is the sister chromatid recombination substrate (see section 2.1.3).
30°C. 39 µl of DMSO is added, and cells incubated for 5 min at 42°C. Cells are pelleted, washed once in sterile water, resuspended in 100 µl of the same, and plated on the appropriate selective media. Plates were typically incubated for three days at 30°C. For targeted integration, plasmid DNA was digested with restriction enzymes and ethanol precipitated prior to transformation.

Yeast cells can be stored for up to four months on sealed agar plates at 4°C. For long term storage, actively growing cells were suspended in 15% sterile glycerol and kept at -70°C indefinitely.

2.1.2 Total genomic DNA isolation

Genomic DNA was isolated from yeast by two methods: by vortexing with glass beads, and by lysis in agarose plugs.

Yeast genomic DNA isolation by the glass bead method was used for all experiments except for CHEF gel analysis. The method was adapted from Hoffman and Winston (1987). Cell were grown on solid media for two days, scraped with a toothpick, and resuspended into 200 µl of extraction buffer (2% Triton X-100, 1% SDS, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) in a screw top 1.5 ml microcentrifuge tube. 100 µl of phenol and 100 µl of chloroform:isoamyl alcohol (mixed at a ratio of 24:1) and 0.3 g of acid washed glass beads (diameter=0.4-0.5 mm) were added. The tubes were vortexed at top speed for 2 to 2.5 min and spun for 5 min at top speed (13 000 rpm) in a microcentrifuge to separate the phases. The aqueous phase was removed and DNA precipitated by adding 400 µl of 100% ethanol for 30 min at -20°C. To collect the DNA, tubes were centrifuged for 15 min at top speed. For Southern analysis, DNA was resuspended in 200 ml TE buffer (10 mM Tris-HCl, 1 mM
EDTA, pH 8.0), and 5 μl of 10 mg/ml RNase A added. After a 10 min incubation at 37°C, DNA was ethanol precipitated and resuspended in 30 μl of TE.

DNA was isolated in agarose blocks using the CHEF genomic DNA plug kit from Bio-Rad (Hercules, CA), following the manufacturer’s instructions. Briefly, 6x10⁷ cells per plug are suspended in 100 μl of 0.75% agarose, and allowed to solidify in a 1.5 mm x 5 mm 85 μl plug mold. Plugs are treated first with lyticase to lyse the cells, then with proteinase K, before being loaded onto a CHEF gel. Plugs are stable at 4°C in wash buffer for up to 3 months.

2.1.3 Recombination assays

Three recombination assays were performed for this thesis. First, an intrachromosomal recombination assay between two inverted ade2 alleles in the ade2-5Δ-TRP1-ade2-n substrate was as described (Rattray and Symington, 1994). Gene conversions, crossovers, or crossovers associated with gene conversions can all restore the ADE2 sequence. Single colonies of B365-14c (wild type) and WXY687 (mms1Δ) were grown on YPD plates for 2-3 days at 30°C. 11 colonies per strain were picked and resuspended in 1 ml sterile water. 100 μl from each colony suspension was pooled, the pool diluted appropriately, and plated onto SD-Trp plates to determine the average total cell number per colony. Each suspension was independently diluted and plated onto SD-Ade media to score the number of Ade⁺ recombinants per colony. The spontaneous recombination rate was determined by the method of the median (Lea and Coulson, 1948), using the following formula: rate=M/N, where N=average number of cells per colony, and M is interpolated from Table 3 in Lea and Coulson (1948). To facilitate interpolation, I graphed the table, as seen in Fig. 2-1. From the graph the formula
**Figure 2-1:** Graph of $r_0$ vs. $r_0/m$, to interpolate $m$ for use in the Lea and Coulsen method of the median calculations.
x=[log(y/0.372)]/0.545 is derived, where y=median number of recombinants, and
x=y/M. Thus this formula allows for the solving of M, and thus the rate can be
calculated. For MMS-induced recombination, single colonies of B365-14c (wild type)
were grown on YPD plates for 3 days at 30°C. Seven colonies per strain were picked
and used to inoculate 2 ml of SD-Trp. Overnight cultures were diluted 10-fold into 10
ml SD-Trp, and incubation continued for 3 hours. 1 ml of culture was removed, diluted
appropriately, and plated onto SD-Trp to determine total number of cells, and onto SD-
Ade to score the uninduced number of recombinants. The remaining 9 ml was split into
three separate tubes, and MMS added to the concentrations indicated. After a 2 hour
incubation at 30°C, cultures were pelleted, washed, diluted, and plated onto SD-Trp and
SD-Ade to determine recombination frequency.

A sister chromatid exchange assay detecting recombination events between non-
identical ade3Δ deletions oriented head to tail was as described (Kadyk and Hartwell,
1992), with the modifications of Paulovich et al. (1998). Briefly, yMP10381 (wild type)
and THY151 (mms1Δ) cultures were grown overnight in YM-1 + 2% glucose, and
plated for single colonies on SC + 2% glucose. After 3 days of growth at 30°C, 19
individual colonies were sliced off of the agar surface using a scalpel blade, transferred
to 3 ml sterile water, vortexed to resuspend the colony, and sonicated. 100 µl from each
colony suspension was pooled, the pool diluted appropriately, and plated onto SC + 2%
glucose to determine the average cell number per colony. Each colony suspension was
independently concentrated in 100 µl sterile water, and the entire sample plated onto SC-
His media to score sister chromatid recombinants. The spontaneous recombination rate
was determined by the method of the median (Lea and Coulson, 1948).
An rDNA recombination assay was modified from that described by Sadoff et al. (1995), in which the loss of an integrated ADE2 gene from the rDNA array is monitored. Strains CY184 and THY153 (mms1Δ) were plated for single colonies on SD-Ade media. After 3 days incubation at 30°C, colonies were picked, resuspended in sterile water, and plated for single colonies on YPD media. After 2 days incubation, five colonies per plate were picked, diluted in sterile water, and plated for single colonies on SC + 2% glucose with half the usual adenine supplement. On these plates, Ade' cells will form colonies, but when the adenine runs out in the media, the adenine biosynthetic pathway will be turned on. Cells defective for Ade2 accumulate a red pigment and thus can be visually selected. After a 5 day incubation at 30°C, the number of colonies formed per original SD-Ade colony were counted; typically this numbered between 1500-3000. Red colonies were picked and streaked onto SD-Ade media to ensure that they were indeed Ade'. The recombination frequency was calculated by dividing the number of Ade' colonies by the total number of colonies on the SC plates.

2.1.4 Spontaneous mutagenesis

Determination of spontaneous trpl-289 reversion and CAN1 forward mutation rates were performed by fluctuation analysis, as described by Drake (1970). Strains were cultured overnight at 30°C in YPD, counted, and subcultured into 10 ml YPD at a density of 20 cells/ml. Five cultures were inoculated per strain. Incubation continued for 3 days at 30°C shaking vigorously (~275 rpm). Cultures were diluted appropriately and plated onto YPD to score for total number of cells, onto SD-Trp to score Trp' revertants, and onto SD-Arg + canavanine to score can1 mutants.
Rate = \((0.4343)(\text{frequency})/[\log(\text{final cell density})-\log(\text{initial cell density})]\), where

\(\text{frequency} = (\text{median number of mutations per ml culture})/(\text{final cell density})\).

### 2.1.5 β-galactosidase assay

The β-gal assays were performed as previously described (Xiao et al., 1993).

Briefly, 0.5 ml of overnight yeast culture was added to 2.5 ml fresh SD selective media and incubation continued for 2 hours. At this time, MMS was added to the indicated concentration, and the cells further cultured for 4 hours. For UV experiments, cells were plated on YPD, irradiated at the indicated dosage, washed off the plates, resuspended into fresh media, and cultured for 4 hours in the dark. One milliliter of culture was used to determine cell concentration at 600 nm. The remaining cells (2 ml) were collected by centrifugation and used for the β-gal assay. The cells were resuspended in 1 ml of buffer Z (60 mM Na$_2$HPO$_4$$\cdot$7H$_2$O, 40 mM NaH$_2$PO$_4$$\cdot$H$_2$O, 10 mM KCl, 1 mM MgSO$_4$$\cdot$7H$_2$O, 40 mM β-mercaptoethanol, pH 7.0). The cells were permeabilized by adding 50 µl of 0.1% SDS and 40 µl of chloroform and vortexed at top speed for 10 seconds. The reaction was initiated by adding 200 µl of 4 mg/ml orthonitrophenyl-β-D-galactoside and incubating at 30°C for 20 minutes. The reaction was stopped by adding 500 µl of 1 M Na$_2$CO$_3$. The tube was centrifuged and the OD$_{420nm}$ of the supernatant measured to determine the β-gal activity using the following equation:

\[ S_{β-gal} = 1000 \left( \frac{\text{OD}_{420nm}}{\text{reaction time (min)} \times \text{culture volume (ml)} \times \text{OD}_{600nm}} \right) \]

The β-gal activity was expressed in Miller units (Guarente, 1983).

### 2.1.6 Cell killing by DNA damaging agents

MMS, camptothecin (CPT), 4-nitroquinoline-N-oxide (4NQO), 1,2;3,4-diepoxybutane (DEB), and hydroxyurea (HU) were purchased from Sigma-Aldrich (St.
Louis, MO). Yeast cells for liquid killing were grown overnight at 30°C in 2 ml of YPD, diluted 10-fold in YPD and the incubation continued for roughly 4 hours until a cell titer of approximately 2x10⁷ cells/ml was reached. Cell cultures were treated with MMS for the time and concentrations as indicated. Samples were removed, washed, diluted and plated onto YPD. For UV killing experiments, cells were diluted and plated onto YPD and then subjected to UV-irradiation in the dark at the given dose. For γ-irradiation, cells were collected by centrifugation, resuspended in sterile water, and exposed to a ⁶⁰Co γ-ray source at a dose rate of 37 rads/second. The plates were incubated for 3 days at 30°C before counting colonies. CPT stock solution was 4 mg/ml in 100% DMSO. 10 mg/ml 4NQO stock solution was made in acetone. HU was added to molten YPD agar to the indicated concentrations.

For a gradient plate assay, 30 ml of molten YPD agar was mixed with different concentrations of reagents to form the bottom layer gradient by pouring on tilted square petri dishes. After brief solidification, the petri dishes were returned flat and 30 ml of the same molten agar without drug was poured. A 0.1 ml sample was taken from an overnight culture, mixed with 0.9 ml 1% molten agar and immediately imprinted onto freshly made gradient plates via a microscope slide. Gradient plates were incubated at 30°C for the time as indicated.

2.1.7 Cell viability assay

Yeast cells were grown overnight at 30°C in 2 ml of YPD, diluted 10-fold in YPD and the incubation continued for roughly 4 hours until a cell titer of approximately 2x10⁷ cells/ml was reached. 100 µl of culture was removed, added to 375 µl of YPD, and 25 µl of 200 mM FUN-1 (Molecular Probes, Inc., Eugene, OR) added. Cells were
incubated for 30 min at room temperature in the dark prior to being spotted on microscope slides. Fluorescence microscopy was performed using a fluorescein filter. Viable cells metabolize FUN-1 into a product that exhibits red fluorescence, whereas non-viable cells appear green.

2.2 Molecular biology techniques

2.2.1 Bacterial culture and storage

The *E. coli* strains DH5α (BRL, Gaithersburg, MD) and NM522 (Pharmacia, Piscataway, NJ) were used for molecular cloning and plasmid preparation. Bacterial cells were cultured in LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl, pH 7.0). Transformed cells were stored indefinitely at -70°C in 10% DMSO in LB + 50 μg/ml ampicillin, and cultured in or plated on LB + 50 μg/ml ampicillin.

2.2.2 Bacterial transformation

Transformation of competent *E. coli* cells was performed either by chemical or electroporation methods. Electroporation was done using an *E. coli* Pulser (Bio-Rad) according to the manufacturer’s instructions. For chemical transformation, competent cells (Chung *et al.*, 1989) were incubated with the plasmid DNA on ice for 30 min, heat shocked at 42°C for 45 sec, incubated for 1 hour in rich SOC media (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 20 mM glucose, 2.5 mM KCl, 10 mM MgCl₂, pH 7.0), and plated on LB + ampicillin plates. Selection for transformants expressing β-galactosidase was performed on LB + ampicillin plates supplemented with 2% IPTG and 4% X-gal to distinguish between blue and white colonies.

2.2.3 Plasmid manipulation
Plasmid manipulation was performed using enzymes from GIBCO/BRL and New England Biolabs as recommended by the manufacturers.

Plasmid amplification and isolation was performed using the miniprep boiling method of Maniatis et al. (1982). Transformed E. coli cells were cultured overnight in 1.5 ml LB+ampicillin at 37°C. Cells were pelleted and resuspended in 350 μl of bacterial plasmid miniprep solution (0.1M NaCl, 10mM Tris-HCl, pH 8, 1mM EDTA, pH 8, 5% Triton X-100), and 20 μl of lysozyme (10 mg/ml in 10mM Tris-HCl, pH 8) was added. After tipping a few times to mix, tubes were placed in a boiling water bath for 45 sec, then spun for 10 min at top speed in a microcentrifuge. The cell debris pellet was removed using a toothpick, and the supernatant ethanol precipitated.

Plasmid pMMS1-6 containing a 10.8 kb insert was isolated from a screening of a YCp50 yeast genomic library for a plasmid that restores wild type MMS resistance to the mms1-1 mutant strain WX15-1c. pBRAHS::URA3, containing the rad52Δ::URA3 disruption cassette, was a gift from Dennis Livingston (University of Minnesota, Minneapolis). pRR330, containing the rad9Δ::hisG-URA3-hisG disruption cassette, was from Robert Schiestl (Harvard University, Boston). CB25, also known as pCT80 (Thrash et al., 1985), containing the top1-7::LEU2 cassette, was from Michael Christman (University of Virginia, Charlottesville). For construction of mms1Δ disruption cassettes, YEpl3 (Broach et al., 1979) was used as the LEU2 donor, YDp-U (Berben et al., 1981) was used as the URA3 donor, and pNKY51 (Alani et al., 1987) was used as the hisG-URA3-hisG donor. Plasmid YEplMAG1-lacZ has been described previously (Xiao et al., 1993). Plasmid pZZ13 containing the RNR3-lacZ reporter gene (Zhou and Elledge, 1992) and pZZ66 containing the dun1Δ::HIS3 disruption cassette

70
(Zhou and Elledge, 1993) were from Steven Elledge (Baylor College of Medicine, Houston). pLG669z (Guarente, 1983) containing CYCI-lacZ was from Leonard Guarente (MIT, Cambridge). Plasmid pWX1812 containing DDII-lacZ was constructed by cloning the 0.65 kb XbaI fragment containing the DDII-MAG1 promoter region into YEp367R (Myers et al., 1986) in the DDII orientation in frame with the lacZ open reading frame. YCpRAD51-lacZ was made by first cloning a 1.2 kb BamHI-EcoRI fragment from YEpR51 (obtained from Dr. L.S. Symington, Columbia University, New York), containing 643 bp promoter and 560 kb coding region of RAD51, into plasmid YEp356R (Myers et al., 1986). The resulting RAD51-lacZ reporter gene was then moved to YCplac33 (Gietz and Sugino, 1988) to make YCpRAD51-lacZ. Multi-purpose plasmids pTZ18R and pTZ19R were purchased from Pharmacia.

To construct the mms1Δ::LEU2, mms1Δ::URA3, and mms1Δ::hisG-URA3-hisG cassettes (Fig. 2-2), the 6.5 kb BgIII-XhoI fragment from pMMS1-6 was cloned into the BamHI-Sall sites of pTZ19R to form p19R-MMS1. The 1.6 kb BamHI fragment within the MMS1 insert of p19R-MMS1 was replaced by the 2.7 kb BgIII fragment from YEp13 to form pWX1503 (mms1Δ::LEU2), by the 1.1 kb BamHI fragment from YDp-U to form pWX1501 (mms1Δ::URA3) or by the 3.8 kb BamHI-BgIII fragment from pNKY51 to form pWX1505 (mms1Δ::hisG-URA3-hisG). In all cases, nucleotides coding for amino acids 305-784 were deleted from the MMS1 open reading frame. For MMS1 disruption, referred to as an mms1Δ mutation, pWX1503 was digested with SacI and XbaI while pWX1501 and pWX1505 were digested with EcoRI prior to yeast transformation. For the yku70Δ::LEU2 disruption cassette, a 1.85 kb yeast genomic DNA fragment containing the entire YKU70 open reading frame was amplified by
**Figure 2-2:** *mms1Δ* disruption cassettes. The 1.6 kb *BamHI* fragment from *MMS1* was replaced by DNA sequence containing *LEU2*, *URA3*, or *hisG-URA3-hisG*.

polymerase chain reaction using oligonucleotides Ku70-1 (5’-GTTCGACACGCCCCCGGGAG-3’) and Ku70-2 (5’-CCGCTCGAGATCCTTCTGACCTTCAGATCTC-3’) as primers. After Smal-XhoI digestion, the fragment was cloned into the Smal and SalI sites of pTZ18R. A 1.3 kb EcoRV-BstBI fragment within the 1.8 kb YKU70 coding region was deleted, the ends filled in with Klenow fragment of E. coli DNA polymerase I, and a BamHI linker ligated in to generate a BamHI site. After BamHI digestion, the 2.7 kb BglII fragment from YEp13 was ligated in to form pKu70Δ::LEU2. The yku70Δ::LEU2 cassette was released by Smal-BglII digestion prior to yeast transformation. To construct the yku80Δ::hisG-URA3-hisG disruption cassette, a 2.4 kb yeast genomic DNA fragment containing the entire YKU80 open reading frame, plus the 0.17 kb upstream and 0.39 kb downstream sequences was amplified by polymerase chain reaction using oligonucleotides Ku80-1 (5’-CAGTGTCGACACAATTTGAC-3’) and Ku80-2 (5’-CCTTTTCGTGAGCTCCGC-3’) as primers. After a SalI-SacI digestion, the fragment was cloned into the SalI and SacI sites of pBluescript. A 1.5 kb XbaI-BamHI fragment within the 1.89 kb YKU80 coding region was deleted, and the ends filled in with Klenow fragment of E. coli DNA polymerase I and ligated to regenerate the BamHI site. After BamHI digestion, the 3.8 kb BamHI-BglII fragment from pNKY51 was ligated in to form pKu80Δ::HUH. The yku80Δ::hisG-URA3-hisG cassette was released by SalI-SacI digestion prior to yeast transformation.

2.2.4 Agarose gel electrophoresis and DNA fragment isolation
For analysis of plasmid and genomic DNA, a 0.8% agarose gel was used. Electrophoresis was performed in 1x TAE (24% Tris base, 5.7% glacial acetic acid, 10% EDTA, pH 8.0) and the gel stained in 0.5 μg/ml ethidium bromide for viewing.

Isolation of DNA fragments from an agarose gel was modified from the method of Wang and Rossman (1994). After restriction enzyme digestion, the sample is electrophoresed through 0.6% agarose and stained with ethidium bromide. The band of interest is excised and chopped into fine pieces. A 0.5 ml microcentrifuge tube is pierced at the bottom, and packed by centrifugation after a small piece of glass wool over which 200 μl of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8)-saturated Sephadex G-10 beads are placed inside. The agarose containing the DNA fragment is placed into the prepared tube, which is placed into another 1.5 ml tube, then spun for 10 min at top speed in a microcentrifuge. The flow through is then treated with phenol/chloroform extraction and ethanol precipitated.

2.2.5 Radioactive labeling of DNA fragments

DNA fragments isolated from an agarose gel or from PCR reactions were used for labeling and hybridizations. The 4.2 kb EcoM-MluI fragment from pWX1507 was used as the MMSI probe for Southern analysis.

A Random Primer Labeling System (Gibco/BRL) was used as per manufacturer’s instructions. Radioactive probe was resuspended in 120 μl water and used as required.

2.2.6 Southern transfer and hybridization

After electrophoresis, the agarose gel was treated for approximately 10 min in 0.25M HCl, 30 min in 0.4M NaOH/0.6M NaCl, and 30 min in 1.5M NaCl/0.5M Tris-
HCl (pH 7.5). The DNA was transferred to a nylon-based membrane (GeneScreen by NEN) using 10x SSC (20x SSC: 3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0) overnight. After transfer, the membrane was washed in 0.4M NaOH followed by 0.2M Tris-HCl (pH7.5)/1x SSC for 1 minute each before UV crosslinking.

Membranes were incubated in prehybridization solution (2x SSC, 10% dextran sulfate, 5x Denhardt’s solution, 50% formamide, 1% SDS) for at least one hour at 42°C. 50 µl of 10 mg/ml sheared salmon sperm DNA and 30 µl of the probe of interest were denatured by boiling for 5 min, and cooled on ice for 5 min before being added to the prehybridization mixture. Hybridization was overnight at 42°C.

The membrane was washed twice for 5 min each at room temperature in 2x SSC/0.1% SDS, followed by twice for 30 min each at 65°C in 0.2x SSC/0.1% SDS. The membrane was exposed to x-ray film at -70°C and developed after an appropriate amount of time.

2.2.7 Pulsed field gel electrophoresis of chromosomal DNA

Cells were grown in 2 ml of YPD for 8 hours at 30°C, used to inoculate a 25-ml YPD culture, and the incubation continued overnight. The next morning, when a density of approximately 2x10⁷ cells/ml was attained, 6x10⁷ cells were removed for DNA isolation. For MMS-treated cultures, MMS was added to the remaining cells to a final concentration of 0.1%, and cells cultured for 1 hour at 30°C. After MMS treatment, cells were collected by centrifugation, washed in sterile distilled water, and resuspended in 0.1M potassium phosphate buffer, pH7, containing 0.1M glucose (same volume as the MMS-treated culture). Another 6x10⁷ cells were removed for DNA isolation, and the remaining cell suspension incubated at 30°C with shaking. Samples of 6x10⁷ cells were
removed after 4, 8, and 12 hours of incubation, and processed for DNA isolation. DNA was isolated in agarose blocks using the CHEF genomic DNA plug kit from Bio-Rad (Hercules, CA), following the manufacturer's instructions.

Pulsed field gel electrophoresis was performed using a Bio-Rad CHEF-DR III system. DNA was electrophoresed through 1% agarose in 0.5x TBE (0.045M Tris-borate, 0.001M EDTA, pH8) for either (a) 20 hours at 6 V/cm, with an angle of 120° and a 50 second switch time, or (b) 24 hours at 4.5V/cm, with an angle of 120° and a switch time ramped from 60-120 seconds, before staining in ethidium bromide. Gels were destained in distilled water overnight at 4°C before viewing.
CHAPTER THREE - RESULTS

3.1 DNA sequence analysis of the MMS1 gene

3.1.1 DNA sequence of MMS1

Prior to my arrival in the laboratory of Wei Xiao, the S. cerevisiae MMS1 gene was cloned by functional complementation of the mms1-1 mutation, and the DNA sequence of a 5.2 kb region including the MMS1 coding region was determined (Fig. 3-1). Possible TATA boxes were found in the 5' non-coding region, and possible poly(A) and transcription termination signals were located in the 3' non-coding region.

3.1.2 Database-assisted analysis of the MMS1 open reading frame

The MMS1 open reading frame (ORF) is 4224 bp long, capable of encoding a protein of 1407 amino acids. I hoped to gain some insight into the function of Mms1 by searching the public databases for homologous sequences and functional motifs. Three possibilities existed: (i) MMS1 encodes a known protein; (ii) MMS1 encodes a protein that has homology to a known protein or proteins, allowing certain assumptions about its function; or (iii) Mms1 shows no homology to known proteins. Using the BLAST program (Altschul et al., 1990) with the BLOSUM-62 matrix, no significant similarity between Mms1 and other polypeptide sequences in the GenBank database (release 115.0) was found. BLAST searches allow for the determination of similarity across an entire protein (or DNA) sequence. Other programs allow the user to search databases of functional protein motifs. A search of the MIPS protein database (Mewes et al., 1997) revealed that there are five putative hydrophobic α-helical regions of length sufficient to span a lipid bilayer (Fig. 3-1). However, no N-terminal signal sequence for membrane
CTCGAGTTTTATGATAATCCCCCTTTATTG

GTCTACTGTACTTTCTAATCTCTGTAGTTATATCTATCTACTCTTTCTGCTCTATTTCTGCTT -601

TTCTTTAGCGATGCTGATTTCGCCCAAAAAAAAAAAAAATTTTATCCTGCTGTTGCTGATGAG -541

GAAATAAGCCAAAAAGAAAAAAAGACAAAGACAAAAAGGTTTGGGCTAGTAAGTAGG -481

CCAGGATATAAAAAAGCAACATAGATTTTCGACTACCCTATTATCTCCTGCAAATTTTACTTTA -421

AGAAGTCTCTATGAAATATACAGGCTAATTTTGGAAACATGAGAAAGGAAAGATGAAGATA -361

CAAGAGTTGCAAGCAGTCAAGAGAGATTTAGGAAGAAAAAGGAAAAAGCAAAAAATATCGATT -301

SacI

TTTATGACTTACAAACTAATAATTTGATATTTTGGAAATATAATTTTGAGAAAGCTGAGCTCAATC -241

TGGTACAATATCCTCAAGTTAGATGTCGCAAAGAAAGAAGAAATAGCATAAAATTCAAAACCA -181

AAGGAAGTGGCAGGCGATTACCCCGCATATTATCGGGAACAGAAAGCCATGTTCTAGAGTGATT -121

TCCACAAATAGGCCCCCTTGCCAGAGATGGGCTCTGCAAAAGAATTGGAAAGAGAAAGAA -61

ATCGCTTCACATCCCGCTTTCTGGGTCTAATTAAGTAGTACCGGTGTCATAATTAGGT -1

ATGCTAGTTGGTGCAGAACCTGATGTTTGAACAGGTATGAACATTATAATTCTGCTGCCGCTG +60

MLGLRTHGLDREHYIIRRPS

GATTATTGGCAAAACTGGCTGCAAGATGCTGGAATCATAAGTCATTCCGAGGTTTCCTCCC +120

DFKELQLQDHLNHKSFVPS

AATTATTATATGTACATCTCAAAGACACACACTGTAGGGAATACACTGAGCTGTTTTTTATCAA +180

NLLIDSTTSWNEPFLYQ

EcoNI

AATACCGAGGGATACGTAATTTTTTGGGTGAGACCTTGTGTAGGGCAAAGCTAGAACCATAATG +240

NEDETEWTWVRPCVGPKLEPSM

ATGTAGCTCTGATATCAGATATCGATTGAAATATTGCTCAAATTTTGTATCAATACTC +300

MMLRYHDSNGQMPQFCYPI

TCAAGTCCGATATATTTTATACAGTTATATTTTGCAGAAAGACCTTCTGAACTG +360

SSPINFKPVLKYILQERSEL

TCAGACGGCCCTGCCAAATATGATAAACACACTAATAGTATGCTTTTTTGACAATTGATAAA +420

SDGFPPQKYNTLIGSLFIDK

AACCCAGAACATTTAGTATCTGAGATATGAAAGCATTGGAGACATAGAAAATAGGACAGT +480

NPETLDDSDIEALDDDIEMSS

GACAGCGCTTTAATTTTTAAAACAATGTTTGAATGCGGCTGGAAGAAATCCAAACAA +540

DSGNVKEPKIELQALEEEIQQ

AAGCAATTTTAAATTAGTATCTCAAACATGGAAATCTTTCACAAAACCGTACACTTCATA +600

KhFSLIVSNNGIFQTGSTSI
ACATAACATACAGTCTGGCATATCTGGGACGATACATAGCTATAAAAACCCAACAACGTTGCAATT
TYIQSGISGSIAIKPNNVAI
hydrophobic?
TTAATATTACTCCTACCCAACAGCTGGTCTACTTTATGTCTATTCTATTTATCCGTTGAGACGGT
+720
LILLTQPSGHLILSPLOLDG
+780
AAAGAGACATATTTTGTGCTAATAATTTGGAAACCTGGGACAAAGAGTCAGTGGACACATTAC
KEYLLQLYNLQGKGGWNIII
+840
AAGCACCAAACGAGAAAGCAGTTTTGTCTTTTATACATAAGGAATCTAGGCAATTTGCAAAAT
KHQNEKQFVLIHIKELGICKF
EcoRI
TTTGAATTCTCATTTACCATTCTACTTTTACATTAACATTTAACATTGACCCGATTCC
+900
FEFHLPFQFLVNNLTLTLDS
+960
GTTATATGAGGAGCATCTTTTTTTCCCAACAAATCTACACTGTATTTAGATCTCTTTACATTT
hydrophobic?
+1020
ATATTTATAACAGCATTATATGAAAGGATAGTCATCTTGTCATAGAATGGACACAC
IFITAIRYERIVYFYIEWNN
+1080
AACGGAAAATAGGAAAAAGAGGTATATCTCAATGGACAGTATTTTGATGTTGAGAAGACTAAAT
+1140
NEIKKKEVYQLTVFDDGEKTN
+1200
ATGACAATCCCATGGCAATATTGCATTTTGTATGCAAAACACCCTAAAGATTCTCTTTTA
MTIPIGLNACLVLVEPTPLKFSL
+1260
GTTTCTGCAAATCATAATTGTGAGAAGCAGCTAATCCACTATCTCCAAATGAGGCT
VSAANQMSGETEFHSFQLKA
pyridoxal phosphate attachment?
CTCGAGGAAATCAGTCATTTTTTTCAGCTCTTTATTGATATATAAAAAACAGAAGACTAAAT
+1320
LKGQIKSSPFAPLLKLKQLQEL
+1440
CACCCACATACATTTAAAAATCCTAAATTGTACCAATATAATCCTCCTGACAGGAAAT
HPTFKFFQCYCTTIISSTSGN
+1500
GACTCCAGATCTCCAGTATTTGATATGCTGGTGTATATTTTTATGATAGACATTTAGGCTAAAT
DSRSRSYVLVVISFSRTLEL
+1560
ACATATCTCTAGAAGATTGGAGATTGATGATAAAAAAGAGCTTATAAGCCTCTTGGAAA
TLSLEDRLRCDKDKDIVIKPLK
+1620
AAATACGATTCGGACACAGCAATTGATAGTCTCCACAGGAGAACTCTCAAATTTTAGCA
NITFKHTDISSTEENSQILA
+1680
TTTACGTCTCTTAAATTATAACACACACACACAGCTCTAAACATCTAGACAGGAAAT
FTSSKFNYHTGNSINDDTRN
+1740
CTTCAGTATTGCTTACCTACCAAAATCGAATAACTCAACCTGCGATATGATTACTAATTACCT
SQQVWLTSPNIAITQPCIDYKLN
+1800
AGGAAAATCCTGATTAATTCGCTACCCATATTAAAAGCATTAAAATATTGAGACACTCTTTAGGATA
RKTQLHILKQFQIFRHLIRI
+1860
TGGAAAAJGTAAAGAACCCTGGTATATGGCTTGTTACAGAGACCTGGGAAATAAACGATTCAAA
WKCKNLDIALLQRGLGINQSN
ACCAGGAGTTGTTATTTTTGGACGACCCGCTGGTCTTTTCTAAAACAGAATAATTTATTTATA
+1920
Figure 3-1: Complete DNA sequence of *MMS1*. A few key restriction sites are noted. Important sequences are underlined: in the 5' non-coding region are possible TATA boxes; in the 3' non-coding region are the putative poly (A) and transcription termination signals, respectively; in the amino acid sequence are the possible hydrophobic α-helices, the “greek key” motif, and the putative pyridoxal phosphate attachment site. The boldface TTG codon with the star (☆) above nucleotide 1400 is mutated to TAG in *mms1-l*. The GenBank accession number for the DNA sequence is U14001, and the *Saccharomyces* Genome Database-assigned locus is yPR164w.
insertion was found. Also of note is the presence of a pyridoxal phosphate attachment site sequence (Fig. 3-1) shared by certain amino acid decarboxylases (Jackson, 1990; Sandmeier et al., 1994). In yeast, only MMS1 and ORF YNL313c bear this sequence. Mms1 also contains the “Greek key” motif (Fig. 3-1) found in beta and gamma crystallins, two eye proteins (Lubsen et al., 1988; Wistow and Piatigorsky, 1988). This motif is purely structural.

The MMS1 gene has been independently identified as KIM3 (killed in mutagen) in a genome-wide screening for mutants sensitive to the DNA cross-linking agents mitomycin C or DEB (Saccharomyces Genome Database), and is designated as yPR164w in the Saccharomyces Genome Database.

### 3.2 Cloning and sequencing of the mms1-l mutation

The original mms1-l mutation rendered cells sensitive to killing by MMS, but not by UV or X radiation (Prakash and Prakash, 1977). It was of interest to determine where the mms1-l mutation was in the MMS1 gene, since the sequence of the mutation might give clues as to regions of functional significance present in the MMS1 ORF. A series of deletions were made in the MMS1 ORF in plasmid pWX1507 (Fig. 3-2), and the linearized plasmids used to transform strain WX15-1c (mms1-l). In S. cerevisiae, the deleted sequence is replaced by chromosomal sequence and the plasmid sealed in a gene conversion process known as gap repair (Orr-Weaver and Szostak, 1983). Transformants were screened for complementation of the MMS-sensitive phenotype of mms1-l on YPD + 0.025% MMS; non-complementing transformants imply that the plasmid was repaired by sequence containing the mms1-l mutation. Transformation
Figure 3-2: Deletions used in gap-repair cloning of the \textit{mms 1-1} mutation. Plasmid pWX1507 was digested with the restriction endonuclease(s) indicated, and the linearized DNA used to transform strain WX15-1c (\textit{mms 1-1}). A failure to complement growth on YPD+0.025\% MMS indicates that the cloned region contains the \textit{mms 1-1} mutation. Asterisk (*) marks the location of the \textit{mms 1-1} mutation.
with four of the linearized plasmids failed to complement for MMS-sensitivity. These were the XbaI, MfeI, PmlI-MfeI, and MfeI-BspEI deleted plasmids (Fig. 3-2). The mms/l-1 mutation was found to be a T→A transversion at nucleotide 1400 (relative to translation initiation) (Fig. 3-3), resulting in a L467→Amber nonsense mutation and a truncated 466-residue Mms1 protein.

3.3 Phenotypes of the mms/lΔ mutant

3.3.1 DNA damage sensitivities of mms/lΔ

The mms/l-1 mutant was originally isolated for its enhanced sensitivity to killing by MMS, but not by UV and X-rays (Prakash and Prakash, 1977). I compared an mms/lΔ mutant with its isogenic wild type strain and found that the mms/lΔ mutant was indeed sensitive to MMS (Fig. 3-4A) and mildly sensitive to both γ-irradiation (Fig. 3-4B) and UV (Fig. 3-4C). To further address the issue of DNA damage sensitivity, wild type and mms/lΔ cells were compared for sensitivity to the UV mimetic agent 4-nitroquinoline-N-oxide (4NQO). 4NQO introduces two types of DNA damage; oxidative base damage that is repaired by base excision repair, and bulky DNA adducts that, like UV-induced DNA damage, are mainly removed by nucleotide excision repair (Friedberg et al., 1995). As shown in Fig. 3-4D, the mms/lΔ mutant is sensitive to 4NQO by a gradient plate assay, although less so than the rad4Δ mutant defective in the nucleotide excision repair pathway. In addition to MMS, the mms/lΔ mutant is also sensitive to other alkylating agents, such as 1-methyl-3-nitro-1-nitrosoguanidine, 1-ethyl-3-nitro-1-nitrosoguanidine, N-nitroso-N-methylurea, and N-nitroso-N-ethyurea (Table 3-1), as
**Figure 3-3:** Sequence of the *mms1-l* mutation. Plasmid recovered from WX15-1c cells transformed with pWX1507Δ*MfeI-BspE*I was used as a template to sequence the *mms1-l* mutation. Arrow indicates the T→A transversion at nucleotide 1400 (relative to translation initiation).
Figure 3-4: Sensitivity of the mms1Δ mutant to various DNA damaging agents. (A) MMS. (B) γ radiation. (C) UV radiation. (D) 4NQO. (E) DEB. Strains used in A, B, and C: (□) DBY747 (wild type); (■) WXY344 (mms1Δ); (O) WXY387 (rad52Δ). In addition, WXY394 (rad4Δ) and WXY347 (mag1Δ) were used in D, E, and F. All strains are isogenic to DBY747. Values shown in liquid killing (A,B,C) are the average of two independent experiments. Gradient plates (D, E, F) were incubated for two days at 30°C before measuring relative growth.
Table 3-1: DNA damaging agent sensitivity spectrum of the mms/Δ mutant.

<table>
<thead>
<tr>
<th>DNA damaging agent^a</th>
<th>DBY747</th>
<th>WXY344 (mms/Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% MMS</td>
<td>+^b</td>
<td>-</td>
</tr>
<tr>
<td>0.5% EMS</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1 µg/ml MNNG</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.05 mg/ml ENNG</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>25 µg/ml methotrexate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1 µg/ml 4NQO</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>3 µg/ml phleomycin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10 µg/ml bleomycin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.1M HU</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

^a Agents were added to solid media (YPD or SD+supplements) to the concentration indicated. MMS, EMS, and t-butyl-hydroperoxide are liquid compounds, and percent is vol/vol. Abbreviations as follows: methyl methanesulfonate (MMS); ethyl methanesulfonate (EMS); 1-methyl-3-nitro-1-nitrosoguanidine (MNNG); 1-ethyl-3-nitro-1-nitrosoguanidine (ENNG); 4-nitroquinoline-N-oxide (4NQO); hydroxyurea (HU).

^b Growth on drug plate as compared to control plate: growth (+); no growth (-); spotty growth (+/-).

WXY344 is isogenic to DBY747.
well as the DNA cross-linking agent 1,2;3,4-diepoxybutane (DEB) (Fig. 3-4E). These results show that MMS1 is not an alkylation-specific DNA repair gene, as previously thought. Treatments with MMS, 4NQO, UV, and DEB will all lead to the formation of many different lesions, so it is possible that Mms1 has a broad substrate specificity for its DNA damage tolerance or repair function. Alternatively, one could speculate that all of the DNA damaging agents mentioned above will form lesions with a common endpoint, such as inhibition of DNA replication, and that perhaps Mms1 is involved in repairing a blocked DNA replication structure.

3.3.2 The mms1Δ mutant has a slow growth phenotype

During the cloning of MMS1 a slow growth phenotype for MD-1 and WX15-1c (both mms1-1) was noticed. Cell growth rates were therefore measured in wild type and its mms1Δ derivative in rich media. Wild type doubling time was calculated to be 1.6 hours, whereas its isogenic mms1Δ mutant doubled in 2.4 hours. Discounting any possibility of a direct role in cell cycle kinetics, two possibilities to account for this are (i) there is a higher rate of cell death in an mms1Δ culture than in the wild type culture and (ii) in the absence of MMS1, endogenous DNA damage accumulates and triggers a DNA damage cell cycle checkpoint thus slowing down cell growth.

The first possibility was assayed as follows: log-phase cultures of FY86 (wild type) and WXY348 (mms1Δ) were incubated with 10 μM FUN-1 in YPD for 30 min in the dark, then examined under a fluorescence microscope for viability. Metabolically active yeast cells fluoresce red, whereas inviable cells fluoresce green. As shown in Table 3-2, deletion of MMS1 does not affect the percentage of viable cells in a log-phase culture of S. cerevisiae.
Table 3-2: Viability of \textit{mms1}\Delta and \textit{rad52}\Delta cells in log phase culture.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable cells/total</th>
<th>% Viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY747 (wt)</td>
<td>398/413</td>
<td>96</td>
</tr>
<tr>
<td>WXY348 (mms1\Delta)</td>
<td>397/417</td>
<td>95</td>
</tr>
<tr>
<td>THY172 (rad52\Delta)</td>
<td>393/422</td>
<td>93</td>
</tr>
</tbody>
</table>

Log phase cell cultures were treated with FUN-1 and examined under a fluorescent microscope for viable staining (red fluorescence). Values are the combined total counts from two independent experiments. All strains were isogenic to DBY747.
To test the second hypothesis, RAD9 was deleted in the mms1Δ background and cells were streaked out on rich media to assay growth rate. The RAD9 protein product is required for the activation of the DNA damage checkpoint, and hence stops the cell cycle in the presence of DNA damage (Weinert and Hartwell, 1988). If mms1Δ causes an accumulation of DNA damage, it is expected that the Rad9-dependent DNA damage checkpoint would be triggered. Thus, deletion of RAD9 in the mms1Δ background would be expected to restore a wild type growth rate to the mms1Δ cells. As seen in Fig. 3-5, deleting RAD9 increased the growth rate of mms1Δ cells to that of the isogenic wild type as judged by colony size. Thus endogenous DNA damage accumulates in mms1Δ mutants, triggering a cell cycle checkpoint. It is interesting in light of this result that not all DNA repair mutants have a slow growth phenotype. Perhaps only certain types of endogenous DNA damage are strong cell cycle checkpoint triggers.

3.3.3 mms1Δ cells have an abnormal cellular morphology

The mms1Δ cells also have an abnormal morphology (Fig. 3-6; Table 3-3); some cells are very large and round, whereas others are hyper-elongated and display a filamentous-like growth pattern of colony formation on solid media. As discussed above, mms1Δ cells might suffer from endogenous DNA damage that alters cell cycle kinetics in these mutants, resulting in elongated doubling times and an abnormal cellular morphology. While not all DNA repair mutants display these morphological phenotypes, recombination-defective mutants such as rad52 do (Fig 3-6). Since recombination-defective mutants accumulate DSBs, perhaps the presence of DSBs in the cell leads to the abnormal morphological phenotype observed for these strains. The rad9Δ mutation not only alleviates the mms1Δ growth phenotype (section 3.3.2), it also greatly reduces
**Figure 3-5:** *rad9Δ* rescues the slow growth of *mms1Δ*. DBY747 (wt), WXY344 (*mms1Δ*), THY123 (*rad9Δ*), and THY124 (*mms1Δ rad9Δ*) cells were streaked onto YPD and incubated for 2 days at 30°C before photographing. All strains were isogenic to DBY747.
**Table 3-3: Abnormal cell morphology for mms1Δ cells**

<table>
<thead>
<tr>
<th>Strain</th>
<th>% abnormal morphology&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY747 (wt)</td>
<td>3.24</td>
</tr>
<tr>
<td>WXY344 (mms1Δ)</td>
<td>33.3</td>
</tr>
<tr>
<td>THY123 (rad9Δ)</td>
<td>3.97</td>
</tr>
<tr>
<td>THY124 (mms1Δ rad9Δ)</td>
<td>14.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are the average of two independent experiments.

Cells were counted as abnormal if they were elongated or larger than normal (~2x normal size or greater). All strains were isogenic to DBY747.
Figure 3-6: Morphology of $mms/\Delta$ cells. Log phase cultures of FY86 (wt) (A.), WXY348 ($mms/\Delta$) (B.), THY172 ($rad52\Delta$) (C.), and THY173 ($mms/\Delta \ rad52\Delta$) (D.) were spotted on slides and viewed using phase contrast microscopy. All strains were isogenic to FY86.
the frequency of abnormally shaped cells seen in mms1Δ cultures (Table 3-3). Hence the abnormal morphology is probably a result of a prolonged duration of a particular phase of the cell cycle.

3.4 Does MMS1 belong to any of the known DNA alkylation repair groups?

3.4.1 MMS1 is likely not a member of the base excision repair pathway

Since MMS1 was initially identified as an alkylation damage-specific gene, epistasis analysis was performed to determine whether MMS1 operates within the base excision repair pathway for the repair of DNA alkylation damage. mms1Δ mag1Δ and mms1Δ apn1Δ double mutants were created and their MMS sensitivities were compared to the corresponding single mutants. As shown in Fig. 3-7, the phenotypic effect of mms1Δ appeared to be additive to both mag1Δ (Fig. 3-7A) and apn1Δ (Fig. 3-7B), defective in 3MeA DNA glycosylase and AP endonuclease; both the mms1Δ mag1Δ and mms1Δ apn1Δ double mutant strains were more sensitive to killing by MMS than the corresponding single mutant strains. This suggests that MMS1 does not belong to the base excision repair pathway.

3.4.2 MMS1 does not likely represent a new member of the nucleotide excision or postreplication repair pathways

Mutants of the RAD3 nucleotide excision repair pathway and RAD6 postreplication repair pathway display various degrees of MMS sensitivity, indicating that both nucleotide excision and postreplication repair play a role in the repair or tolerance of DNA alkylation damage in addition to UV-induced damage (Broomfield et al., 1998; Friedberg et al., 1995; Xiao et al., 1996). With this in mind, I undertook an
Figure 3-7: Epistasis analysis of \( mms1\Delta \) with DNA repair mutations. (A) \( mms1\Delta \) and \( mag1\Delta \). (B) \( mms1\Delta \) and \( apn1\Delta \). (C) \( mms1\Delta \) and \( rad4\Delta \). (D) \( mms1\Delta \) and \( rad6\Delta \).

Strains used: (□) DBY747 (wt); (□) WXY344 (\( mms1\Delta \)); (O) JC8901 (\( mag1\Delta \)); (●) WXY406 (\( mms1\Delta mag1\Delta \)); (△) WXY105 (\( apn1\Delta \)); (▲) THY162 (\( mms1\Delta apn1\Delta \)); (▽) WXY394 (\( rad4\Delta \)); (▼) THY170 (\( mms1\Delta rad4\Delta \)); (<) WXY376 (\( rad6\Delta \)); (▲) THY132 (\( mms1\Delta rad6\Delta \)). All strains were DBY747 derivatives. Values shown are the average of at least two independent experiments.
MMS killing experiment to determine whether or not MMS1 operates within these pathways. As mms1Δ mutants show only a minor UV sensitivity (Fig. 3-4C) I did not expect to find epistasis with mutants of these pathways. Similar to the results seen for mms1Δ mag1Δ or mms1Δ apn1Δ double mutants, the MMS sensitive phenotype of mms1Δ is additive to that of both rad4Δ (Fig. 3-7C) and rad6Δ (Fig. 3-7D), suggesting that Mms1 does not operate in the nucleotide excision or postreplication repair pathways for DNA damage resistance.

3.4.3 MMS1 represents a novel branch of the RAD52 group recombinational repair pathway

In addition to their sensitivity to ionizing radiation, mutants of the RAD52 group display a marked sensitivity to killing by MMS (Friedberg et al., 1995). Although mms1Δ cells are sensitive to killing by MMS, they display no γ-sensitivity (Fig. 3-4B), so I did not expect to find that MMS1 belonged to the RAD52 group of recombinational repair genes. Attempts to perform epistasis analysis between mms1Δ and RAD52 pathway mutations failed in the DBY747 background because of the reduced viability of the double mutants. I observed a synthetic poor growth phenotype for both mms1Δ rad50Δ and mms1Δ rad52Δ strains (Fig. 3-8), while all single mutants were relatively healthy (although growing slower than the wild type). This phenotype was reflected in a low level of colony formation on rich media. I suspect that there is a hidden mutation in DBY747, which renders mms1Δ recombination double mutants almost non-viable, as this mutant combination is viable in the FY86 genetic background. As shown in Fig. 3-9, rad52Δ is epistatic to mms1Δ for MMS sensitivity; the mms1Δ rad52Δ strain is no more sensitive to MMS than the single rad52Δ mutant. This was not anticipated, as all
Figure 3-8: recombination mms1Δ double mutants have a synergistic growth phenotype in the DBY747 genetic background. DBY747 (wt), WXY344 (mms1Δ), WXY561 (rad50Δ), THY113 (mms1Δ rad50Δ), WXY387 (rad52Δ), and THY171 (mms1Δ rad52Δ) cells were streaked onto YPD and incubated for two days at 30°C before photographing. All strains were isogenic to DBY747.
Figure 3-9: *rad52Δ* is epistatic to *mms1Δ* for MMS sensitivity. Strains used: (□) FY86 (wt); (■) WXY348 (*mms1Δ*); (○) THY172 (*rad52Δ*); (●) THY173 (*mms1Δ rad52Δ*). Values shown are the average of two independent experiments. All strains were isogenic to FY86.
other RAD52-group mutants are extremely sensitive to killing by γ-irradiation, whereas
mms1Δ cells are not (Fig. 3-4B).

3.5 Evidence of elevated spontaneous DNA damage and genomic instability in
mms1Δ cells

3.5.1 Elevated spontaneous recombination in mms1Δ cells

If mms1Δ and rad52Δ mutants suffer from similar defects, then it would be
anticipated that the mms1Δ mutation affects certain recombination events. For an
intrachromosomal inverted repeat assay measuring recombination between two ade2
heteroalleles (Rattray and Symington, 1994), the observed recombination rate for the
mms1Δ strain was ten-fold higher than that of the corresponding wild type strain (Table
3-4). Conversely, deletion of MMS1 did not cause a measurable increase in either the
rate of sister chromatid exchange (Table 3-5), which would be induced to bypass DNA
polymerase-blocking lesions, or recombination within the rDNA array (Table 3-6),
which would use direct repeats as the homologous substrates. It is possible that Mms1
function is under either cell cycle or locational constraints, explaining the lack of an
effect on sister chromatid exchange or rDNA recombination. Alternatively, the exact
recombinational substrate created in the mms1Δ background might be amenable to only
a certain recombination event(s), specifically to recombination between inverted repeat
sequences. Since the rDNA array contains a tandem set of direct repeats, recombination
in this region of the genome might be through the single-strand annealing pathway, and
it is possible that MMS1 does not affect single-strand annealing. Regardless, the
observation of hyper-recombinant activity in the inverted repeat assay indicates that ss
Table 3-4: Intrachromosomal recombination between inverted \textit{ade2} heteroalleles.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of Ade$^+$ (x10$^4$)</th>
<th>Fold increase$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B365-14c (wt)</td>
<td>2.5$^a$</td>
<td>1</td>
</tr>
<tr>
<td>WXY687 (\textit{mms1}$\Delta$)</td>
<td>25.3</td>
<td>10.1</td>
</tr>
</tbody>
</table>

$^a$ Results are the average of three independent experiments.

$^b$ Relative to the wild type level.

WXY687 is isogenic to B365-14c.
Table 3-5: Sister chromatid exchange between ade3 heteroalleles.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate of Ade⁺ (x10⁶)</th>
<th>Fold increase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>yMP10381 (wt)</td>
<td>2.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>THY151 (mms1Δ)</td>
<td>1.51</td>
<td>0.63</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are the average of two independent experiments.

<sup>b</sup> Relative to the wild type level.

THY151 is isogenic to yMP10381.
Table 3-6: Measurement of *ADE2* deletion by recombination within the rDNA array.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Frequency of Ade² (x10⁻²)</th>
<th>Fold increaseᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY184 (wt)</td>
<td>1.42 (0.35)</td>
<td>1</td>
</tr>
<tr>
<td>THY153 (<em>mms lΔ</em>)</td>
<td>1.22 (0.47)</td>
<td>0.86</td>
</tr>
</tbody>
</table>

ᵃ Results are the average of three independent experiments, with standard deviation shown in brackets.

ᵇ Relative to the wild type level.

THY153 is isogenic to CY184.
gaps or DSBs accumulate in the \textit{mms1}Δ cells, and supports the hypothesis that Mms1 plays a role in the repair of these lesions.

\textbf{3.5.2 Expression of DNA damage inducible genes is elevated in the mms1}Δ \textbf{background}

If \textit{mms1}Δ cells indeed have an elevated level of endogenous DNA damage, then DNA-damage-inducible genes might be activated in the absence of exogenous damaging agents. To quantify the effect of the \textit{mms1}Δ mutation on the transcription of DNA-damage-inducible genes, β-gal activities of \textit{MAG1-lacZ} and \textit{RNR3-lacZ} transformants were analyzed in the wild type and isogenic \textit{mms1}Δ strains. As seen in Fig. 3-10, \textit{MAG1-lacZ} and \textit{RNR3-lacZ} transcription is elevated in the \textit{mms1}Δ mutant by 2 and 5 fold, respectively. In contrast, the non-inducible control \textit{CYC1-lacZ} did not show an elevated expression in the mutant background. These results are consistent with previous observations (Zhu and Xiao, 1998) that \textit{RNR3} induction is more sensitive to DNA damage than is \textit{MAG1} induction. These data supports the hypothesis that the \textit{mms1}Δ mutant contains an elevated level of DNA damage in its genome. At least some of these lesions, if left unrepaired, are recombinogenic, suggesting that they are, or can be processed into, DNA strand breaks.

\textbf{3.6 The mms1}Δ \textbf{mutation renders cells sensitive to replication-induced DNA damage}

Taking into account that unlike known \textit{RAD52} group mutants, \textit{mms1}Δ is sensitive to a variety of DNA damaging agents but not to ionizing radiation, I propose that \textit{MMS1} may
Figure 3-10: The effect of \textit{mms1}Δ mutation on the \textit{lacZ} fusion gene expression.

DBY747 (wild type) and WXY344 (\textit{mms1}Δ) cells were transformed with YEpMAG1-
lacZ (\textit{MAG1-lacZ}), pZZ13 (\textit{RNR3-lacZ}) or pLG669z (\textit{CYC1-lacZ}), β-gal activity was
measured and expressed in Miller units, and the relative induction of \textit{mms1}Δ
transformants to the corresponding DBY747 transformants is presented. The results are
the average of two (\textit{CYC1-lacZ}) or three (\textit{MAG1-lacZ} and \textit{RNR3-lacZ}) independent
experiments with standard deviation indicated by error bars. WXY344 is isogenic to
DBY747.
specifically protect cells from replication-dependent DNA strand breaks. Cells defective in the AP endonucleases encoded by APNI (Popoff et al., 1990) and APN2 (Johnson et al., 1998) will accumulate abasic lesions in their DNA. The Ntg1 and Ntg2 glycosylases have an associated AP lyase activity that appears to play a role in the processing of abasic sites (Swanson et al., 1999, You et al., 1999; W. Xiao, unpublished results). Thus, it is expected that the apnlΔapn2Δ double mutant would accumulate not only AP sites, but also SSBs. Both AP sites and SSBs interfere with DNA replication, presumably leading to DSB formation. If Mms1 acts to protect cells from replication-dependent DNA damage, it is expected that the mmslΔapnlΔapn2Δ triple mutant would display a synergistic growth defect. Indeed, while both the mmslΔapnlΔ and mmslΔapn2Δ double mutants were relatively healthy as assessed by their ability to form colonies on rich media, the mmslΔapnlΔapn2Δ triple mutant exhibited a synthetic poor growth phenotype (Fig. 3-11). One could argue that apnlΔapn2Δ double mutants accumulate AP sites, and that Mms1 is required for abasic site repair. However, MMS1 is not a member of the base excision repair pathway for DNA alkylation repair (Figs. 3-7A and 3-7B), and therefore the mutant is probably not sensitive to the presence of AP sites in the DNA per se. Thus, it is most likely that the mmslΔ mutation renders cells sensitive to lesions which interfere with proper replication fork progression.

The above experiment provides only circumstantial evidence that Mms1 helps to protect cells against replication-dependent DNA damage. In order to solidify this hypothesis, three additional experiments were performed.
Figure 3-11: Synthetic growth phenotype of the \textit{mms1\Delta apn\Delta apn2\Delta} mutant strain.

DBY747 (wt), WX344 (\textit{mms1\Delta}), WX105 (\textit{apn1\Delta}), THY162 (\textit{mms1\Delta apn1\Delta}),
WX791 (\textit{apn2\Delta}), THY163 (\textit{mms1\Delta apn2\Delta}), WX814 (\textit{apn1\Delta apn2\Delta}), and THY164
(\textit{mms1\Delta apn1\Delta apn2\Delta}) cells were streaked onto YPD and incubated for 2 days at 30°C
before photographing. The \textit{apn1\Delta/apn2\Delta} status is indicated underneath each plate. All
strains were isogenic to DBY747.
3.6.1 \textit{mms1}Δ cells are sensitive to killing by camptothecin

Camptothecin (CPT) is a Top1 inhibitor that traps Top1 in the cleavage complex, producing a protein-bound SSB in the DNA (Pommier \textit{et al.}, 1998). DNA replication can convert this single-stranded nick into a DSB (Ryan \textit{et al.}, 1991; Tsao \textit{et al.}, 1993). Hence, camptothecin treatment provides an effective system to produce replication-dependent DSBs. All of the cellular effects of camptothecin are due to its inhibitory effect on Top1. As shown in Fig. 3-12A, \textit{mms1}Δ cells are sensitive to camptothecin in a Top1-dependent manner. This is reminiscent of recombination-defective mutants (e.g., \textit{rad52}) that are also sensitive to killing by camptothecin treatment (Eng \textit{et al.}, 1988; Nitiss and Wang, 1988), and indeed the epistatic relationship between \textit{mms1}Δ and \textit{rad52}Δ holds true for camptothecin sensitivity (Fig. 3-12B). While deletion of \textit{TOP1} alleviated the camptothecin sensitive phenotype of the \textit{mms1}Δ mutant, it does not affect MMS-sensitivity (Fig. 3-12A). This indicates that not all of the phenotypes observed for \textit{mms1}Δ mutants are related to trapped Top1 complexes. In order to determine which pathways are involved in repairing camptothecin-mediated DNA damage, I screened a number of DNA repair mutants for camptothecin-sensitivity. As shown in Figures 3-12C\&D, while \textit{mms1}Δ and \textit{rad52}Δ were sensitive to camptothecin, neither the postreplication repair mutant \textit{rad18}Δ, the non-homologous end-joining mutants \textit{yku70}Δ and \textit{yku80}Δ, nor the nucleotide excision repair mutants \textit{rad1}Δ, \textit{rad2}Δ, \textit{rad4}Δ, and \textit{rad10}Δ displayed sensitivity to killing by camptothecin. As expected, all available \textit{RAD52}-group mutants were camptothecin-sensitive (Fig. 3-12E). This data shows that the repair of camptothecin-induced DNA damage is probably exclusively

109
Figure 3-12: Camptothecin-sensitivity of various DNA repair mutants. (A) Top1-dependent camptothecin sensitivity of mms1Δ mutants. Ten-fold serial dilutions beginning with $10^7$ cells/ml of DBY747 (wild type), WXY344 (mms1Δ), THY161 (mms1Δ top1), and WXY387 (rad52Δ) log phase cultures were spotted onto YPD, YPD+10 μg/ml CPT, and YPD+0.01% MMS. Plates were incubated for three days at 30°C before photographing. All strains were isogenic to DBY747. (B) rad52Δ is epistatic to mms1Δ for camptothecin-sensitivity. Ten-fold serial dilutions beginning with $10^7$ cells/ml of FY86 (wild type), WXY348 (mms1Δ), THY172 (rad52Δ), and THY173 (mms1Δ rad52Δ) log phase cultures were spotted onto YPD, YPD+0.25 μg/ml CPT, YPD+1 μg/ml CPT, and YPD+5 μg/ml CPT. Plates were incubated for three days at 30°C before photographing. All strains were isogenic to FY86. (C) Non-homologous end-joining mutants are not camptothecin-sensitive. Ten-fold serial dilutions beginning with saturated cultures ($\sim 10^8$ cells/ml) of DBY747 (wild type), WXY347 (mms1Δ), THY156 (yku70Δ), and THY158 (yku80Δ) were spotted onto YPD and YPD+10 μg/ml CPT. Plates were incubated for two days at 30°C before photographing. All strains were isogenic to DBY747. (D) Nucleotide excision repair and postreplication repair mutants are not camptothecin-sensitive. Ten-fold serial dilutions beginning with saturated cultures ($\sim 10^8$ cells/ml) of DBY747 (wild type), WXY344 (mms1Δ), WXY379 (rad1Δ), WXY555 (rad2Δ), WXY394 (rad4Δ), WXY537 (rad10Δ), and WXY482 (rad18Δ) were spotted onto YPD and YPD+10 μg/ml CPT. Plates were incubated for two days at 30°C before photographing. All strains were isogenic to DBY747. (E) Recombination-defective mutants are camptothecin-sensitive. Ten-fold serial dilutions beginning with saturated cultures ($\sim 10^8$ cells/ml) of LSY391 (wt), LSY399 (xrs2Δ),
LSY395 \((rad^{50}\Delta)\), LSY401 \((rad^{51}\Delta)\), LSY387 \((rad^{52}\Delta)\), LSY404 \((rad^{54}\Delta)\), LSY406 \((rad^{55}\Delta)\), LSY408 \((rad^{57}\Delta)\), and THY174 \((mms1\Delta)\) were spotted onto YPD and YPD+10 \(\mu\)g/ml CPT. Plates were incubated for two days at 30°C before photographing. All strains were isogenic to LSY391.
recombinational in nature.

3.6.2 mms1Δ cells are sensitive to DNA polymerase-induced DNA damage

DNA strand breaks can also be induced by a faulty DNA polymerase instead of by treatment with a damaging agent. POL3 (CDC2) encodes the catalytic subunit of DNA polymerase δ. A cdc2-2 mutation induces DNA damage at the restrictive temperature, as indicated by the triggering of the G2/M DNA damage cell cycle checkpoint (Weinert and Hartwell, 1993) and the appearance of Holliday junctions during S phase (Zou and Rothstein, 1997). Deletion of either RAD52 or MMS1 in the cdc2-2 background decreases the survival of the cdc2-2 strain after a four hour incubation at the non-permissive temperature (Table 3-7). Since Rad52 is required for the formation of Holliday junctions and recombinational rescue of the DNA template after cdc2-2-induced DNA damage (Zou and Rothstein, 1997), it is conceivable that the DNA lesions acquired by the cdc2-2 mutant at the non-permissive temperature are mainly DSBs.

3.6.3 The mms1Δ mutation renders cells sensitive to hydroxyurea treatment

Hydroxyurea (HU) inhibits ribonucleotide reductase activity and therefore depletes the pool of dNTPs available for DNA synthesis (Collins and Johnson, 1981). The end result is an S phase block with some DNA damage due to aberrant replication, including recombinogetic lesions as indicated by the hydroxyurea sensitivity of rad52 mutants (Allen et al., 1994; Fig. 3-13). The mms1Δ mutant also displays a marked hydroxyurea sensitivity (Fig. 3-13). Together, the camptothecin-sensitivity, hydroxyurea-sensitivity, and decreased survival of a cdc2-2 strain at the non-permissive
Table 3-7: Survival of *cdc2-2* strain derivatives after incubation at restrictive temperature

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>6613-53a</td>
<td><em>cdc2-2</em></td>
<td>37^b</td>
</tr>
<tr>
<td>THY109</td>
<td><em>cdc2-2 mms1Δ</em></td>
<td>8.2</td>
</tr>
<tr>
<td>WXY433</td>
<td><em>cdc2-2 rad52Δ</em></td>
<td>9.0</td>
</tr>
</tbody>
</table>

^a Log phase cultures grown at 23°C were shifted to 37°C for 4 hours. Cells were plated and incubated 3-4 days at 23° to score for survival.

^b Results are the average of three independent experiments. All strains were isogenic to 6613-53a.
Figure 3-13: Hydroxyurea sensitivity of $mms1\Delta$ mutants. Ten-fold serial dilutions beginning with $10^7$ cells/ml of (a) DBY747 (wild type), (b) WXY344 ($mms1\Delta$), and (c) WXY387 ($rad52\Delta$) log phase cultures were spotted onto YPD (left) and YPD+40mM HU (right). Plates were incubated for two days at 30°C before photographing. All strains were isogenic to DBY747.
temperature induced by the \textit{mms1\Delta} mutation indicates that Mms1 plays a specific role in the Rad52-dependent repair of replication-mediated DNA strand breaks.

\textbf{3.7 Spontaneous mutagenesis in the \textit{mms1\Delta} background}

Many DNA repair mutants display a spontaneous mutator phenotype. A number of unrepaired DNA lesions will inhibit DNA replication, and be bypassed in an error-prone manner by a mutagenic DNA polymerase such as Pol\(\zeta\). Two types of spontaneous mutagenesis assays were performed: the Trp reversion assay, where a specific point mutation or select extragenic suppressor mutations will revert the \textit{trp1-289} allele to wild type, and the canavanine resistance forward mutation assay, where inactivation of the arginine permease gene (\textit{CAN1}) allows for growth on the toxic substance canavanine. \textit{CAN1} can be inactivated by a number of mutagenic events, including base substitutions, frameshifts, small deletions, or gross chromosomal rearrangements (i.e. translocations, deletion of a chromosomal arm, inversions), though in wild type cells the majority of mutations are single base substitutions (Chen \textit{et al.}, 1998). The absence of \textit{MMS1} resulted in a roughly 10-fold increase in the level of spontaneous Trp reversion (Table 3-8) observed in the DBY747 background, and this was additive to Trp reversion rate of the \textit{apn1\Delta} strain (Table 3-8). Surprisingly, the \textit{mms1\Delta} mutation had no effect on the rate of canavanine resistance, even in the \textit{apn1\Delta} background (Table 3-8). Since canavanine resistance can occur by many different types of mutations in the \textit{CAN1} gene, there is a higher basal level mutation rate in the canavanine resistance assay as compared to the Trp reversion assay, but it is expected that mutators will affect the mutation rate in both assays.
Table 3-8: Spontaneous mutagenesis: *trp1-289* reversion and *CAN1* forward mutation rates.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Allele</th>
<th>Mutation rate</th>
<th>Fold increase&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBY747 (wt)</td>
<td><em>trp1-289</em></td>
<td>1.02x10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>CAN1</em></td>
<td>4.48x10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>WXY344 (<em>mms1Δ</em>)</td>
<td><em>trp1-289</em></td>
<td>9.91x10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>9.72</td>
</tr>
<tr>
<td></td>
<td><em>CAN1</em></td>
<td>4.45x10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>0.99</td>
</tr>
<tr>
<td>WXY105 (<em>apn1Δ</em>)</td>
<td><em>trp1-289</em></td>
<td>5.73x10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>5.62</td>
</tr>
<tr>
<td></td>
<td><em>CAN1</em></td>
<td>2.34x10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>5.22</td>
</tr>
<tr>
<td>THY162 (<em>mms1Δ apn1Δ</em>)</td>
<td><em>trp1-289</em></td>
<td>5.16x10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>50.6</td>
</tr>
<tr>
<td></td>
<td><em>CAN1</em></td>
<td>3.53x10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>7.88</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are the average of four to six independent experiments.

<sup>b</sup> Relative to the wild type level.

All strains were isogenic to DBY747.
3.8 Deleting the \textit{YKU80} gene encoding a DNA end-binding protein can partially rescue the DNA damage sensitivity of \textit{mms1Δ}

Homologous recombination is but one way to repair a DSB, non-homologous end-joining is another. In yeast, non-homologous end-joining is not the primary pathway for the repair of DSBs, and non-homologous end-joining mutants are not sensitive to ionizing radiation. Non-homologous end-joining mutants display weak (Milne \textit{et al.}, 1996) to negligible (Siede \textit{et al.}, 1996) MMS sensitivity, indicating that this pathway is not involved in the repair of MMS-induced DSBs. In the genetic background used in this study, non-homologous end-joining mutants are not MMS-sensitive, and the \textit{yku80Δ mms1Δ} double mutants are actually less sensitive to killing by MMS than is the \textit{mms1Δ} single mutant (Fig. 3-14). It is possible that the activity of the non-homologous end-joining pathway sensitizes \textit{mms1Δ} cells to MMS damage. The yKu80 protein will bind to a free DNA end, providing a nucleation site for the non-homologous end-joining proteins to bind. Since the rescuing effect was seen with \textit{yku80Δ} and not \textit{yku70Δ}, perhaps end-binding interferes with correct repair of certain types of DNA damage in the absence of Mms1.

3.9 \textit{mms1Δ} cells are proficient in the repair of MMS-induced DSBs

Homologous and nonhomologous recombination mechanisms are directly involved in the repair of DSBs. Since \textit{MMS1} belongs to the \textit{RAD52} pathway, I wanted to assess the ability of the \textit{mms1Δ} mutant to repair MMS-induced DSBs. In order to assay this, isogenic wild type, \textit{mms1Δ}, and \textit{rad52Δ} strains were treated with 0.1% MMS for 1 hour to induce a high level of DSBs, and allowed to recover in a potassium
Figure 3-14: MMS-sensitivity of $mms/l\Delta$ cells is partly alleviated by deleting the non-homologous end-joining gene $YKU80$. Strains used: (□) DBY747 (wt); (■) WXY344 ($mms/l\Delta$); (○) THY156 ($ku70\Delta$); (●) THY157 ($mms/l\Delta ku70\Delta$); (△) THY158 ($ku80\Delta$); (▲) THY159 ($mms/l\Delta ku80\Delta$). All strains were DBY747 derivatives. Values shown are the average of three independent experiments.
phosphate buffer for up to 24 hours. Incubation in the phosphate buffer ensured that the cells would not divide during the recovery period, and viability was not affected by this incubation. DNA was isolated at various times, resolved by pulsed field gel electrophoresis and visualized by ethidium bromide staining. As shown in Fig. 3-15A, MMS treatment of yeast cells induced many DSBs and sheared the genomic DNA, yet wild type and mms/Δ cells readily recovered from genomic DNA fragmentation within 12 hours. As expected, rad52Δ cells did not recover their chromosomes as quickly as the wild type strain did; the background recovery was presumably due to the actions of the non-homologous end-joining pathway. In case the hidden mutation in DBY747 affects the DSB repair activity of mms/Δ cells, the experiment was repeated in the FY86 genetic background. Again, no DSB repair defect was observed for mms/Δ cells (Fig. 3-15B).

A defect in the prevention of MMS-induced DSBs may be revealed by a comparison between wild type and mms/Δ genomic DNA immediately following a relatively low dose exposure of cells to MMS. Log phase cells were exposed to a series of low doses of MMS for 1 hour, and genomic DNA was immediately isolated and resolved by pulsed field gel electrophoresis (Fig. 3-16). Through computer-assisted quantitation of the DNA, I was unable to detect a difference in the level of DSBs induced in wild type and mms/Δ cells. While I could not detect a difference in the level of DSBs induced or repaired between wild type and mms/Δ cells after MMS treatment, it remains possible that the pulsed field gel electrophoresis method used lacks the sensitivity required to do so, especially if the mms/Δ mutation is responsible for only a small proportion of the observed DSBs induced or repaired.
Figure 3-15: *mms1Δ* cells are not deficient in the repair of MMS-induced DSBs. (A) DSB repair in the DBY747 genetic background. Log-phase cultures of DBY747 (wt, Lanes 1-5), WX344 (*mms1Δ*, Lanes 6-10), and WXY387 (*rad52Δ* Lanes 11-15) were either untreated (Lanes 1, 6 and 10) or treated with 0.1% MMS for 1 hour. Cells were resuspended in a phosphate buffer (0.1M potassium phosphate, pH7, 0.1M glucose) to prevent cell division. Samples were either immediately taken (Lanes 2, 7 and 12), or incubated at 30°C for 4 hrs (Lanes 3, 8, and 13), 8 hrs (Lanes 4, 9, and 14) and 12 hrs (Lanes 5, 10, and 15), and processed for CHEF gel analysis. Survival rates were 33.2% for wt, 0.630% for *mms1Δ*, and 0.00357% for *rad52Δ*. (B) DSB repair in the FY86 genetic background. Log-phase cultures of FY86 (wt, Lanes 1-5), WX348 (*mms1Δ*, Lanes 6-10), and THY172 (*rad52Δ* Lanes 11-15) were either untreated (Lanes 1, 6 and 10) or treated with 0.05% MMS for 1 hour. Cells were resuspended in a phosphate buffer (0.1M potassium phosphate, pH7, 0.1M glucose) to prevent cell division. Samples were either immediately taken (Lanes 2, 7 and 12), or incubated at 30°C for 4 hrs (Lanes 3, 8, and 13), 8 hrs (Lanes 4, 9, and 14) and 12 hrs (Lanes 5, 10, and 15), and processed for CHEF gel analysis.
Figure 3-16: MMS-induced genomic DSBs in wild type and mms/Δ cells. Log-phase cultures of DBY747 (wt, Lanes 1-4) and WXY344 (mms/Δ, Lanes 5-8) were either untreated (Lanes 1 and 5) or treated with 0.02% MMS (Lanes 2 and 6), 0.04% MMS (Lanes 3 and 7), or 0.06% MMS (Lanes 4 and 8) for 1 hour. Samples were removed immediately after treatment and processed for CHEF gel analysis. WXY344 is isogenic to DBY747.
3.10 MMS-induced DSBs are dependent on cellular metabolism but not on DNA replication

It is thought that MMS-induced chromosomal aberrations are dependent on DNA replication (Schwartz, 1989). In order to confirm this for MMS-induced DSBs, I ran a series of CHEF gels. First, MMS induces DSBs in DNA only if cells themselves are treated. As seen in Fig. 3-17A, chromosomal DNA in agarose plugs treated with 0.1% MMS for 1 hour yields no DSBs, as compared to similarly treated log phase cells. This shows that the induction of DSBs by MMS requires cellular metabolism. Next, I examined the role of DNA replication in producing MMS-induced DSBs. First, log phase and stationary phase cell cultures were compared for the accumulation of MMS-induced DSBs. As shown in Fig. 3-17B, there is no detectable difference in DSB induction in stationary phase versus log phase cells. Because it is possible that a low level of DNA replication was occurring in stationary phase cells, I examined the induction of DSBs by MMS in α-factor treated G1-arrested cells. Again, DSBs were detected in the absence of DNA replication (Fig. 3-17C). Production of DSBs occurs very rapidly, as after 10 minutes in 0.3% MMS chromosomal degradation had already occurred. These results indicate that MMS-induced DSBs are not reliant upon DNA replication. However, it is possible that a subset of the DSBs are replication-dependent in log phase cycling cells, but are masked by a high background of replication-independent breaks.

If MMS-induced DSBs are mainly replication-independent, they are likely caused as a byproduct of DNA repair. I examined the role of Mag1, the main DNA glycosylase responsible for initiating the repair of MMS-induced alkyl lesions. As
Figure 3-17: DSB formation by MMS requires cellular metabolism. (A) MMS does not induce chromosomal DSBs to DNA *in vitro*. Chromosomal DNA was isolated from DBY747 cells, embedded in agarose blocks, and treated with 0.1% MMS for 1h (Lane 1) or 3 hours (Lane 2). (B) No detectable difference in the amount of MMS-induced DSBs produced in log phase cells versus stationary phase cells. Log- (Lanes 1 and 2) or stationary-phase (Lanes 3 and 4) cultures of DBY747 cells were either untreated (Lanes 1 and 3) or treated with 0.025% MMS for 1 hour (Lanes 2 and 4). Samples were processed for CHEF gel analysis immediately after treatment. (C) MMS-induced DSBs in G1-arrested cells. Log phase SX46A cells were arrested in G1 by α-factor (Lane 1) and treated with 0.3% MMS for 10 minutes (Lane 2). Samples were processed for CHEF gel analysis immediately after treatment.
shown in Fig. 3-18A, DSBs are still induced in \textit{mag1}Δ cells, as well as in \textit{apn1}Δ cells. Fig. 3-15A shows that \textit{rad52}Δ cells, defective for recombinational repair, accumulate DSBs in response to MMS treatment. I also examined \textit{mre11}Δ cells, defective for the recombination-associated exonuclease activity (Fig. 3-18B). Again, this mutation had no effect on the accumulation of MMS-induced DSBs. Nucleotide excision repair endonuclease mutants were not tested. It is possible that nucleotide excision repair, base excision repair, and recombinational repair would all have to be disabled before a decrease in DSBs would be observed. These three pathways overlap in the repair of MMS-induced DNA damage, and if one pathway is defective, the others can compensate to a certain degree.

3.11 MMS induces recombination events in \textit{S. cerevisiae}

If MMS induces DSBs in the \textit{S. cerevisiae} genome, then it is expected that treatment of cells with MMS will also induce recombination events. I tested this hypothesis using the intrachromosomal inverted repeat recombination assay of Rattray and Symington (1994). As shown in Table 3-9, MMS induces recombination events in a dose-dependent manner. A five-fold increase in MMS concentration only results in less than a two-fold increase in recombination frequency. The basal level of recombination is already high for this assay (frequency of roughly $6.5 \times 10^{-3}$), so it is possible that the number of events plateaus, and very little increase is seen with increased MMS treatment.
Figure 3-18: MMS-induced DSBs in DNA repair mutants. (A) DSB formation in base excision repair mutants. Log phase cultures of isogenic JC8901 (magΔ) (Lanes 1 and 2) and WXY105 (apnΔ) were either untreated (Lanes 1 and 3) or treated with 0.025% MMS for 1 hour (Lanes 2 and 4). Samples were processed for CHEF gel analysis immediately after treatment. (B) DSB formation in mre11Δ cells. A log phase culture of MCY27 (mre11Δ) cells was either untreated (Lane 1) or treated with 0.1% MMS for 1 hour (Lane 2). Samples were processed for CHEF gel analysis immediately after treatment.
Table 3-9: MMS-induced intrachromosomal recombination between inverted ade2 heteroalleles in strain B365-14c.

<table>
<thead>
<tr>
<th>% MMS</th>
<th>Frequency of Ade(^+) (x10(^{-3}))</th>
<th>Fold increase(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.47 (1.89)(^a)</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>32.9 (4.65)</td>
<td>5.1</td>
</tr>
<tr>
<td>0.025</td>
<td>38.6 (7.5)</td>
<td>6</td>
</tr>
<tr>
<td>0.05</td>
<td>53.9 (12.6)</td>
<td>8.3</td>
</tr>
</tbody>
</table>

\(^a\) Results are the average of three or four independent experiments with standard deviations shown in the bracket.

\(^b\) Relative to the uninduced (0% MMS) level.
3.12 *MMS1* is involved in the transcriptional regulation of *MAG1* following DNA damage

3.12.1 *MMS1* is required for *MAG1* induction following DNA damage

Transcription of *MAG1* is induced by DNA alkylating agents (Chen *et al.*, 1990), as well as other DNA damaging agents such as 4NQO and UV radiation (Chen and Samson, 1991). The *MAG1* promoter has been well characterized, and upstream activating (UAS) and repressing (URS) sequences identified (Xiao *et al.*, 1993). Residing immediately upstream of *MAG1* is a divergently transcribed DNA damage inducible gene *DDII*. *DDII* is coregulated with *MAG1* through an 8 bp tandem repeat that acts as a UAS required for basal level expression and partial induction of both genes, but both *DDII* and *MAG1* contain their own URS (Liu and Xiao, 1997). As well, a *MAG1*-specific UAS lies within the *DDII* open reading frame (Liu *et al.*, 1997), and is required for full-level induction of *MAG1* (Liu *et al.*, 1997; Xiao *et al.*, 1993).

*MMS1* appears to be a key positive regulator of *MAG1* transcription. β-gal specific activities of *MAG1-lacZ* and *DDII-lacZ* transformants were determined in the wild type and isogenic *mms1Δ* strains treated with various concentrations of MMS. As seen in Fig. 3-19A&B, both *MAG1-lacZ* and *DDII-lacZ* were induced in wild type cells; however, in the isogenic *mms1Δ* mutant, *MAG1-lacZ* induction was less than 2-fold over its basal level while *DDII-lacZ* induction was only reduced by about two-fold. The effect of *mms1Δ* on *MAG1* induction is not limited to an MMS-induced signal, as UV-induced *MAG1* expression is also reduced in the *mms1Δ* mutant. At 40 J/m² *MAG1-lacZ* induction was reduced from 5.7 fold in wild type cells to 1.7 fold over wild type basal level in the mutant (Fig. 3-19C).
Figure 3-19: The effect of mms1Δ on expression of (A) and (C) MAG1-lacZ (YEpMAG1-lacZ), (B) DD11-lacZ (pWX1812), (D) RNR3-lacZ (pZZ13), and (E) RAD51-lacZ (YCPRAD51-lacZ). MMS or UV treatment was as described in Materials and Methods. ( ) DBY747 (wt); (■) WX347 (mms1Δ) for (A), (B), (C), (D), WXY344 (mms1Δ) for (E). β-gal specific activity was expressed in Miller units, and relative induction calculated compared to β-gal specific activity of untreated DBY747 cells. The values were the average of two or three independent experiments. All strains were isogenic to DBY747.
Since \textit{MMS1} is required for the DNA damage inducible transcription of \textit{MAG1}, it may affect the expression of other genes that are induced by a similar spectrum of DNA damaging treatments. The effect of the \textit{mms1}\textDelta mutation on \textit{RNR3} and \textit{RAD51} induction was investigated. While basal level \textit{RNR3-lacZ} expression is consistently elevated in the \textit{mms1}\textDelta mutant as compared to in the isogenic wild type, the maximum level of induction was correspondingly increased (Fig. 3-19D). Similarly, the \textit{mms1}\textDelta mutation had little effect on the expression of \textit{RAD51-lacZ} in response to MMS treatment (Fig. 3-19E). Thus the \textit{mms1}\textDelta mutation does not seem to significantly affect \textit{RNR3} and \textit{RAD51} gene expression.

\textbf{3.12.2 mms1}\textDelta \textit{is epistatic to dun1}\textDelta

Since both \textit{MMS1} and \textit{DUN1} (Liu, 1997) are required for the DNA damage inducible transcription of \textit{MAG1}, it was of interest to determine their relationship with respect to alkylation damage resistance. \textit{MMS1} was deleted in a \textit{dun1}\textDelta strain and the double mutant was compared to the \textit{mms1}\textDelta and \textit{dun1}\textDelta isogenic single mutants for MMS sensitivity. The results show that \textit{mms1}\textDelta is epistatic to \textit{dun1}\textDelta, as no further increase in MMS sensitivity was observed in the double mutant as compared to the single \textit{mms1}\textDelta mutant (Fig. 3-20). This result supports a hypothesis that \textit{MMS1} and \textit{DUN1} are in the same pathway for transcriptional induction of \textit{MAG1}.

In order to extend these results, I wanted to determine if this epistatic relationship held for other agents causing replication-dependent DNA damage. I found that \textit{dun1}\textDelta cells were not sensitive to camptothecin (Fig. 3-21), indicating that Dun1 does not play a role in all aspects of Mms1 function. These results show that the epistasis between \textit{mms1}\textDelta and \textit{dun1}\textDelta probably only applies to transducing the DNA
Figure 3-20: \textit{mms1}\Delta is epistatic to \textit{dun1}\Delta for MMS sensitivity. Strains used: (□) DBY747 (wt); (■) WXY344 (\textit{mms1}\Delta); (○) THY142 (\textit{dun1}\Delta); (●) THY143 (\textit{mms1}\Delta \textit{dun1}\Delta). All strains were isogenic to DBY747. Values shown are the average of two independent experiments.
Figure 3-21: The *dun1Δ* mutant is not camptothecin-sensitive. Ten-fold serial dilutions beginning with saturated cultures (~$10^8$ cells/ml) of DBY747 (wild type), WXY344 (*mms1Δ*), THY142 (*dun1Δ*), and THY143 (*mms1Δ dun1Δ*) were spotted onto YPD and YPD+10 µg/ml CPT. Plates were incubated for two days at 30°C before photographing. All strains were isogenic to DBY747.
damage signal for the transcriptional response to DNA damage.
CHAPTER FOUR - DISCUSSION

4.1 *mms1*-1 is a nonsense mutation

The *mms1*-1 mutation, isolated by Prakash and Prakash (1977), renders cells sensitive to killing by MMS, but not by UV or X-rays. Since MMS induces DNA strand breaks, many MMS-sensitive mutants are also γ- or X-ray-sensitive. Unless the mutant is defective specifically in the repair of the alkyl lesions induced by MMS (e.g. base excision repair mutants), it would be rare to see MMS sensitivity without ionizing radiation sensitivity. The *mms2*-1 mutation showed the same sensitivity pattern as *mms1*-1, but when the *MMS2* gene was disrupted, cells were rendered UV-sensitive as well (Broomfield *et al.*, 1998).

It was of interest to sequence the *mms1*-1 mutation. If *mms1*-1 was a missense mutation, then its location might provide a clue as to a functional domain in the Mms1 protein. In addition, if *mms1*-1 was a missense mutation then we might expect there to be differences in phenotypes between *mms1*-1 and *mms1Δ*, as seen with *mms2*-1 and *mms2Δ* (Broomfield *et al.*, 1998). Cloning and sequencing of *mms1*-1 revealed it to be a nonsense mutation, truncating the protein at 466 amino acids (of 1407 residues in wild type). The difference in UV sensitivities between what was observed for *mms1Δ* and what was reported for *mms1*-1 indicates that the *mms1*-1 allele might retain some activity. It is also possible that *mms1*-1 is a partial loss-of-function mutation in the MD-1 strain due to a low level of translational read through, or that the observed difference was simply due to the different genetic backgrounds of the two strains (MD-1 versus DBY747).
4.2 \textit{mms1}\(\Delta\) is not sensitive exclusively to DNA alkylation damage

The \textit{mms1}\(\Delta\) mutant is sensitive to killing by a wide variety of DNA alkylating agents, as would be expected of an MMS-sensitive mutant. It was interesting to find that it is also sensitive to other chemical DNA damaging agents, such as 4-NQO and DEB. 4-NQO is considered a UV-mimetic agent, as it produces bulky adducts that are mainly repaired by the nucleotide excision repair pathway. Oxidative DNA damage is also produced by 4-NQO treatment; this is typically repaired by the base excision repair pathway. DEB produces intra- and inter-strand DNA crosslinks. Intra-strand crosslinks are likely repaired by nucleotide excision repair, and inter-strand crosslinks by a combination of nucleotide excision repair and recombinational repair (Bessho \textit{et al.}, 1997). Mms1 may have multiple repair functions or a broad substrate range, or these agents all lead to a common biological endpoint, such as a transcription or replication block, and Mms1 repairs/tolerates that situation.

4.3 \textit{MMS1} is a member of the \textit{RAD52} epistasis group

I have shown that \textit{mms1}\(\Delta\) cells are sensitive to killing by a variety of DNA damaging agents, including UV and UV-mimetic agents, methyl- and ethylating agents and DNA cross-linking agents. Interestingly, \textit{mms1}\(\Delta\) cells are not hypersensitive to \(\gamma\) radiation. This phenotype distinguishes \textit{MMS1} from all other known DNA repair pathway genes. For example, the base excision repair pathway mutants (e.g., \textit{mag1} and \textit{apn1}) are sensitive to base-damaging agents, but not to UV and \(\gamma\)-rays; the nucleotide excision repair pathway mutants (e.g., \textit{rad4}) are extremely sensitive to UV and 4NQO,
but display little sensitivity to MMS and ionizing radiation; the major postreplication repair pathway mutants (e.g., rad6) are sensitive to a broad range of DNA damaging agents including MMS, UV, and ionizing radiation; and the recombination repair pathway mutants (e.g., rad50 and rad52) are extremely sensitive to MMS and ionizing radiation, but are moderately sensitive to UV (Friedberg et al., 1995). It was therefore surprising to find that rad52Δ is epistatic to mms1Δ for MMS sensitivity.

mms1Δ cells also share other phenotypes with rad52-group mutants. Cell growth rate is affected by the mms1Δ mutation. Doubling time increases from 1.6 to 2.4 hours, and is a consequence of endogenous DNA damage triggering the RAD9-dependent cell cycle checkpoint. Apparently not every kind of endogenous damage can trigger the checkpoint, because many DNA repair mutants do not grow slowly. However, the recombination-deficient rad52-group mutants do have a growth phenotype, indicating that endogenous DNA strand breaks probably trigger the DNA damage checkpoint. The mms1Δ mutation also leads to an abnormal morphological phenotype; some cells in a log phase culture are large and round, or grossly elongated. Again, not all DNA repair mutants appear this way, so the abnormal morphology cannot be purely a consequence of the presence of endogenous DNA damage. However, rad50Δ and rad52Δ mutants have a similar abnormal shape. It is possible that mms1Δ accumulates the same kind of lesion that the recombination mutants do, namely DSBs, and that this changes cellular metabolism such that morphology is affected. These two phenotypes provide circumstantial supporting evidence that MMS1 and RAD52 operate in the same pathway for DNA damage repair.
4.4 Replication-dependent DNA strand breaks and MMS1

In order to reconcile the fact that the mms1Δ mutant is sensitive to many DNA damaging agents which can indirectly cause DSBs, but not to X- and γ-rays, and yet belongs to the RAD52 epistasis group, I propose that MMS1 may play a specific role in the repair of replication-dependent DNA strand breaks. Many DNA lesions, including abasic sites, alkylated bases, and regions of abnormal DNA secondary structure such as cruciforms, protein-bound sites, and interstrand DNA crosslinks (Bierne and Michel, 1994), are able to inhibit DNA replication. The stalled replication fork might give rise to a replication-dependent DSB. In contrast, ionizing radiation-induced DSBs are considered replication-independent. While it is true that SSBs produced by ionizing radiations could be converted into DSBs by DNA replication, probably very few of these lesions persist into S phase due to the triggering of the G1/S DNA damage checkpoint (Siede et al., 1993). I examined the effect of MMS1 deletion under three conditions thought to induce replication-dependent DSBs: camptothecin treatment, where ssDNA nicks produced in the leading strand replication template lead to DSBs; cdc2-2 cells incubated at the restrictive temperature, which induces Holliday junction formation; and hydroxyurea treatment, where replication failure induces recombinogenic lesions. Camptothecin-induced lesions begin as SSBs, and it is conceivable that the cdc2-2- and hydroxyurea-induced lesions do as well. These SSBs could then be converted to DSBs via DNA replication. The results are consistent with a notion that Mms1 is involved in the recombination-mediated repair of replication-dependent DNA damage.

4.5 Spontaneous DNA damage in the mms1Δ background
The \textit{mms1}\Delta mutation induces a spontaneous hyper-recombination phenotype in yeast cells for certain recombination events. Other \textit{RAD52}-group mutants, such as \textit{rad50, rad51, rad54, rad55, rad57, mre11,} and \textit{xrs2}, are hyper-recombinant in certain assays (see, for example, Alani \textit{et al.}, 1990; Ivanov \textit{et al.}, 1992; Ajimura \textit{et al.}, 1993; McDonald and Rothstein, 1994). The inverted repeat recombination assay of Rattray and Symington (1994) is negatively affected by mutations in the \textit{RAD52} group. The \textit{rad52} mutation reduced the recombination rate to 0.03\% that of the wild type, while the \textit{rad51} (Rattray and Symington, 1994) and \textit{rad59} (Bai and Symington, 1996) mutations reduced the rate to roughly 25\% wild type. Mutations in \textit{RAD54, RAD55,} and \textit{RAD57} reduced the recombination rate to roughly 5\% that of wild type (Rattray and Symington, 1995). Combining \textit{rad54, rad55,} or \textit{rad57} with \textit{rad51} yields a recombination rate equal to that of \textit{rad51} (Rattray and Symington, 1995), while the rates of \textit{rad51} and \textit{rad59} are additive (Bai and Symington, 1996). The \textit{rad52} mutation is epistatic to all other recombination mutations for this assay. Recombination restoring the \textit{ADE2} gene in this assay can occur by gene conversion, crossing over, or gene conversion with an associated cross-over (Rattray and Symington, 1994). Various \textit{RAD52}-group mutations affect the distribution of events differently. Deleting \textit{MMS1} led to a hyper-recombinant phenotype for this particular recombination assay; the \textit{mms1}\Delta strain had a rate 10-fold higher than the wild type. Thus for this assay, \textit{MMS1} is the only \textit{RAD52}-group gene that plays a recombination-suppression role. Along with its lack of \(\gamma\)-sensitivity, this phenotype sets \textit{mms1}\Delta apart from the other \textit{RAD52}-group mutations. It is not known if the \textit{mms1}\Delta mutation affects the distribution of possible recombination events in this assay.
The sister chromatid exchange assay of Kadyk and Hartwell (1992) can detect gene conversion and reciprocal recombination events between sister chromatids. A rad52 mutation reduces spontaneous sister chromatid exchange to roughly 15% that of wild type, whereas a rad50 mutation only reduces it to 70% of wild type (Kadyk and Hartwell, 1993). Deleting MMS1 does not affect the rate of spontaneous sister chromatid exchange. This was surprising, because sister chromatid exchange is proposed to be the recombinational means of repairing or tolerating replication-blocking DNA lesions (Kadyk and Hartwell, 1993; Paulovich et al., 1998), and I propose that MMS1 is involved specifically in this type of recombination event. The damage-induced sister chromatid exchange events assayed by members of Hartwell’s laboratory are induced by UV-irradiation. It is possible that UV-induced lesions and those repaired/tolerated by Mms1 invoke different recombination events, and that Mms1-reparable lesions do not result in increased spontaneous sister chromatid exchange in this assay. As well, it is possible that although mms1Δ did not affect spontaneous sister chromatid exchange, it might affect damage-induced events by the same assay.

The rDNA::ADE2 “pop-out” assay of Sadoff et al. (1995) measures recombination within the rDNA array. The ADE2 gene can in theory be lost by a number of different events, including unequal sister chromatid exchange and intrachromosomal gene conversion (Christman et al., 1988). Recombination within the rDNA array appears to be independent of RAD52, as rad52 mutants are actually elevated 3-fold for loss of an integrated URA3 marker (Christman et al., 1988). Interestingly, loss of TOP1 elevates the recombination frequency 30-200 fold over wild type (Christman et al., 1988; Sadoff et al., 1995). Thus topoisomerase I plays the role of
recombination suppressor within the rDNA repeats. Deletion of MMS1 did not affect recombination in this assay. Some of the recombination events expected to lead to the loss of the integrated ADE2 gene would be intrachromosomal events, but unlike those assayed in the Rattray and Symington assay, the intervening DNA sequence must be lost. Thus it is possible that Mms1 affects only a specific intrachromosomal recombination event where no sequence is lost. Alternatively, Mms1 might be excluded from the nucleolus, and therefore have no effect on rDNA recombination.

The mms1Δ mutant also exhibits a spontaneous mutator phenotype for trp1-289 reversion. Many DNA repair mutants are mutators, as many unrepaired DNA lesions are either mutagenic due to base mispairing or bypass by mutagenic DNA polymerases. It was unexpected that mms1Δ did not increase the rate of canavanine resistance, as this is a forward mutation assay and is expected to yield rates higher than seen with the trp1-289 reversion assay. It is possible that the sequence or chromosomal context of the trp1-289 mutation renders it susceptible to the mms1Δ effect. Since mms1Δ was additive to apn1Δ for MMS sensitivity, I expected that mms1Δ would also be additive to apn1Δ for trp1-289 reversion rate. This was indeed the case.

4.6 Possible mechanism(s) of Mms1 function in recombination

MMS treatment of cells can lead to the production of AP sites through the actions of the base excision repair pathway. If unrepaired, AP sites are lethal lesions; they can be bypassed by the mutagenic polymerase Rev3 (Johnson et al., 1998) but not by replicative DNA polymerases. The fact that base excision repair rad52 and rad52 rev3 strains are sensitive to killing by oxidative agents, but base excision repair,
rad52Δ, or rev3Δ alone are not (Swanson et al., 1999) suggests that in addition to translesion bypass by Rev3, AP sites or a subsequent lesion arising from AP sites such as an SSB can be repaired by a recombination event. As AP sites block replicative polymerases, recombination could be required to rescue a collapsed replication fork.

AP sites can also affect topoisomerase activity. They can trap Top1 in a cleavage complex when present immediately 3' to the Top1 cleavage site, similar to the action of camptothecin (Pourquier et al., 1997C). The resulting protein-bound ssDNA nick could potentially be converted into a DSB through the action of the DNA replication machinery (Ryan et al., 1991; Tsao et al., 1993). AP sites also enhance the cleavage activity of topoisomerase II (Kingma et al., 1995) and the DSBs produced can be rendered irreversible through the action of DNA replication or transcription (D’Arpa et al., 1990; Zhang et al., 1990; Howard et al., 1994). Further processing of AP sites by AP endonucleases creates a temporary SSB in the DNA backbone. SSBs or gaps opposite a Top1 cleavage site lead to the formation of irreversible cleavage complexes and Top1-bound DSBs without the need for collision with a replication fork (Pourquier et al., 1997A).

The sensitivity of mms1Δ mutants to both MMS and camptothecin treatments could be explained by a few possibilities. As both AP sites and camptothecin have the ability to trap Top1 in the cleavage complex, it is possible that Mms1 is specifically involved in repairing Top1-mediated DNA damage. This cannot be true, however, as deletion of TOP1 in the mms1Δ background does not alleviate the MMS-sensitive phenotype of the mms1Δ mutant. Another possibility is that Mms1 is required for the
repair of DNA replication-dependent DSBs in general, whether caused by a trapped topoisomerase or another polymerase-blocking lesion.

In *E. coli*, the link between replication and recombination is well established. A model was proposed whereby a Holliday junction forms at blocked replication forks through the annealing of the newly synthesized strands of DNA. RuvAB binds to this structure and catalyzes branch migration, creating a double stranded tail recognized by RecBCD. RecBCD initiates homologous recombination, creating a structure recognized by the primosome, which reinitiates DNA replication. Thus, in wild type cells, RuvAB and RecBCD might catalyze replication fork repair without the production of a DSB (Seigneur et al., 1998). It is possible that in *S. cerevisiae* Mms1 acts with recombination proteins to repair replication-dependent DNA damage without actual DSB formation, analogous to the situation described above for *E. coli*. In this case, the absence of Mms1 would lead to DSB formation and the hyper-recombinant phenotype observed.

However, the sensitivity of *mms1Δ* cells to camptothecin, an agent known to produce DSBs, suggests that the substrate of Mms1 is in fact a DSB. Since *mms1Δ* mutants are not sensitive to ionizing radiation, Mms1 is not required for the direct repair of all DSBs. Replication-dependent DSBs might require additional processing before recombinational proteins can use them as a substrate. Alternatively, it is possible that Mms1 acts to attract recombination proteins to the site of a replication-dependent DSB, or that Mms1 might increase the accessibility of the damaged site to recombination proteins, possibly by removing the replication apparatus or reversing the replication fork. The *RAD6* postreplication repair pathway is thought to mediate the tolerance of DNA damage acquired during S phase, but the lack of camptothecin sensitivity in *rad6* mutants
suggests that not all S phase-dependent DNA damage is tolerated by postreplication repair. I propose that at least some of these lesions are repaired by Mms1-mediated recombination events.

4.7 Mms1 transduces the DNA damage signal for MAG1-induction

I have found that, apart from its role in repairing replication-dependent DNA damage, MMS1 is required for the DNA damage-induced transcription of MAG1, but not for that of the divergently transcribed gene DDI1. As well, deleting MMS1 has a minimal effect on the induction of RNR3 and RAD51. The findings in this study showing that MAG1-lacZ is not induced in the mms1Δ mutant contrasts with those of Chen and Samson (1991), who found that MAG1 is induced in the mms1-1 mutant in response to MNNG treatment. This discrepancy could be due to an allele-specific phenotype of mms1-1. Since mms1-1 is a nonsense mutation, the original mms1-1 mutant strain used by Chen and Samson may contain a leaky amber suppressor that allows some expression of MMS1 and hence MAG1 induction. Another possibility is that specific lesions produced by MNNG but not by MMS, such as O\(^6\)MeG or methylphosphotriesters (Beranek, 1990), trigger a signal that is transduced through an MMS1-independent pathway, leading to induction of MAG1 in the mms1-1 mutant. Thus MMS- and UV-produced signals would be transduced through Mms1 while MNNG-specific signals would be transduced by an as of yet unknown pathway. Examination of MAG1 induction in response to MNNG treatment in the mms1Δ strain has not been performed.
The protein kinase Dun1 is a key regulator of RNR gene induction in response to DNA damage; Dun1 is phosphorylated in a Rad53-dependent manner, and Dun1 phosphoprotein presumably activates downstream effectors (possibly Crt1) that in turn induce the transcription of various genes. Some downstream effectors of Dun1 may be involved in the transactivation of a subset of DNA damage inducible genes. This argument is supported by the fact that while both RNR2 and MAG1 induction requires DUN1 (Zhou and Elledge, 1993; Liu, 1997), the transcriptional induction of RNR2 in response to DNA damage can occur in the absence of de novo protein synthesis (Elledge and Davis, 1989), whereas the induction of MAG1 requires new proteins to be synthesized (Chen and Samson, 1991). Thus, in addition to the Dun1 protein kinase, MAG1 induction requires at least one other signal transducer. Three arguments support the assertion that Mms1 might be this protein. First, DUN1 is required for the induction of both RNR3 and MAG1, while MMS1 is not required for RNR3 induction. Secondly, the dun1 mutant is strongly sensitive to both UV and MMS (Zhou and Elledge, 1993), whereas the mms1 mutant has only a minor UV-sensitivity. Finally, mms1Δ is epistatic to dun1Δ with respect to MMS sensitivity, indicating that Mms1 acts within the Dun1 pathway. Based on the above analyses, I propose that the DUN1 pathway branches into an arm for RNR gene induction and one for MAG1 induction, and that MMS1 functions in the latter arm. Other alternatives are that an MMS1-dependent signal transduction pathway could feed into the DUN1-dependent pathway downstream of a branch leading to the induction of RNR3 or that an MMS1-dependent modification of Dun1 could cause Dun1 to phosphorylate downstream effectors specific for the induction of MAG1.
Figure 4-1: Proposed model for the requirement of *MMS1* in the DNA damage transcriptional response of *MAG1*. The DNA damage signal is presumably transmitted through Pol2 to the Dun1 protein kinase, which in turn modifies downstream effectors to directly affect the transcription of certain genes. The Mms1-independent branch of this pathway leads to *RNR* gene induction; another Mms1-dependent branch leads to *MAG1* induction.
4.8 Could Dun1 play a direct role in DNA repair?

The \textit{dun1} (DNA damage-uninducible) mutation was isolated in a screen for mutants unable to induce RNR3 expression after a DNA damaging treatment (Zhou and Elledge, 1993). This study also showed that the mutant was sensitive to killing by hydroxyurea, MMS, and UV. A \textit{dun1} strain was also shown to be partially defective in the G2 DNA damage checkpoint (Pati \textit{et al.}, 1997). Fasullo \textit{et al.} (1999) further explored the DNA damage sensitivities of the \textit{dun1\Delta} strain, and showed that \textit{rad1\Delta} is epistatic to \textit{dun1\Delta} and \textit{rad52\Delta} is synergistic with \textit{dun1\Delta} for UV sensitivity. The authors suggest that Dun1-induced genes participate in the \textit{RAD3} nucleotide excision repair pathway, and channel lesions away from recombinational repair. Data in the paper also shows that for \textit{\gamma}-sensitivity, \textit{rad1\Delta} is epistatic to \textit{dun1\Delta}, and \textit{dun1\Delta} is additive to \textit{rad52\Delta}. No data for MMS-sensitivity was presented or discussed. The \textit{dun1\Delta} mutant is hyper-recombinant for homologous recombination in a diploid strain, and this phenotype is dependent on \textit{RAD52} (Fasullo \textit{et al.}, 1999). The mutant is also hyper-recombinant for intrachromosomal recombination between direct repeats of \textit{ade2} flanking \textit{URA3} (gene conversion), but displays a decreased rate of \textit{URA3} “pop-outs”. UV-induced sister chromatid exchange and heteroallelic recombination events in diploids are also increased in \textit{dun1\Delta} mutants, consistent with the synergistic increase in UV-sensitivity in the \textit{dun1\Delta rad52\Delta} double mutant. Neither MMS nor \textit{\gamma}-radiation increased induced recombination over the wild type levels.

I have shown that \textit{mms1\Delta} is epistatic to \textit{dun1\Delta} for MMS-sensitivity. Both \textit{MMS1} and \textit{DUN1} are required for \textit{MAG1} induction in response to MMS treatment. However, epistasis results showing that \textit{MMS1} belongs to the \textit{RAD52} pathway indicate
that Mms1 is more than just an inducer of *MAG1* transcription. As well, *mms1Δ* is sensitive to killing by replication-dependent DNA damaging treatments, and *MAG1* has no bearing on survival in these cases. It is possible that Dun1 actively participates with Mms1 in DNA damage repair. Dun1 is a protein kinase, and phosphorylation of certain replication or repair proteins might facilitate the Mms1 replication-dependent DNA damage repair pathway. However, the *dun1Δ* mutant is not sensitive to camptothecin, suggesting that it does not play a role in the Mms1- and Rad52-dependent repair of replication-dependent DNA lesions. Although there is no data on the relationship between *dun1Δ* and *rad52Δ* with respect to MMS-sensitivity, it seems likely that the two mutations would be additive. Thus the epistatic relationship between *dun1Δ* and *mms1Δ* probably only applies to transducing the DNA damage signal for gene induction, and not to the direct role of Mms1 in DNA repair. If Mms1 plays two roles in the cell, one in *MAG1*-induction and one in Rad52-dependent DNA repair, and Dun1 only participates in *MAG1*-induction, then the *mms1Δ dun1Δ* double mutant is expected to be no more sensitive to MMS than the *mms1Δ* single mutant, as was observed.

4.9 MMS induces DNA DSBs that are dependent on cellular metabolism

An interesting observation that came out of this study is that MMS causes DSBs in DNA. MMS has been referred to as an X-ray mimetic agent, because recombination-defective mutants are MMS-sensitive, however to the best of my knowledge it had never been shown that MMS does indeed cause DSBs. It had been shown that MMS induces SSBs (Eastman and Bresnick, 1978; Pavlis et al., 1978); however, these studies were
performed under alkaline conditions, and could overestimate the number of frank strand breaks due to alkaline cleavage of abasic sites.

The induction of DSBs by MMS depends upon cellular metabolism, as chromosomal DNA treated with MMS in vitro is not sheared. Although the many chemicals and enzymes used in preparing the chromosomal DNA plugs are able to penetrate the agarose, it is possible that MMS cannot, and thus has no effect on DSB formation in vitro.

As DSB induction depends upon cellular metabolism, I wanted to find the process responsible. Since MMS is proposed to cause replication-dependent DNA damage, and is an S-phase dependent clastogen (Schwartz, 1989), I first examined the role of active DNA replication in producing the DSBs. In both stationary phase cells and α-factor G1-arrested cells, MMS still produced DSBs. It is possible that in stationary phase, a low level of DNA replication is still occurring, but α-factor-arrested cells were only in G1 during the period of MMS treatment. Another possibility, however unlikely, is that during the period in which cells are incubating in EDTA on ice prior to DNA isolation, a low level of DNA replication occurs. It is most likely that the majority of DSBs occur as repair enzymes cleave the DNA during excision repair, with a subset of DSBs possibly being replication-dependent. I screened the base excision repair mutants mag1Δ and apn1Δ, the postreplication repair mutant rad18Δ, and the recombination mutants rad52Δ and mre11Δ, and all showed DSBs upon MMS treatment. It is quite possible that a number of excision genes would have to be mutated in order to see a decrease in DSB production by MMS.
In a related study, McHugh et al. (2000) used CHEF gels to examine DSBs induced by interstrand crosslinking agent nitrogen mustard. Interstrand DNA crosslink repair is likely initiated by nucleotide excision repair, and non-crosslinking monoadducts are removed by either base excision repair or nucleotide excision repair. They observed that both the nucleotide excision repair endonuclease deficient rad1 rad2 double mutant, and the rad4 mag1 double mutant (unable to excise monoadducts), accumulate DSBs to the same level as the wild type strain. They also screened rad27 and mre11 mutant strains, both of which are sensitive to killing by nitrogen mustard, but these strains also accumulate DSBs at wild type levels. Thus, the protein(s) responsible for DSB formation in response to both MMS and nitrogen mustard treatment remain unknown.
References


Broach, J.R., J.N. Strathern, and J.B. Hicks. 1979. Transformation in yeast:
development of a hybrid cloning vector and isolation of the CAN1 gene. Gene 8:
121-133.

Broomfield, S., B.L. Chow, and W. Xiao. 1998. MMS2, encoding a ubiquiting-
conjugating-enzyme-like protein, is a member of the yeast error-free postreplication

Brusky, J., Y. Zhu, and W. Xiao. 2000. UBC13, a DNA-damage-inducible gene, is a
member of the error-free postreplication repair pathway in Saccharomyces cerevisiae.

Budd, M.E., and J.L. Campbell. 1995. DNA polymerases required for repair of UV-

Buhl, S.N., R.B. Setlow, and J.D. Regan. 1972. Steps in DNA chain elongation and

Buttin, G., and M. Wright. 1968. Enzymatic DNA degradation in E. coli: its
relationship to synthetic processes at the chromosome level. Cold Spring Harb.

Calmels S., H. Ohshima, M. Crespi, H. Leclerc, C. Cattoen, and H. Bartsch. 1987. N-
nitrosamine formation by microorganisms isolated from human gastric juice and
urine: biochemical studies on bacteria-catalysed nitrosation, p. 391-395. In H.
Bartsch, I. O'Neill, and R. Schulte-Hermann (ed.), The relevance of N-nitroso
compounds to human cancer. IARC Scientific, Lyon, France.

Capaldo-Kimball, F., and S.D. Barbour. 1971. Involvement of recombination genes in

Smith, (ed.), Genetic recombination. American Society for Microbiology,
Washington, D.C.

Saccharomyces cerevisiae replication and recombination defective mutants. Nature

Chen, C., K. Umezu, and R.D. Kolodner. 1998. Chromosomal rearrangements occur in
S. cerevisiae rfa1 mutator mutants due to mutagenic lesions produced by double-
Chen, J., B. Derfler, and L. Samson. 1990. *Saccharomyces cerevisiae* 3-methyladenine DNA glycosylase has homology to the AlkA glycosylase of *E. coli* and is induced in response to DNA alkylation damage. EMBO J. 9: 4569-4575.


Klein, H.L. 1988. Different types of recombination events are controlled by the RAD1 and RAD52 genes of *Saccharomyces cerevisiae*. Genetics 120: 367-377.


DNA phosphodiesterase that repairs topoisomerase I complexes. Science 286: 552-
555.

Pourquier, P., A.A. Pilon, G. Kohlhagen, A. Mazumder, A. Sharma, and Y. Pommier.
1997A. Trapping of mammalian topoisomerase I and recombinations induced by
damaged DNA containing nicks or gaps. Importance of DNA end phosphorylation

Bjornsti, and Y. Pommier. 1997B. Induction of reversible complexes between
eukaryotic DNA topoisomerase I and DNA-containing oxidative base damages. J.
Biol. Chem. 274: 8516-8523.

Pourquier, P., L.-M. Ueng, G. Kohlhagen, A. Mazumder, M. Gupta, K.W. Kohn, and Y.
Pommier. 1997C. Effects of uracil incorporation, DNA mismatches, and abasic sites
on cleavage and religation activities of mammalian topoisomerase I. J. Biol. Chem.
272: 7792-7796.

Prado, F., and A. Aguilera. 1995. Role of reciprocal exchange, one-ended invasion
crossover and single-strand annealing on inverted and direct repeat recombination in
yeast: different requirements for the RAD1, RAD10, and RAD52 genes. Genetics
139: 109-23.

interstrand cross-linking and DNA strand break formation associated with alkylated
DNA. Carcinogenesis 3: 425-431.


Prakash, L., and S. Prakash. 1977. Isolation and characterization of MMS-sensitive

Preston, B.D., B. Singer, and L.A. Loeb. 1986. Mutagenic potential of O-tri-
methylthymine in vivo determined by an enzymatic approach to site-specific

replication origin near a double-stranded DNA break. Genes Dev. 8: 554-62.

Apo1 apurinic endonuclease/3'-diesterase: repair of oxidative and alkylation DNA
Rattray, A.J., and L.S. Symington. 1994. Use of a chromosomal inverted repeat to demonstrate that the RAD51 and RAD52 genes of *Saccharomyces cerevisiae* have different roles in mitotic recombination. Genetics 138: 587-595.


