Mechanisms Ensuring Genomic Integrity In Yeast

By

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Obtaining my PhD has been an adventure, to say the least. I've learned many lessons about being a scientist and, perhaps more importantly, about being a person. And while I did not identify a candidate gene, determine its function, and then provide a cure based on its function as I had nievely outlined in my graduate school application, I did learn a lot. For this great accomplishment I have a number of people to thank.

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LIST OF ABBREVIATIONS

8-oxo-G–8-hydroxyguanine
AP-apurinic/apyrimidic
BER-base excision repair
CPD-cyclopyrimidine dimers
CTH-C-terminal homology
DNA-deoxyribonucleic acid
FaPy-2,6 diamino-4-hydroxyuracil-5-N-methyl formamidopyrimidine
HNPCC-hereditary non-polyposis colon cancer
IDL-insertion-deletion loops
MLH-MutL homolog
MMR-mismatch repair
MSH-MutS homolog
MSI-microsatellite instability
NER-nucleotide excision repair
ORF-open reading frame
PCR-polymerase chain reaction
TCR-transcription coupled repair
TLS-translesion synthesis
UV-ultraviolet light

ABSTRACT

DNA mismatch repair (MMR) acts on DNA mispairs associated with replication, chemical damage, or recombination. The yeast MMR protein, Mlh1p, is a member of the highly conserved MutL protein family necessary for the formation of the repair complex. To identify other proteins involved in MMR, a two-hybrid system was employed to screen a yeast expression and genomic library for proteins that interact with *S. cerevisiae* Mlh1p.

Three proteins interacting with Mlh1p in the two-hybrid screen were characterized: Mlh2p, a MutL homolog, M86p, a protein of unknown function, and Ntg2p, a DNA glycosylase/AP-lyase involved in base excision repair (BER). Analysis indicates that neither M86p, nor Mlh2p, is required for mutation avoidance, normal meiotic progression, or homeologous recombination. Loss of *NTG2* did not result in increased microsatellite instability at the frameshift allele *hom3-10*, effect meiosis or homeologous recombination. However, strains lacking Ntg2p, Ntg1p, an *NTG2* homolog, and Apn1p, an AP-endonuclease, showed a 20-fold increase in *hom3-10* reversion relative to wild type, implicating Ntg1p, Ntg2p, and Apn1p in preventing frameshifts in mononucleotide runs.

The human genome contains over 50,000 microsatellites, simple repeat sequences of mono-, di-, or tri-nucleotides. Microsatellites, although inherently mutable compared to simple sequence DNA, show increased instability in certain inherited and sporadic forms of cancer. Such microsatellite instability (MSI) is most notably associated with defects in MMR genes. As a second project, I characterized the role of multiple DNA

repair pathways in MSI induced by the DNA damaging agents, UV light and hydrogen peroxide, in the *hom3-10* reversion assay. In wild type cells, *hom3-10* reversion was increased in response to UV light and hydrogen peroxide, 88-fold and 100-fold, respectively. UV light induced *hom3-10* reversion was slightly decreased in a *rev3Δ* and *rev1Δ* strain, indicating that translesion synthesis is responsible for some induced frameshifts. UV mutagenesis was increased in a *rad52* mutant, suggesting that UV-induced frameshifts are repaired by *RAD52*-dependent recombinational repair. *hom3-10* reversion, in response to hydrogen peroxide, is increased in the BER mutant, *ntg1 ntg2 apn1* implicating these proteins in the repair of frameshifts associated with oxidative damage. In a *rad1* mutant, *hom3-10* reversion is decreased, implying that the presence of Rad1p is mutagenic in response to oxidative damage. These data suggest a role for TLS and recombinational repair in UV-induced mutagenesis, and a role for Rad1p and Ntg1p, Ntg2p, and Apn1p in oxidation-induced mutagenesis.

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Estimates suggest that mutations arising from replication errors of the human genome occur only every 10^{10} base pairs (Kunkel, 1992). However, proofreading-proficient DNA polymerase error rates are about 10^{-6} to 10^{-7} (Kunkel, 1992). In order to overcome the high error rate of DNA polymerase, organisms have developed a specialized system, referred to as DNA mismatch repair (MMR). Absence of MMR can result in an increased mutation rate in humans and mice leading to cancer.

Mutations in the mammalian MMR genes have been associated with hereditary nonpolyposis colon cancer (HNPCC) and sporadic colon and endometrial cancer (reviewed in Lynch, et al., 1997). HNPCC, also known as Lynch Syndrome, is characterized by tumors of the colon, endometrium, stomach, urinary tract and ovaries (Lynch, et al., 1997). Patients are typically heterozygous for a recessive mutation in one of the MMR genes, with somatic inactivation of the second allele leading to a mutator phenotype and tumorigenesis. Microsatellite instability (MSI), characterized by frameshifts of one repeat unit, is found in over 90% of HNPCC colorectal tumors (Lynch, et al., 1997).

The mutator phenotype suggests an explanation for why tumors can arise in MMR deficient cells. One hypothesis is that tumor progression occurs by generating a large number of random mutations and then selection occurs for clones that have malignant properties (Loeb, et al., 1974). Consistent with this idea, alterations in simple repeats have been found in genes involved in growth regulation (i.e. TGFß2, BAX, etc.) in the tumors from HNPCC patients (Lynch, et al., 1997).

Approximately 70% of HNPCC patients have mutations in MMR genes, with the majority of mutations occurring in *MSH2* or *MLH1* (Peltomaki and de la Chapelle, 1997). However, 30% of HNPCC patients do not have an apparent defect in the known MMR genes, leaving the possibility that other proteins are involved in MMR and the development of HNPCC-associated tumors. In addition, promoter methylation of *MLH1* has been found in a large proportion of sporadic gastrointestinal tumors with MSI (Veigl, et al., 1998).

Identification of additional proteins involved in MMR will allow us to determine how cells recognize and repair replication errors, possibly allowing for identification of candidate genes involved in HNPCC.

Bacterial MMR

Mutant strains of *E. coli* were isolated that displayed increased mutation frequencies. The original "mut" strain *(mutT)* was isolated in 1954 (Treffers, 1954). Other *mut* strains were identified that showed increased sensitivity to antibiotics and/or increased reversion of auxotrophic markers (reviewed in Cox, 1976). Isolation and characterization of *mut* strains led to the identification of the MutHLS-dependent, methyl-directed, DNA mismatch repair system. This pathway is involved in the repair of base-base mispairs and small insertion/deletion loops (IDLs) of up to four bases that can occur during replication due to misincorporation, replication slippage or as a normal part of homologous recombination (reviewed in Horst, et al., 1999, and Modrich and Lahue, 1996).

Transient hemi-methylation of the parental strand allows the repair machinery to identify the newly synthesized strand (Wagner and Meselson,

1976). As shown in Figure 1-1, the MutS homodimer binds to the DNA mismatch. A homodimer of MutL then couples MutS mismatch recognition to activation of the latent endonuclease MutH in an ATP-dependent manner (Grilley, et al., 1990). Activated MutH incises the undermethylated strand at the nearest GATC sequence. This is followed by UvrD helicase unwinding the DNA and exonucleolytic action by one of four exonucleases, RecJ, ExoI, ExoVII, or ExoX, in either a 5′ or 3′ direction, removing the mismatch and generating a gap (Viswanathan and Lovett, 1998). DNA polymerase III holoenzyme and DNA ligase repair the single strand gap (Lahue, et al., 1989).

Until recently, a biochemical activity for MutL had not been documented. Recent studies have shown that the MutL homodimer has ATP-binding and hydrolysis activities that are required for MMR (Aaltonen, et al., 1993; Aronshtam and Marinus, 1996; Ban and Yang, 1998b; Ban and Yang, 1998a; Ban, et al., 1999). In addition, MutL interacts with MutS, MutH, and UvrD, suggesting that it might have a role in promoting the formation of a more efficient repair complex (Sancar and Hearst, 1993).

In addition to a role in mutation avoidance associated with DNA replication, the MMR proteins are also involved in preventing homeologous recombination, a genetic exchange between similar, but non-identical, sequences. In *E. coli*, mutations in *mutH, L, S,* and *U* result in an increase in homeologous conjugational recombination between *E. coli* and *S. typhimurium* (Rayssiguier, et al., 1989).

The bacterial MMR proteins are also required for normal transcription-coupled repair (TCR) of ultraviolet (UV) damage *in vivo*. TCR is the selective removal of DNA damage from the transcribed strand of an active gene. Cyclobutane pyrimidine dimers (CPDs) resulting from UV light are repaired more efficiently from the transcribed strand. In MutS or MutL deficient *E.*

coli, the TCR of CPDs is abolished (Mellon, et al., 1996). However, all CPDs are eventually repaired, suggesting that MutS and MutL are necessary for directing repair to the transcribed strand, but are not absolutely required for the removal of CPDs (Mellon, et al., 1996).

Genetic and biochemical elucidation of the *E. coli* system of MMR has laid the foundation for understanding MMR in higher organisms. However, in eukaryotes MMR appears to be more complex, involving multiple MutS and MutL homologs that show both specialization and overlap of function. Interestingly, MutH homologs have not been identified in any other organisms besides *E. coli*. The lack of MutH homologs, coupled with the lack of DNA methylation in some organisms, including *S. cerevisiae*, suggests that other prokaryotes and eukaryotes have different strand discrimination mechanisms.

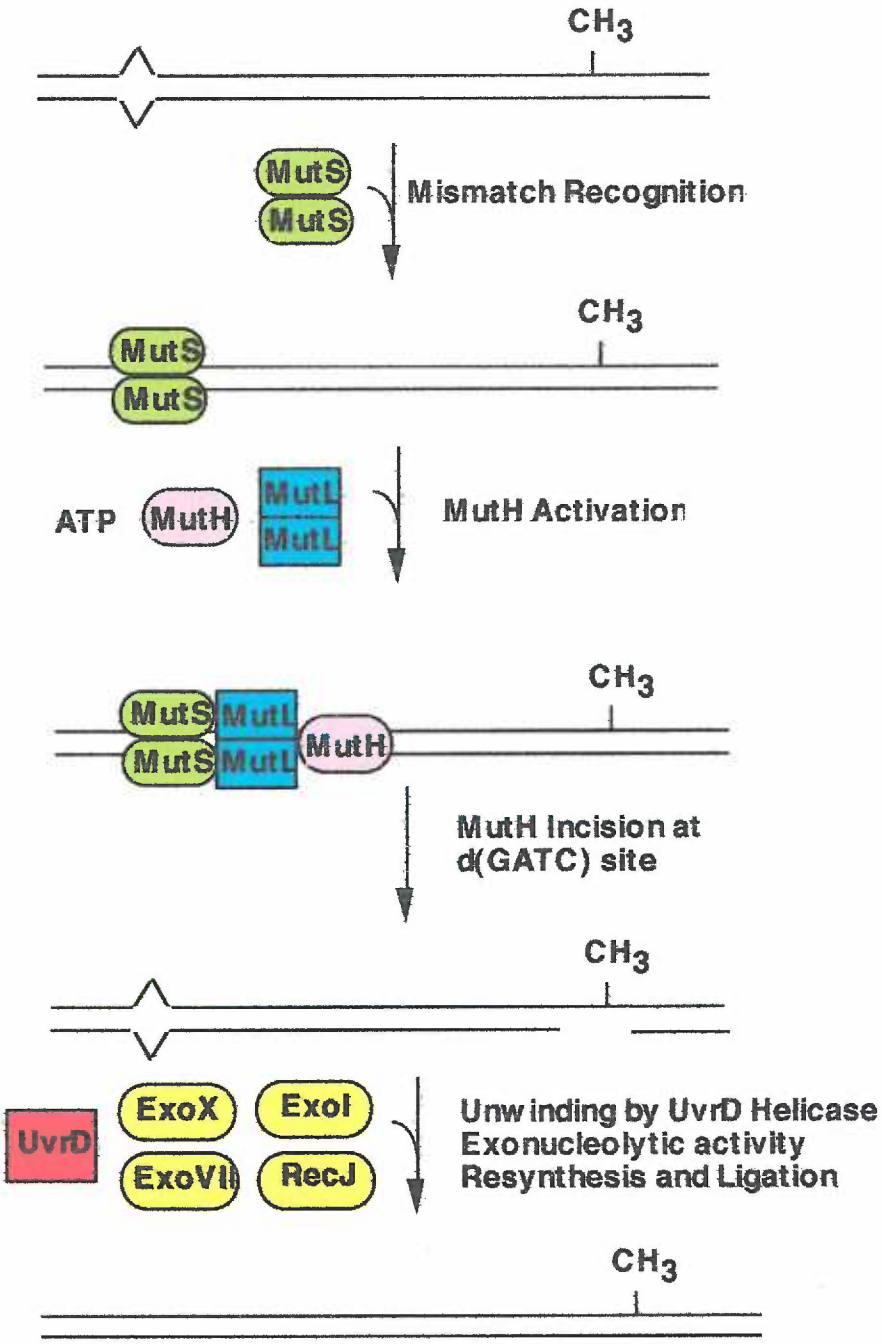


Figure 1-1. Bacterial MMR

MMR in S. cerevisiae

Analogous to the *E. coli* system, *S. cerevisiae* employs MutS and MutL homologs for MMR. Recognition and binding of the mismatch occurs by one of two different heterodimeric MutS-homolog (MSH) complexes, Msh2p-Msh6p (MutSα) or Msh2p-Msh3p (MutSβ) (Figure 1-2). MutSα has a stronger affinity for base/base mispairs, while MutSβ prefers IDLs of 2-4 basepairs (reviewed in Kolodner and Marsischky, 1999). MutSα has ATP binding and hydrolysis activities (Iaccarino, et al., 1998) that, based upon mutational analysis, are required for MMR *in vitro* (Iaccarino, et al., 1998) and *in vivo* (Alani, et al., 1997). The ATPase activity of MutSα is proposed to promote protein interactions and translocation along DNA (Blackwell, et al., 1998 and Gradia, et al., 1999).

MMR in yeast also requires two MutL complexes, MutLα, a heterodimer of Mlh1p (MutL homolog 1) and Pms1p (Post-meiotic segregation increased 1), or MutLβ, a heterodimer of Mlh1p and Mlh3p (Figure 1-3). MutLα is involved in processing the majority of DNA mismatches with MutSα and MutSβ, while MutLβ processes some IDLs, specifically in conjunction with MutSβ (Flores-Rozas and Kolodner, 1998 and Figure 1-3). All of the MutL homologs contain highly conserved ATP binding and hydrolysis motifs found in known ATPases, such as Hsp90, and GyrB (Bergerat, et al., 1997; Ban and Yang, 1998a; Ban and Yang, 1998b; Ban, et al., 1999). The necessity of ATP binding and/or hydrolysis by the MutL proteins is unclear, but studies from bacteria suggest that ATP binding is necessary for conformational changes in MutL that could facilitate interaction with other MMR proteins (Ban, et al., 1999). In yeast, the formation of the ternary

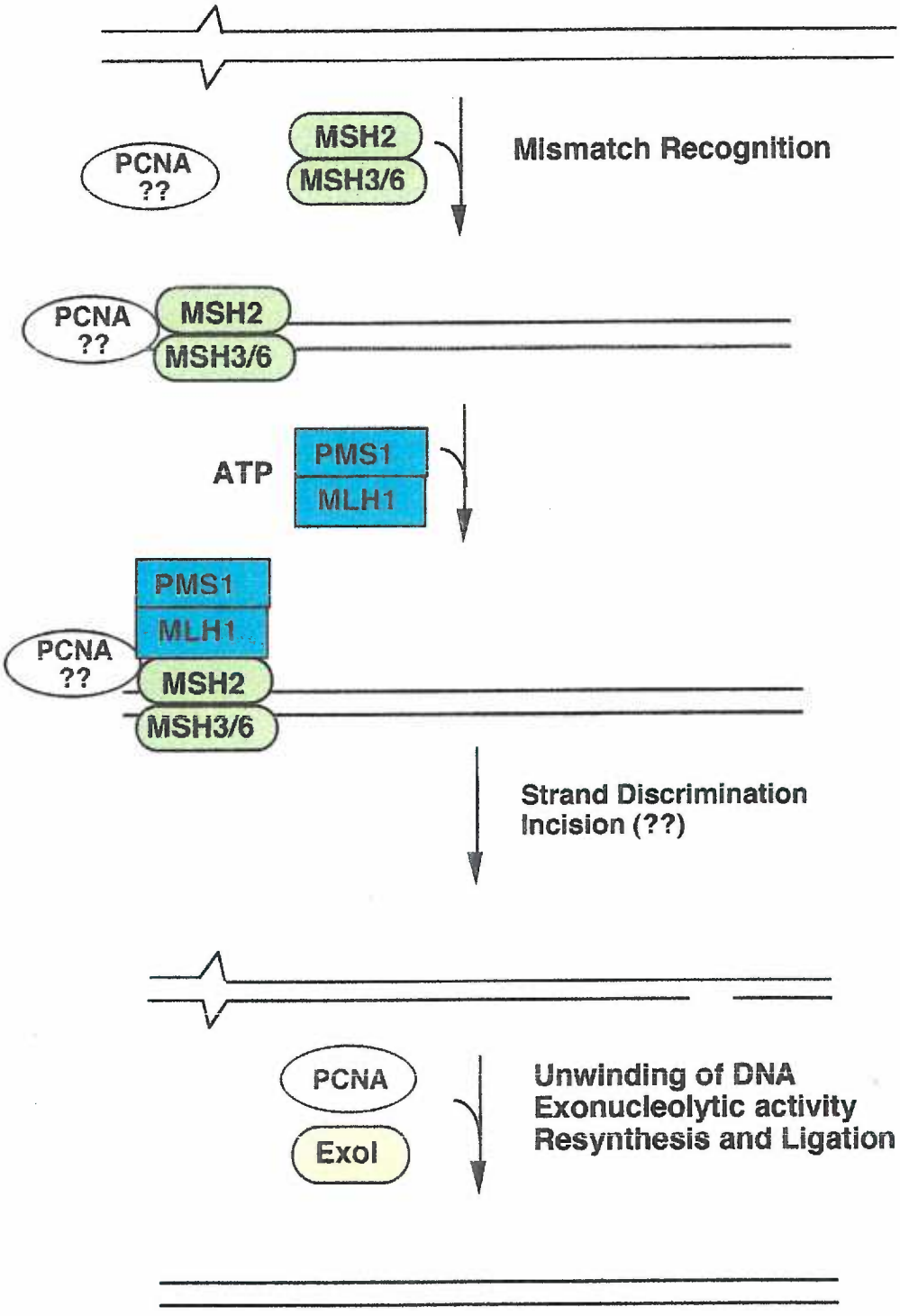


Figure 1-2. Schematic representation of yeast MMR

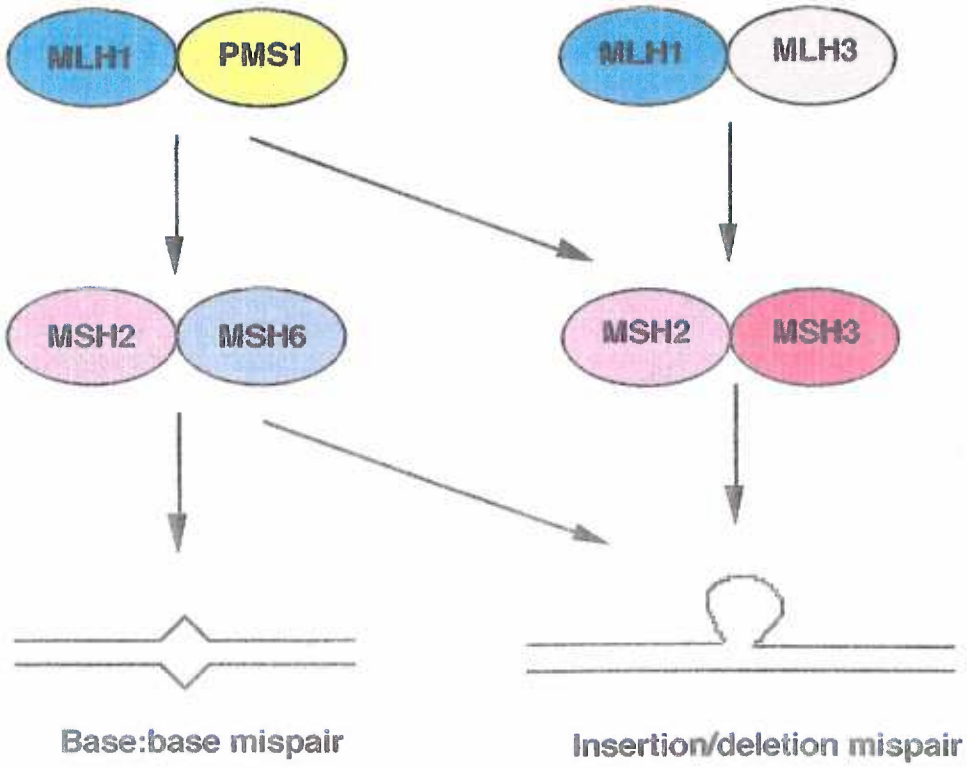


Figure 1-3. Substrate specificity of MutL proteins and MutS proteins. Adapted from Kolodner and Marsischky, 1999.

complex of Msh2p-Msh6p and Mlh1p-Pms1p requires ATP-binding (Habraken, et al., 1998). Additionally, studies from our laboratory have found that mutations in the putative ATPase motif of yeast MutLs lead to an increased mutation rate (P. Tran, unpublished data). Interestingly, a number of missense mutations in the ATPase domain of *MLH1* have been identified in HNPCC patients (Liu, et al., 1996, Viel, et al., 1998).

Other proteins necessary for MMR in yeast and/or mammals include proliferating cell nuclear antigen (PCNAp), Exonuclease 1 (Exo1p), and DNA polymerase ∂ (Pol∂p).

PCNAp, the processivity factor for DNA polymerases ∂ and ε, is essential for DNA replication and is involved in nucleotide excision repair (NER). In addition, PCNAp is involved in MMR at two steps: the initiation of DNA repair and resynthesis (Umar, et al., 1996 and Gu, et al., 1998). PCNAp interacts with both Mlh1p and Msh2p (Umar et al, 1996), possibly linking the MMR apparati to the replication machinery and targeting it to the newly synthesized strand of DNA.

The double-stranded 5′-3′ exonuclease, ExoIp, interacts with both Mlh1p and Msh2p (Tishkoff, et al., 1997 and Simon and Liskay, unpublished results). ExoIp is likely to be one of the exonucleases responsible for excision in the MMR reaction. The *exo1* mutant has a mutator phenotype that is significantly lower then the mutation rates seen in *msh2*, *mlh1*, or *pms1* deficient strains (Tishkoff, et al., 1997). Since there are four exonucleases required for MMR in bacteria (Viswanathan, 1998), it is likely that eukaryotes require multiple exonuclease for MMR, which would explain why *exo1* mutants do not show a strong mutator phenotype.

Other MutS homologs, whose functions are less clear, include *MSH1*, *MSH4*, and *MSH5*. In *S. cerevisiae,* Msh1p is involved in maintaining the mitochondrial genome (Reenan and Kolodner, 1992a and Chi and Kolodner, 1994). *MSH4* and *MSH5* do not appear to play any role in mutation

avoidance, but are necessary for normal levels of crossing over during meiosis (Ross-Macdonald and Roeder, 1994).

Another MutL homolog in yeast, *MLH2*, does not appear to have a role in mutation avoidance and will be discussed further in Chapter 4.

Phenotypes of MMR Deficiency in Saccharomyces cerevisiae

I. Mutation Rates in MMR mutants

Various metabolic markers and plasmids can be used to evaluate mutation rates and spectrum in yeast. Reversion of a mutation in a metabolic marker, such as *hom3-10* or *lys2BglII*, resulting in a functional protein, allows determination of the rate at which frameshift mutations occur. Other assays can report a wide spectrum of mutations through the loss of a gene product, such as Can1p, a protein required for arginine transportation into yeast cells. Yeast harboring defective Can1p are resistant to the toxic arginine analog, canavanine, and mutations can be determined by sequencing Can^r yeast DNA. Other ways to identify mutations in yeast include introducing a plasmid that can report a multitude of mutational events. Yeast strains deficient in MMR exhibit increased mutations rates in most of these assays. However, microsatellites, repeats of mono-, di-, or tri-, nucleotides are particularly unstable, presumably due to slippage by DNA polymerase during replication. If the associated-exonuclease activity of the polymerase does not repair the resulting frameshift, then it is usually repaired by MMR. In MMR deficient strains microsatellite instability is greatly increased, up to 10,000-fold in a mononucleotide run of fourteen adenines inserted into the LYS2 gene (Tran, et al., 1997). In addition, increases in transitions (purine to

purine or pyrimidine to pyrimidine) and transversions (purine to pyrimidine or pyrimidine to purine) are observed in MMR deficient strains (Kunz, et al., 1998).

Not only do MMR proteins play a role in avoiding mutations due to replication errors, but they also appear to have a role in protecting against spontaneous oxidative damage. One study found that *Δmsh2* strains grown in an anaerobic environment have a decrease in mutation rate up to 60-fold (Earley and Crouse, 1998). Another study found that a yeast strain deficient in both the MMR protein Msh6p and Ogg1p, a glycosylase involved in repairing oxidative damage, exhibits a synergistic increase in signature mutations of spontaneous oxidative damage (Kolodner, 1999). In addition, MutSα binds the oxidatively damaged base-pairs A:G^{O} and C:G^{O} *in vitro* (Kolodner, 1999).

II. Increased PMS

Increased post-meiotic segregation (PMS) is also observed in yeast strains deficient in MMR. MMR repair was first hypothesized to account for observations regarding fungal genetic recombination (Holliday, 1964). Some fungi contain an ascus of eight spores, each a product of meiosis. For a heterozygous marker A, the expected Mendelian segregation is 4A:4a. However, when non-reciprocal transfer of genetic material occurs, a 6A:2a or 2A:6a segregant pattern is observed. In addition, a rare pattern of segregants of 5:3 or 3:5 is observed, and is referred to as PMS. These non-Mendelian segregants are classified as aberrant segregations (Figure 1-4). In yeast, with the absence of MMR, spores contain heteroduplex DNA which result in sectored colonies.

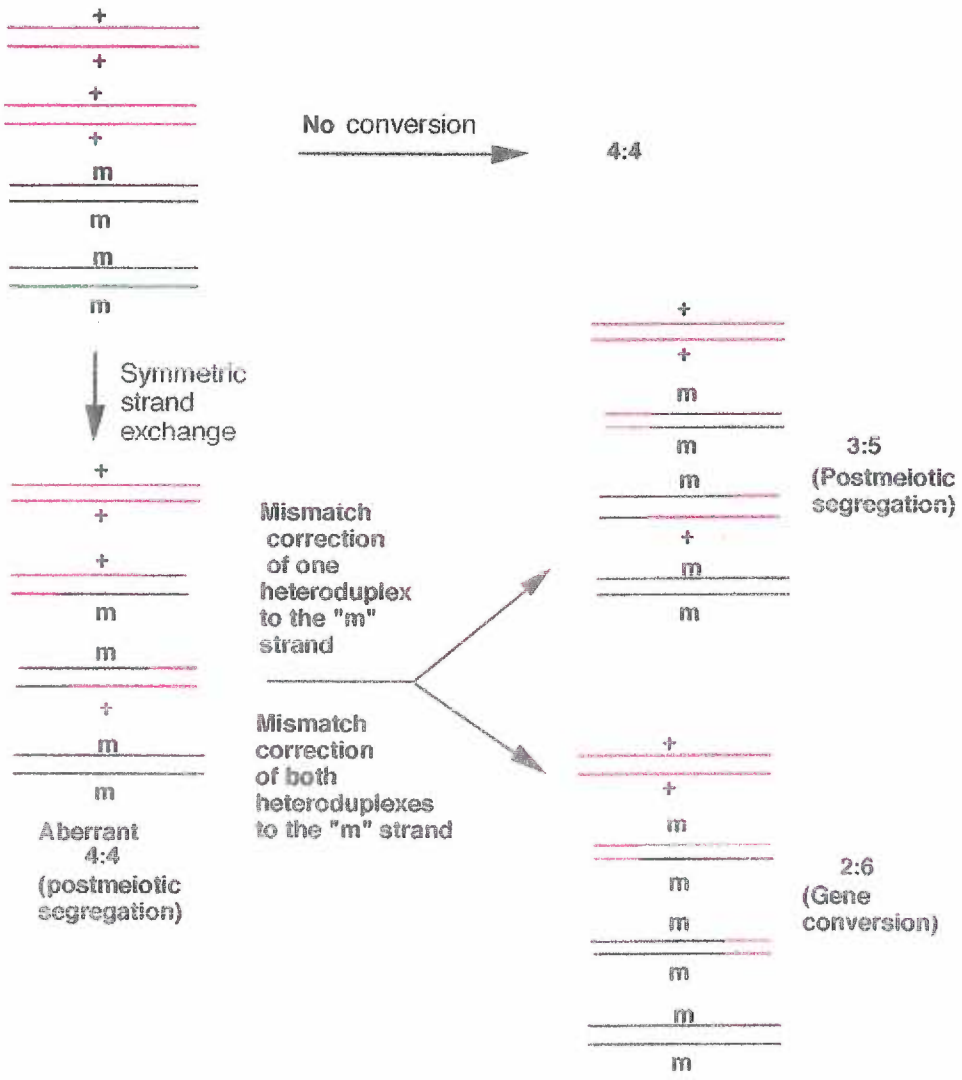


Figure 1-4. PMS.
Illustration of how MMR deficiency results in aberrant segregation, 3:5 or 5:3. Figure adapted from Friedberg et al, 1995 (Friedberg, 1995) and represents only symmetric strand exchange. Note that 5:3 and 6:2 segregants are not shown.

III. Spore Inviability

Decreased spore viability is seen in strains in which exchange between homologs in meiosis is decreased or abolished, resulting in improper segregation of homologs (Guthrie, 1991). In MMR deficient strains, including *pms1*, *mlh1*, and *msh2* there is decreased spore viability that is thought to result from the accumulation of recessive lethal mutations in diploids (Kramer, et al., 1989b; Reenan and Kolodner, 1992b). However, *mlh1* deficient strains have even lower spore viability then other MMR mutants due to decreased crossing-over (Hunter and Borts, 1997) implicating a unique role for Mlh1p in crossing-over.

IV. Recombination and MMR

Another phenotype associated with defects in MMR is increased mitotic homeologous recombination (Datta, et al., 1997 and Chapter 3, this work). Similar to bacteria, MMR in *S. cerevisiae* is involved in preventing recombination between slightly divergent sequences during mitotic recombination.

In addition, *MLH1* deficiency in yeast results in reduced crossing-over, high PMS, and increased non-Mendelian segregation, suggesting that Mlh1p plays a role during meiotic recombination in both crossing-over and gene conversion (Hunter and Borts, 1997). Supporting studies in Mlh1 -/- mice show that crossing over between homologs during meiosis is reduced 10- to 15-fold (Baker, et al., 1996 and Woods, et al., 1999).

V. Transcription coupled repair

In addition to roles in mutation avoidance and recombination, yeast and mammalian MMR proteins are involved in TCR (Leadon and Avrutskaya, 1998). Furthermore, MMR proteins are necessary for TCR of DNA damage resulting from oxidative and UV damage in bacteria and mammalian cells. Yeast lacking both Mlh1p and Pms1p are deficient in the repair of thymine glycols, a product of oxidative damage (Leadon and Avrutskaya, 1998), but, surprisingly, are proficient for TCR of UV damage (Sweder, et al., 1996).

VI. Increased sensitivity to anticancer drugs

In yeast, toxicity of the DNA cross-linking agents, cisplatin, carboplatin and doxorubicin (Durant, et al., 1999), is partially dependent on the presence of MMR proteins, suggesting that MMR recognizes DNA adducts. The increased resistance of MMR deficient cells is relevant to cancer since DNA cross-linking agents are commonly used for cancer treatment and tumors lacking functional MMR might not respond to treatment with these agents.

Mammalian MMR

MMR in mammals shares common features with the yeast system, but the ability to analyze patients and animal models allows one to study the direct relationship between MMR deficiency and cancer. As mentioned

earlier, MMR defects have been associated with HNPCC, including mutations in hMLH1, hPMS2 (yPMS1), hPMS1 (yMLH3), hMSH2, and hMSH6. Tumor DNA samples from HNPCC patients with germline MMR gene mutations (MLH1 and MSH2), exhibit global MSI and increased base substitutions (Parsons, et al., 1993). In mouse cell lines homozygous for MMR gene mutations, MSI is reduced by introduction of a chromosome or plasmid bearing a wild type copy of the MMR gene (Aquilina, et al., 1997; Umar, et al., 1997; Umar, et al., 1998; Buermeyer, et al., 1999a), indicating that MMR deficiency directly results in MSI. MMR-defective human tumor and mouse cell lines also exhibit tolerance to a number of DNA-damaging agents, including alkylating agents, 6-thioguanine, cisplatin, gamma irradiation, and topoisomerase inhibitors (reviewed in Buermeyer et al., 1999b), indicating a role for MMR in promoting cell death in the presence of extensive DNA damage. Mammalian MMR defective cell lines also show defective TCR of UV damage, similarly to *E. coli* MMR mutants (Leadon, 1997).

Mice homozygous for MMR gene mutations in Mlh1 and Msh2 develop HNPCC-like tumors and other tumors (reviewed in Buermeyer, 1999, in press). However, Pms2 $^{-/-}$ mice have a different spectrum of tumors than Mlh1 or Msh2 defective mice, and Pms1$^{-/-}$ mice remain tumor free (Prolla, et al., 1998). These different tumor spectra suggest that, in mice, loss of Mlh1 or Msh2 results in complete inactivation of MMR, while loss of Pms2 does not completely inactivate MMR, perhaps because Pms1 can partially fulfill the function of Pms2.

It is interesting that while MMR proteins are expressed in most tissues, only certain tissues are prone to tumors in patients harboring MMR defects. This could indicate that systems redundant with MMR act to repair defects in certain tissues. Another possibility is that DNA polymerases are less error-

prone in some tissues than others, resulting in an overall decreased mutation rate. Similarly, MMR activity might be enhanced in tissues that remain tumor-free.

Thesis Prospectus

As outlined above, loss of MMR proteins can result in tumorigenesis. Identification of other proteins that have a role in MMR may implicate candidate genes involved in HNPCC or other spontaneous cancers. One way to identify proteins that are involved in MMR is to search for proteins that interact with the known MMR proteins. Since the MutLs are involved in recruiting known proteins to the MMR repair complex, they are likely candidates for interacting with additional, yet unidentified, proteins.

In my research, I primarily used the yeast MutL homolog, Mlh1p, in two-hybrid screens to identify candidates involved in MMR. "Interacting" proteins were characterized by determining the region of Mlh1p they interact with and by analyzing strains deficient in candidate proteins for phenotypes consistent with MMR deficiency.

Since the two-hybrid assay can result in false positives I chose to characterize interactors that were obvious candidates in MMR, such as exonucleases, endonucleases, or helicases and proteins that had a unique interaction with Mlh1p. Proteins of interest obtained in the screens included the MutL homologs Pms1p, Mlh2p, Mlh3p, Pol30p, the yeast PCNA homolog, the Exo1p exonuclease, the DNA glycosylase/AP lyase Ntg2p, and M86p, a protein that requires the C-terminal of Mlh1p for interaction (Table 1-1). I focused my efforts on characterizing the interactions with Mlh2p, Ntg2p, and M86p. Mlh3p (Flores-Rozas and Kolodner, 1998), Exo1p (Tishkoff, et al.,

1997), and Pol30p (Umar, et al., 1996 and Gu, et al., 1998) involvement in MMR has previously been described.

In addition to studying the proteins that interact with Mlh1p, I examined the induction of MSI by DNA damaging agents in multiple DNA repair mutants to define the repair pathways that influence induced MSI.

GENE	FUNCTION
PMS1	MMR
NTG2	DNA glycosylase involved in BER
POL30	PCNA homolog, involved in MMR and replication
M86	Unknown
MLH2	MutL homolog
FUN20	Essential gene of unknown function
M15	Unknown
MLH3	MMR
M29	ATPase subunit homology/unknown
M76	Putative ATP-dependent RNA helicase
CHS1	Chitin Synthase
UBPD	Ubiquitin Carboxyl-terminal hydrolase 13
EXO1	MMR
SMT4	Suppressor of MIF2
CHC1	Clathrin heavy chain

Table 1-1. Proteins identified in two-hybrid screens using yeast Mlh1p bait.

CHAPTER 2: MATERIALS AND METHODS

Strains, Media, and Reagents

I. Bacteria

E. coli strain DH10B (Gibco) was used for all plasmid manipulations. *E. coli* strain MC1066 provided by Mike Forte was used to select for library plasmids, pGAD and pGAD-CAN, in the two-hybrid studies. *E. coli* strain MC1066 was grown on M9 plates (0.4% glucose, 0.02% tryptophan, 0.01% uracil, 1mM $MgSO_4$, 0.1mM $CaCl_2$, 100mg/ml ampicillin, 6% Na_2PO_4, 3% KH_2PO_4, 1% NH_4Cl, 0.5% $NaCl_2$, 0.003% $CaCl_2$), which allows for selection of bacteria carrying a plasmid that confers ampicillin resistance and leucine auxotrophy. DH10B cells were grown on LB or LB media containing 100mg/ml ampicillin.

II. *S. cerevisiae*

The *S. cerevisiae* strains used in this study are listed in Appendix A. Strain GCY35 was a gift from Gray Crouse. Michael Hampsey generously provided the YMH strains. Hannah Klein provided the two mating types of W303, 579-10A and 580-10D. The *hom3-10* allele was introduced into W303 using the two-step replacement plasmid pK8 provided by Richard Kolodner, and was verified by the inability of the strain to grow on complete synthetic media (CSM) lacking threonine. The yeast strains L40 and AMR70 (Vojtek, 1993), used for two-hybrid analysis, were provided by Mike Forte. SJR strains were generously provided by Sue Jinks-Robertson. Southern blot analysis and/or PCR confirmed the *Δntg2*, *Δntg1*, *Δmlh2*, and *Δrev3* strains. The *Δmsh2* and *Δmlh1* strains were verified by mutator phenotype. The *Δrad1*

strains were determined by sensitivity to ultraviolet light. The *Δapn1* strains were verified by sensitivity to MMS.

Yeast were grown nonselectively on YPD (1% Bacto-yeast extract, 2% Bactopeptone, 2% glycerol, 2% agar). Auxotrophic yeast were selected on CSM media lacking an amino acid or base (0.7% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, 2% agar, and 0.09% dropout mix (Bio101) lacking the amino acid used for selection). Canavanine plates used for mutational analysis contained CSM lacking arginine and supplemented with 30mg/L canavanine (Sigma). Revertants of the YMH strains were selected on YPDG medium (Hampsey, 1991), containing 1% yeast extract, 2% peptone, 3% glycerol, 0.1% glucose and 2% agar. To select for loss of *URA3*, cells were grown on CSM containing 5-fluoroorotic acid (5-FOA, US Biologicals) as described (Boeke, et al., 1987). Mitochondrially impaired strains were identified by inability to grow on YP Glycerol media (2% yeast extract, 2% peptone, 3% glycerol). Diploids were sporulated on media containing 0.2% CSM, 0.1% yeast extract, 2% potassium acetate. For two-hybrid analysis, cells were selected on appropriate drop out plates supplemented with succinic acid (0.12% yeast nitrogen base, 0.5% ammonium sulfate, 1% succinic acid, 0.6% sodium hydroxide) (Hollenberg, 1994). Yeast assayed for homeologous recombination were grown in YPD supplemented with 2% glycerol and 4% galactose (YPDGG) and plated on CSM with 2% glycerol and 4% galactose, but deficient in histidine (SGG-his). All yeast cultures were grown at 30°C.

Plasmids

All DNA manipulations were carried out using standard protocols (Ausubel, et al., 1992). Plasmid pBTM-yMLH1 and deletion derivatives have been described previously (Pang, et al., 1997).

The *MO37* (a.k.a. *NTG2*) targeting vector, *Mo37-hUh*, was constructed by PCR amplification of surrounding sequence 5' and 3' to the *NTG2* open reading frame (ORF) and the two arms were cloned into plasmid RDK3353 containing hisG direct repeats separated by *URA3* (Alani, et al., 1987) (Figure 2-1). The 5' arm, amplified using the primers Mo37 lt-forward, 5'-AGACGCCCGCGGCCTGATGACGATATAAAG-3' (SacII site underlined) and, Mo37 lt-reverse, 5'-AGACGCTCTAGACCTACTTTCCTCTCTCAT-3' (XbaI site underlined), was inserted into the SacII/XbaI site of RDK3353 . The 3' arm, amplified using primers, Mo37 rt-forward, 5'-AGACGCGGATCCGCATTGGTTGGACACGGT-3' (BamHI site underlined) and Mo37 rt-reverse, 5'-AGACGCGAATTCACTCCAACCCTAAAAGGGC-3' (EcoRI site underlined), was inserted into the BamHI/EcoRI site of RDK3353. The complete *MO37* targeting vector, *MO37-hUh*, was digested with EcoRI/SacI prior to transformation. The *YAB5 (NTG1)* targeting vector, *YAB5-hUh*, was also cloned into RDK3353 using a similar strategy to the cloning of *MO37-hUh* (Figure 2-4). The 5' arm, amplified using Yab5-lt forward, 5'-AGACGCCCGCGGTAGAGACAGATCTCACGACAGC-3' (SacII site underlined) and Yab5 lt-reverse, 5'-AGACGCTCTAGAGCATATTTTTTTTTTTTTGGG-3' (XbaI site underlined), was cloned into the SacII/XbaI site of RDK3353. The 3' arm, amplified using primers Yab5-rt forward, 5'-AGACGCGAATTCAAGAAACTATGGTCAAACTGG-3' (EcoRI site underlined) and Yab5-rt reverse, 5'-AGACGCGTCGACAAGATCTTACCTGCTGTGCAG-3' (SalI site underlined), was cloned into the EcoRI/SalI site of RDK3353. The complete targeting vector, *YAB5-hUh*, was digested with KpnI/SacI prior to transformation.

The *RAD1* targeting vector used to construct the *rad1Δ* strains was kindly provided by Lee Hartwell. The *mlh2::LEU2* targeting plasmid was constructed by Tom Prolla (Prolla, 1994). Plasmids *mlh1ΔURA3* ,*mlh1ΔLEU2* , and *mlh2::LEU2* constructed by Tom Prolla were used to create the *mlh1* and *mlh2* deletion strains (Prolla, et al., 1994). The *apn1Δ* strains were constructed using the hisG-*URA3*-hisG plasmid pSCP108, provided by Bruce Demple.

The *msh2hUh* vector, a gift from Gray Crouse, was used to create the *msh2Δ* strains.

M86-hUh, constructed by Jeff Simon, was used to create *Δm86* strains. *M86-LEU2-TV* was constructed by removing URA3 from *M86-hUh* with NheI, filling in the ends, and inserting the 2.2kb HpaI fragment containing the *LEU2* selectable marker from YEp13. Prior to transformation, the *M86-LEU2-TV* was digested with XbaI/XhoI.

Δrev3 and *Δrev1* strains were constructed using the Rev3 and Rev1 hisG-URA3-hisG targeting vectors, pYPG101and pSF3, respectively, provided by David Hinkle.

M86-pJAS was constructed by linearizing pJAS with EcoRI and ligating to an EcoRI fragment containing the complete *M86* open reading frame (ORF).

Two-Hybrid Screen and Analysis

I. Library Screen

The two-hybrid screen used in these studies is a modified version of the system devised by Stan Fields (Fields and Song, 1989 and Figure 2-3). Interaction between the LexAp DNA binding and Gal4p activation domains drives *GAL1* and *HIS3* promoters fused to multiple lexA binding sites, resulting in expression of lacZ and His3p, respectively. Therefore, protein interaction can be detected in cells by growth on media lacking histidine and β-galactosidase activity. Yeast *MLH1* was fused to the DNA binding domain of *LEXA* on a tryptophan containing plasmid (yMLH1-pBTM). *S. cerevisiae* strain L40, containing yMlh1-pBTM, was transformed using a modified lithium acetate transformation (Vojtek, 1993) with an efficiency of 10^3 to 10^4 transformants per microgram using either an *S. cerevisiae* cDNA library fused to GAL4 activation domain on a plasmid with the leucine marker, provided by S. Elledge, or a randomly sheared genomic library cloned into pGAD.CAN provided by S. Fields. Potential interactors were detected by growth on media lacking tryptophan and leucine, selecting for both the library and bait plasmids; leucine, lysine and uracil, selecting for the appropriate strains; and histidine, which identifies interacting proteins. The β-galactosidase assay described below was used as a second test for interaction.

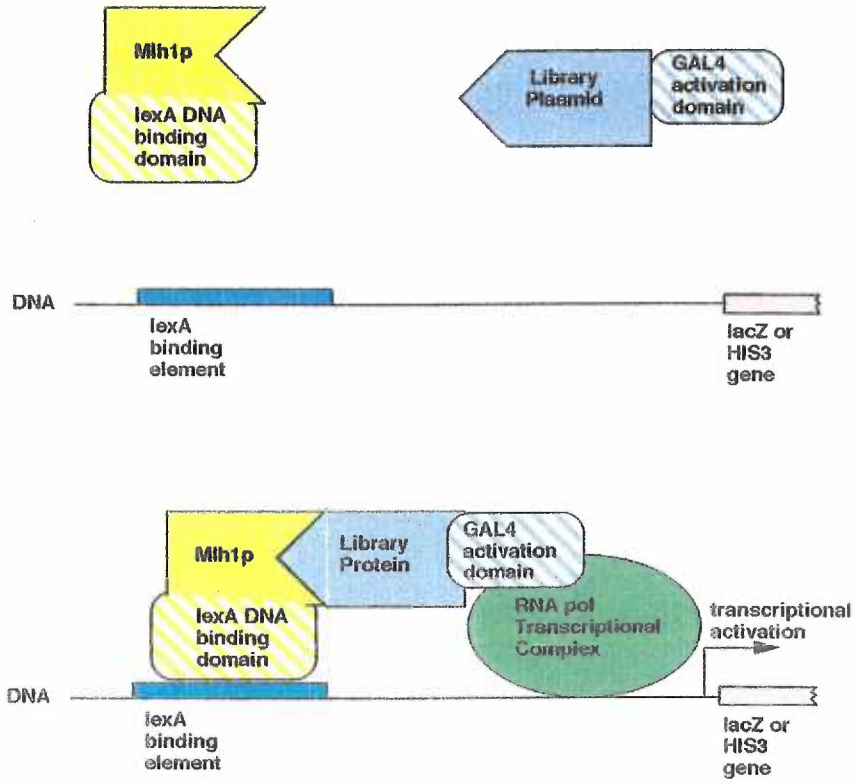


Figure 2-3. Two-hybrid scheme

II. β-galactosidase assay: Color filter method and β-galactosidase activity

Ura-Leu-Lys (-THULL For the color filter method, transformant colonies, surviving on –Trp-His-) or mated diploids, were lifted onto #1 Whatman filter circles, frozen in liquid nitrogen, and incubated at 30°C in 0.7 ml Z -buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7.0) with 0.75mg X-gal (Miller, 1972). To quantify β-galactosidase activity,

cells from minus THULL plates were suspended in 0.5 ml Z-buffer with 0.64mg o-nitrophenyl-β-D-galactosidase substrate per ml, permeablilized with chloroform, and incubated at 30°C. Reactions were stopped with Na_2CO_3. B-galactosidase activity was calculated using the equation Units/h=[(OD_{420}/OD_{600})•60]/min (Miller, 1972).

III. Mating Analysis

The "library" plasmid recovery was facilitated by first isolating DNA from L40 strains using a yeast DNA miniprep and subsequently recovered by transformation and selection of amp^S, leu^- MC1066 bacteria. Plasmid DNA was isolated from amp^r, leu^+ MC1066 by alkaline lysis and transformed into the *S. cerevisiae* strain AMR70 using the Frozen-EZ Yeast Transformation Kit (Zymo Research). Transformants were mated to L40 strains on YPD, and replica plated to -URA-TRP-LEU plus succinic acid plates to test for mating efficiency and -THULL plates to test for interaction.

IV. DNA Sequencing

Inserts of library members showing positive interaction were sequenced using an Automated Sequencer (Vollum Institute) with the primers GAL4F-2942 5'-CGTTTGGAATCACTACAGGG-3' and GAL4R-2673 5'CGGGGTTTTTCAGTATCTACG-3'. DNA sequences were compared to the Genbank and Saccharomyces Genome (SGD) databases.

Genotyping Strains

To construct the *Δntg2* yeast strains, *Mo37-hUh* was digested with EcoRI/SacI, and transformants were selected on CSM lacking uracil. Genomic DNA was isolated from transformants using a yeast genomic prep (Ausubel, et al., 1992) and analyzed for *NTG2* disruption by PCR or Southern

analysis (Southern, 1975) (Figure 2-1 and 2-2). DNA was digested with HindIII/XbaI, loaded onto a 1% TAE agarose gel, and transferred to Hybond+ nylon membrane. A probe, generated by amplifying genomic DNA 5' to the targeting arm using the primers Mo37 probe-for 5'-AAGCTGCGAGACAACACC-3' and Mo37 probe-rev 5'-GTAGATACGGACGACGGAAC-3', was purified using a Qiaquick column (Qiagen), labeled using the Random Prime Labeling Kit (Clonetech), and exposed to the membrane resulting in an untargeted band at 8.9 kb and a targeted band at 5.8 kb (Figure 2-2). For PCR genotyping, DNA isolated from *URA*$^+$ transformants was amplified using the primers Mo37 untargeted 5'-GGGAAATTCTCTCGTTACCTGG-3', hisG 5'-ACCATGAGCTTCAATACCCTG-3', and Mo37 anchor 5'-ATGTATTGGGCGCTTTGC-3' (95°C 1', 56°C 1', 72°C 2', 40 cycles) resulting in a 1 kb untargeted band or a 650 bp targeted band (Figure 2-2). To generate *Δntg1* strains, *Yab5-hUh* was digested with KpnI/SacI, transformed into yeast using the LiAc protocol, selected on CSM lacking uracil, and confirmed by Southern analysis and/or PCR similar to the *Δntg2* strains (Figure 2-4). For Southern analysis, DNA was digested with SphI/BamHI, and probed with a PCR product generated from amplifying genomic DNA 5' to the targeting arm with primers Yab5 probe for 5'-AAAATTCAATCTGGCACGGC-3' and Yab5 probe rev 5'-TGCTCCTGAGTTGAACCCG-3', resulting in a untargeted fragment of 11.2 kb or a 7.9 kb targeted band (Figure 2-5). For PCR genotyping of the *Δntg1* strains, Yab5 untargeted 5'-CAGGTCCTTCTTGGGGTG-3', hisG (above), and Yab5 anchor 5'-GTGCCCGTGGTATCGTTAG-3' (95°C 1', 56°C 1', 72°C 2', 40 cycles) were used to generate a 1 kb untargeted fragment strains or a 500 bp targeted fragment (Figure 2-5).

Δrev3 strains were genotyped by Southern using a probe generated with primers yREV3.997F 5--TAGAGATATAAAAAAGG-3' and yREV3-253R 5'-GAATAGTACAAACACAG-3' resulting in a 5.2 kb untargeted band or a 4.4 kb targeted band.

MLH2 targeting was detected by Southern by digesting genomic DNA with EcoRI and probing with a 300 bp fragment generated by PCR of genomic DNA with MLH2-3' and MLH2 rev, resulting in a 9.1 kb untargeted band or a 5.7 kb targeted band (Figure 2-6).

M86 targeting was determined by Southern and PCR (Figure 2-7). For detecting targeting by Southern, genomic DNA was digested with SmaI and probed with M86 probe, created by amplifying genomic DNA with M86 probe for 5'-CAACCCGGTGCCTGGGAG-3' and M86 probe rev 5'-CATGCTTTCTCTTGCGGGGC-3' (45s@95°C, 45s@55°C, 45s@72°C, 30 cycles, PFU polymerase) resulting in a 6.7 kb untargeted band or a 5 kb targeted band. PCR was used to detect targeting using the primers M86 reverse, M86 forward, and hisG 5'-ACCATGAGCTTCAATACCCTG-3' (1'@ 95°C, 1'@55°C, 2'@72°C, 40 cycles) resulting in a 800 bp untargeted fragment or a 600 bp targeted fragment.

In Southerns used to detect *ntg1Δ*, *m86Δ*, and *mlh2Δ*, distinct bands at unexpected sizes were identified (Figures 2-2, 2-5, and 2-6). The identity of these bands is unknown, but could be genomic DNA recognized by the probe in other places in the genome. Search of the Saccharomyces Genome Database (SGD) with the probe sequences reveals that similar DNA sequences are indeed located in other portions of the yeast genome.

Mutation Analysis

For mutational analysis using *hom3-10* reversion or *CAN1* forward mutation, strains were grown to saturation in 10 ml YPD. Approximately $2X10^8$ or $2X10^5$ cells, for wild type and mutator strains, respectively, were incubated for 3 days on CSM plates lacking threonine or supplemented with canavanine and total colonies were counted. The number of total cells in the culture was determined by plating serial dilutions of the saturated culture on CSM. For each experiment, at least 8 independent cultures were tested.

To determine the nature of the *hom3-10* revertants genomic DNA was prepared from the revertants (Ausubel, et al., 1992), and used for PCR of the *hom3-10* region using primers HOM3 FOR 5'- CTTTCCTGGTTCAAGCATTG-3' and HOM3 REV 5'-GGATCAGCAGTAAATATACC-3'. PCR reactions were purified using Qiaquick columns (Qiagen) and sequenced using primer HOM3-seq.

In the *CYC1* assay strains were grown in 100 ml YPD shaking for 48 hours. To determine the number of revertants the culture was pelleted and the cells were resuspended in the remaining media and plated on five 100mm X 150mm YPDG plates. The YPDG plates were incubated for 7 days at 30°C. For each experiment, at least 4 independent cultures were tested.

Mutation rates were determined using method of the mean (Lea and Coulson, 1948) by the program Chipmunk Basic Rate (Nicholson Jr., 1997).

Survival Curves and Mutation Induction

To assay induced mutation and survival of cells treated with hydrogen peroxide or menadione 10ml YPD cultures were grown to an OD_{600} between 0.6-1.0 10 ml and were treated with varying concentrations of hydrogen peroxide (3% stock diluted to 0.3% in distilled water) or menadione (SIGMA) shaking in the appropriate medium for 1 hour at 30°C. Hydrogen peroxide treatment was performed in YPD, while cells treated with menadione were harvested and washed before exposure and resuspended in 100mM potassium P_i buffer, pH 7.4. After treatment, cells were washed, and dilutions were plated to determine cell survival. To determine the number of induced mutants, cells were incubated on selection plates. Mutation frequencies are expressed per survivor.

To assay UV induced mutagenesis and survival yeast cells were grown to an OD_{600} of approximately 0.6. Cells were pelleted and resuspended in TE. Varying volumes were plated on CSM or CSM lacking threonine and treated with UV light delivering 1 J/m^2 /second for the appropriate time (0-80 seconds). Plates were placed in a light proof box to inhibit photoreversal and incubated for 2 days.

Tetrad Analysis

Strains of opposite mating types (579-10Dα X 580-10Aa) were "co-patched" onto YPD plates and incubated for 2 days. Cells were streaked onto YPD plates to isolate individual colonies. Individual colonies were patched onto YPD plates, replica plated to YPD plates with mating testers BY26 and BY27,

grown overnight, and replica plated to complete drop-out media. Diploid strains are able to grow on complete drop-out media. Yeast unable to mate with either tester were considered as diploid and used for tetrad analysis. A diploid colony was patched onto a sporulation plate and incubated at room temperature until >80% of the cells had asci formation (4-7 days). Cells were suspended in 0.3 ml distilled water containing a 0.01 dilution of glusulase (NEN), incubated for 5 minutes at room temperature, and placed on ice. The asci were dissected using a Micro Video Instruments, Inc. tetrad dissection system with individual spores plated on YPD and subsequently incubated for 3-4 days. The product of at least 10 tetrads was analyzed for each strain.

Petite Formation (Mitochondrial Deficiency)

Approximately 100 colonies grown on YPD were replica plated to YP glycerol plates and YPD to determine fraction of petite cells.

Complementation studies

M86-pJAS, or the empty vector pJAS, was transformed into MW3317-21A, and the *Δmlh1* and *mlh1F766A* MW3317-21A derivatives constructed by Qishen Pang, using the LiAc transformation protocol. Transformants were selected on CSM-trp and then patched to CSM-trp and grown for 2 days. Patches were then replica plated to CSM-trp-thr to detect *hom3-10* reversion.

Homeologous Recombination

Homeologous recombination assays were performed as described (Datta, et al., 1997 and Chapter 3) on at least 8 independent colonies in SJR486 and SJR381 strain backgrounds. Two-day old colonies were used to innoculate YPDGG medium followed by incubation at 100 RPM for 2 days at 30°C. Cells were harvested, washed with water, and resuspended in 1 ml water. 100 µl aliquots were plated on SGG-his plates. Total viable cells were determined by plating serial dilutions on SGG plates. Colonies formed after 3-4 days at 30°C were counted and the number of total recombinants/total cells for each independent culture was processed by the Chipmunk Basic Rate program (Nicholson Jr., 1997) to determine the recombination rate using the method of the mean (Lea and Coulson, 1948).

Mutator Assay of Plasmid Bearing Strains

Wild type or *Δntg1Δntg2Δapn1* strains were treated with plasmid Mlh1-pJAS (Pang, et al., 1997) using standard lithium acetate protocol, and transformants selected on CSM lacking tryptophan. Eight transformants were grown overnight in liquid culture of CSM-trp. Total cells were determined by plating serial dilutions on CSM. *hom3-10* revertants were assayed by plating on CSM-thr. Mutation rates were calculated as previously described.

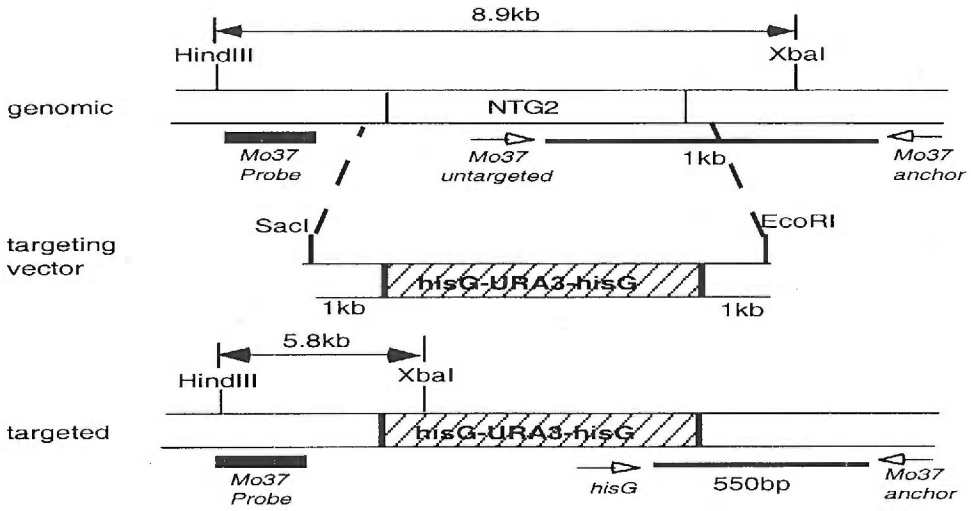


Figure 2-1. Ntg2 targeting scheme and detection.
Filled in arrows represent fragments detected by Southern. Open arrows are primers used for PCR targeting detection. Solid line represents probe used in Southern.

A. B.

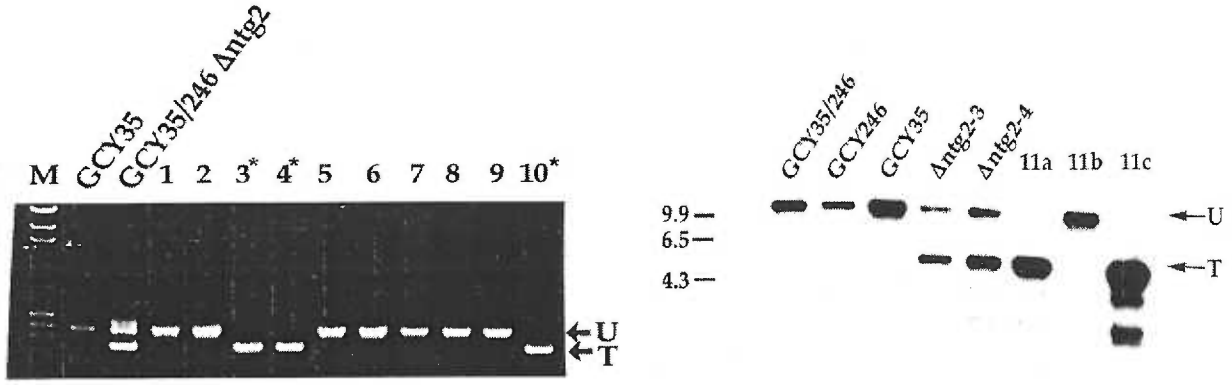


Figure 2-2. Southern and PCR genotyping of Δntg2 strains.
(A) PCR genotyping: M: marker, GCY35 parent, GCY35/246 diploid heterozygous for *Δntg2*, lanes 1-10 represent URA$^+$ transformants, * indicates targeted transformants. (B) Southern detection: GCY35 and GCY246 are parents, *Δntg2-3* and *Δntg2-4* are diploids heterozygous for *ntg2* mutation. 11a, 11b and 11c represent meiotic haploid products of *Δntg2-3*. U=untargeted, T=targeted.

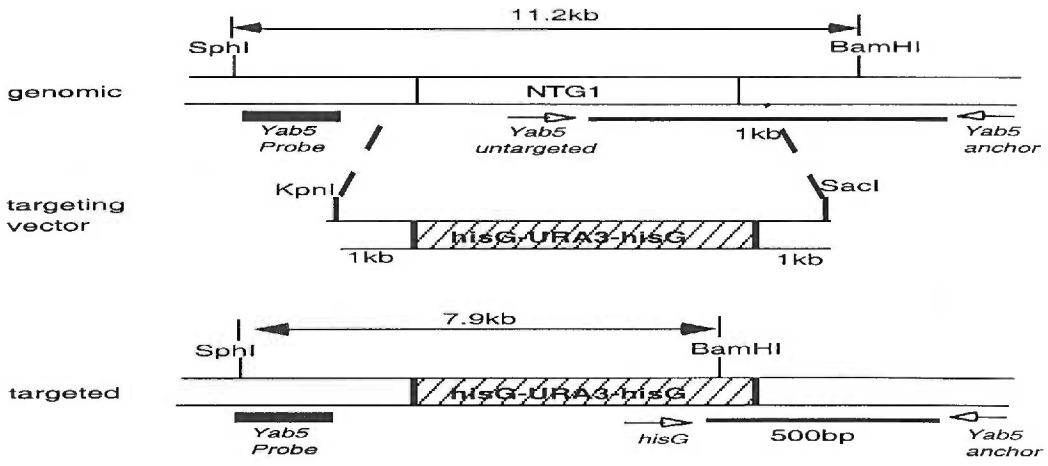


Figure 2-4. Ntg1 targeting scheme and detection.
Filled in arrows represent fragments detected by Southern. Open arrows are primers used for PCR targeting detection. Solid line represents probe used in Southern.

A. B.

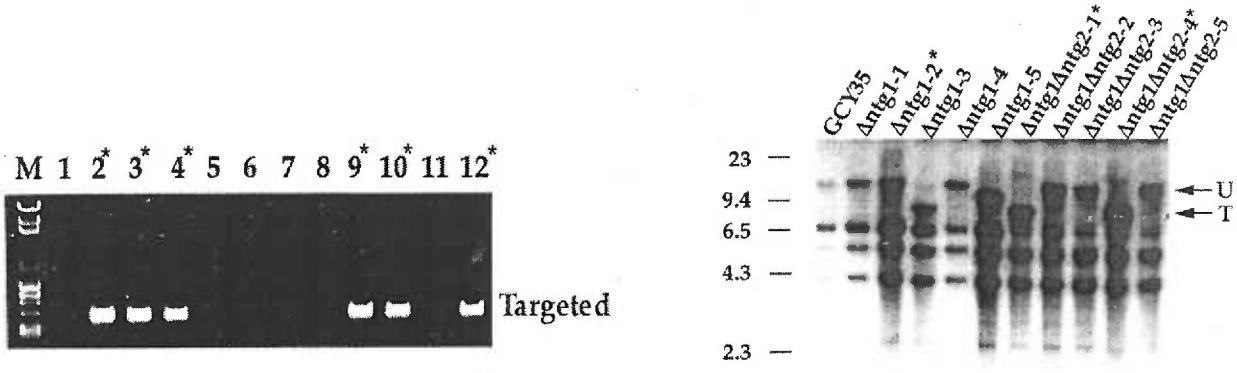


Figure 2-5. Southern and PCR genotyping of Δntg1 *strains.*
(A) PCR using targeted primers. M: marker, Lane1: GCY35 parent, Lanes 2-10 *URA*$^+$ transformants. * indicates targeted transformants. (B) Southern detection. GCY35: parent, *Δntg1-1* to *-5 URA*$^+$ transformants, *Δntg1Δntg2-1* to *-5* are *URA*$^+$ transformants in GCY35*Δntg2* background. * indicates targeted transformants. U=untargeted, T=targeted.

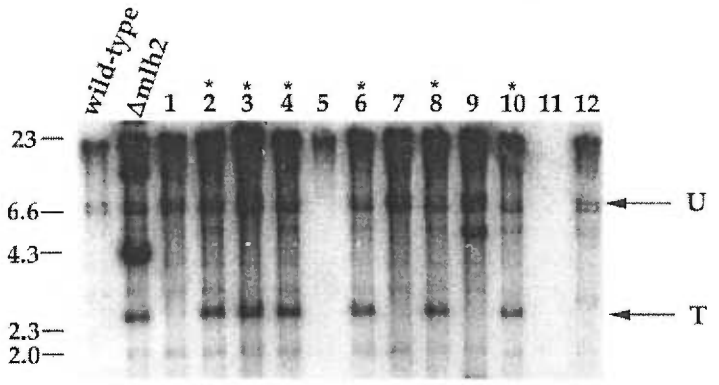


Figure 2-6. Southern genotyping of Δmlh2 strains.
Wild type strain is parent SJR486. *Δmlh2* is a known *mlh2* mutant in SK1 background, 1-12 represent *LEU*$^+$ transformants. Untargeted band is the top band in a doublet. * indicates targeted transformant. U=untargeted, T=targeted.

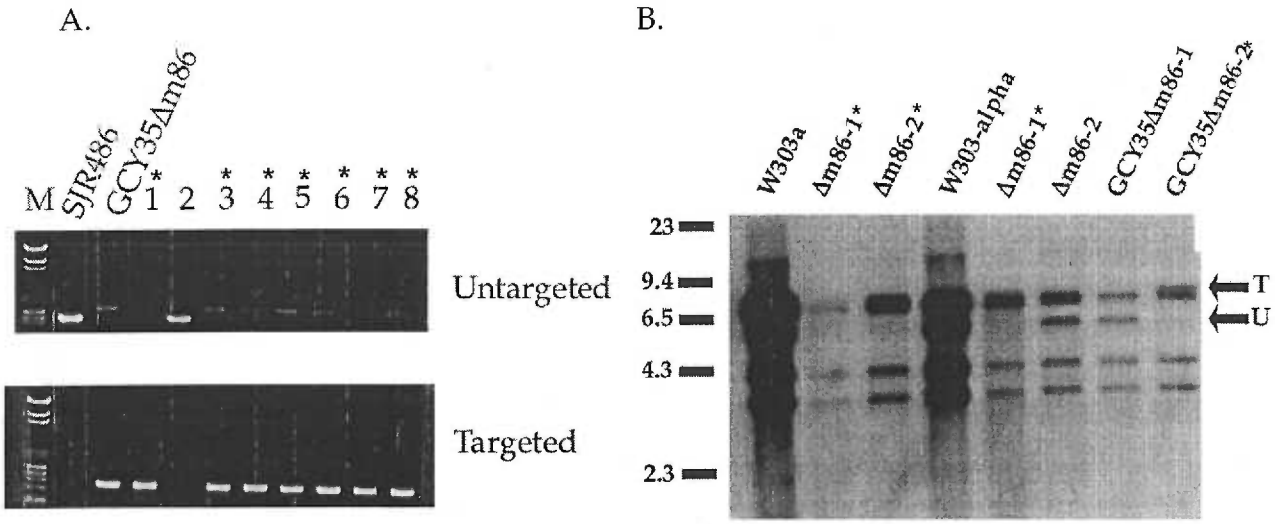


Figure 2-7. Southern and PCR genotyping of Δm86 *strains.*
(A) PCR genotyping in SJR486, parent, GCY35*Δm86*, known mutant, SJR486 *URA*$^+$ transformants (Lanes 1-8). (B) Southern detection, W303a parent, *Δm86-1* and -2 *URA*$^+$ transformants, W303α parent, *Δm86-1* and -2 *URA*$^+$ transformants, and *GCY35Δm86-1* and -2 *URA*$^+$ transformants. Targeted band is top part of doublet indicated by T. T=targeted, U=untargeted.
* indicates targeted transformants. Light band in untargeted PCR appears to be an artifact specific to targeted *Δm86* transformants.

CHAPTER 3: CHARACTERIZATION OF M86p

Mlh1 Structure

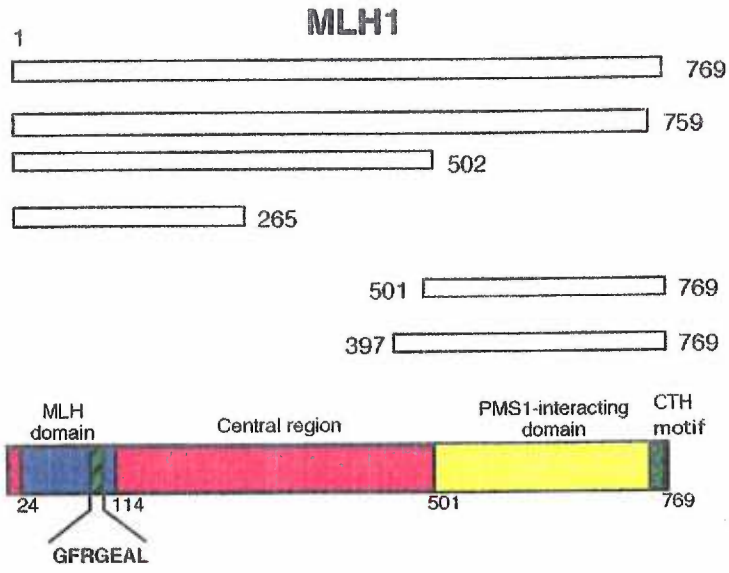
Genetic and biochemical analysis have implicated three regions of Mlh1p necessary for mutation avoidance: The MLH domain, the Pms1-interactive domain, and the CTH motif (Pang, et al., 1997) (Figure 3-1). The MLH domain contains the highly conserved ATPase motif, and most mutations in these residues result in a mutator phenotype, but do not affect interaction with Pms1p (Pang, et al., 1997 and P. Tran, personal communication). Disruption of the region of Mlh1p that interacts with Pms1p also leads to an increased mutation rate (Pang, et al., 1997). Mutations in the C-terminal homology (CTH) motif, which is absolutely conserved between yeast, mice and humans, results in an increased mutation rate, but does not interfere with the Mlh1p/Pms1p interaction. Interestingly, overexpression of the CTH motif results in a slight mutator phenotype, possibly by titrating out proteins, other than Pms1p, that are necessary for mutation avoidance (Pang, et al., 1997).

Proteins obtained in the two-hybrid library screens were tested for interaction with various fragments of Mlh1p to determine the domain(s) of Mlh1p that were required for interaction (Figure 3-1).

M86

M86p is a protein that interacted with Mlh1p in a screen of both the cDNA and randomly sheared genomic libraries. Full length M86p interacts with full-length Mlh1p and the C-terminus of Mlh1p (501-769) (Figure 3-1).

Interaction was slightly decreased between M86p and Mlh1p harboring mutations in the putative ATPase domain (G98A and R97A, not shown). There was no interaction between M86p and the Mlh1p-Pms1p complex (PMS1:CoMLH1), suggesting that the Mlh1p-Pms1p heterodimer does not interact with M86p, and that M86p interacts with regions of Mlh1p that also interact with Pms1p. Interestingly, the fragment of Mlh1p that is lacking the C-terminal homology (CTH) domain (1-759) does not interact with M86p (Figure 3-1). M86p was the only Mlh1p-interacting protein identified that requires the CTH domain of Mlh1p for interaction. The CTH domain of Mlh1p is required for mutation avoidance, but not for interaction with Pms1p (Pang, et al., 1997) implicating a potential role for M86p in MMR. M86p does not interact with the other MMR proteins, Pms1p, Mshp, Mlh2p, Msh3p, or Msh6p.



	β-Galactosidase activity	
	PMS1	M86
1–769	1.87	11.8
1–759	1.94	<0.02
1–502	<0.02	<0.02
1–265	<0.02	<0.02
501–769	7.21	12.4
397–769	0.57	<0.02

Figure 3-1. Mlh1p and M86p Interactions in Two-Hybrid Assay. Interaction in the two-hybrid system was determined by measuring β-galactosidase activity as described in material and methods. Pms1-Mlh1 values were determined previously by Q. Pang in our laboratory. *MLH1* figure adapted from Pang, et al., 1997.

M86

M86p (Accession #YNL218W) shares 32% identity with *E. coli* ycaj, a hypothetical protein of unknown function that is highly conserved in *C. burnetti, H. influenza, S. pombe*, mouse, and humans. M86 motifs include a P-loop nucleotide binding motif and the SCR (strictly conserved region) motif conserved among polymerase accessory proteins (Figure 3-2). In *S. cerevisiae* M86 shares identity with Replication factor C, 40kDa (RFC3, #YNL0533) (28% in 234 residues in the N-terminus, see Figure 3-2) and some similarity with RFC2, with the highest similarity in the ATP/GTP binding site motif of RFC2 and RFC3.

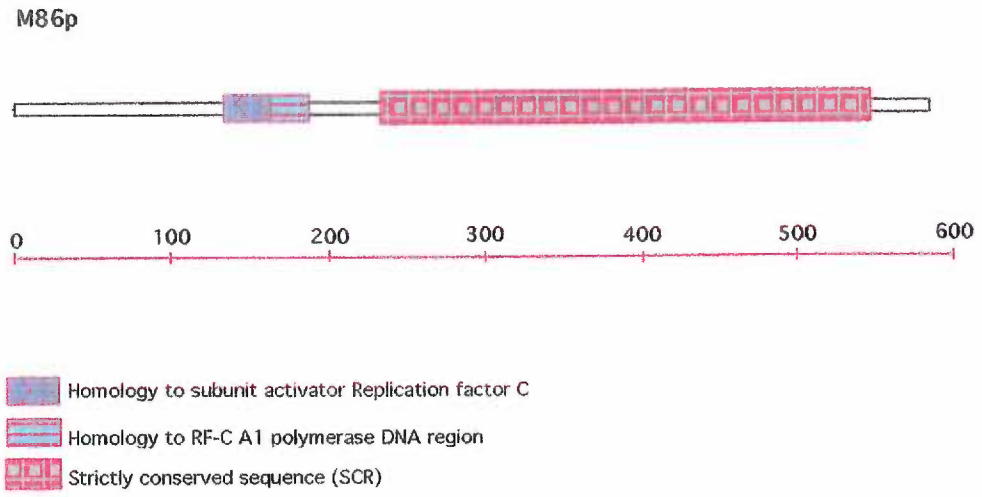


Figure 3-2. M86 Homology.
M86 homology to other proteins as predicted by ProDom (http://protein.toulouse.inra.fr/prodom/prodom.html). Unfilled boxes represent areas that do not demonstrate significant homology to proteins in the database.

Analysis of Δm86 Strains

Strains deficient in M86p were analyzed for MMR phenotypes including mutator phenotype, low spore viability, and increased homeologous recombination.

***M86* disruption does not result in a mutator phenotype.**

First, *Δm86* strains were assayed for microsatellite instability at either *hom3-10*, which measures -1 frameshifts in a mononucleotide run of 7As, and in the dinucleotide repeat $(CA)_{25}$, a plasmid based system that also reports -2 frameshifts (Petes, et al., 1997). The mutation rate in the *Δm86* strain was the same as wild type in both the *hom3-10* and the $(CA)_{25}$ reporter assays (Table 3-1), suggesting that M86p is not essential for mutation avoidance. In addition, canavanine forward mutation, which measures both frameshifts and base changes, was the same in both *M86* null and wild type strains.

Strain	*hom3-10*	$(CA)_{25}$	CAN^r
wild type	$1.2X10^{-8}$ (1)	$2.1X10^{-5}$ (1)	$8.3X10^{-8}$ (1)
Δm86	$4.8X10^{-7}$ (0.4)	$1.7X10^{-5}$ (0.8)	$1.1X10^{-7}$ (1.3)

Table 3-1. Mutation rates in *Δm86* strains.
Mutation rates in *m86* null strains were determined by fluctuation test as described in Chapter 2 in at least 8 independent transformants. Fold effects compared to wild type are in parenthesis.

Spore viability is not affected by M86 deficiency

Strains that are deficient in certain MMR proteins show effects on spore viability for several reasons: First, strains deficient in Mlh1p show decreased levels of crossing over, leading to spore inviability (Hunter and Borts, 1997). Second, two MutS homologs, *MSH4* and *MSH5*, have no role in mutation avoidance, but are required in meiotic processes. The absence of *MSH4* and *MSH5* results in a failure to segregate chromosomes during the first meiotic division resulting in decreased spore viability (Ross-Macdonald and Roeder, 1994; Hollingsworth, et al., 1995; de Vries, et al., 1999; Edelmann, et al., 1999). Third, MMR deficient diploids are hypothesized to accumulate recessive lethal mutations that can result in spore inviability (Kramer, et al., 1989a; Prolla, et al., 1994). M86p deficient strains have 100% spore viability, indicating that M86p deficient strains do not show the meiotic characteristics of MMR deficiency (Figure 3-3).

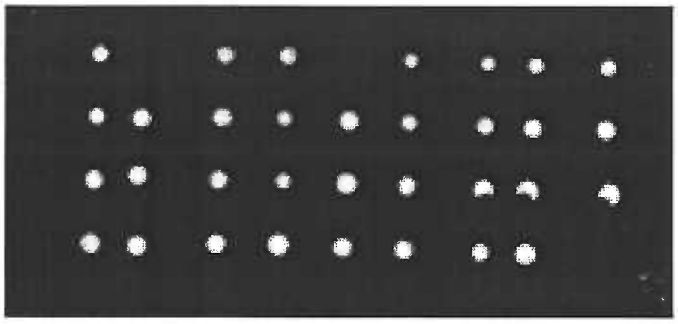


Figure 3-3. M86 tetrad analysis.
Each column represents a single meiotic event, resulting in four spores. Columns with missing spores were due to technical problems and not inviability.

Homologous and homeologous recombination

Certain MMR proteins are involved in regulating recombination between similar, but non-identical substrates, referred to as homeologous recombination. In a yeast assay that measures mitotic recombination between diverged sequences (Figure 3-4), recombination between 91% identical substrates is reduced 41-fold in wild type strains relative to perfectly matched substrates (Datta, et al., 1996). However, in *Δpms1* and *Δmsh2* strains, recombination of the 91% identical substrate is reduced only 4-fold and 1.4-fold, respectively (Datta, et al., 1996) relative to the 100% identical substrate showing that Msh2p and Pms1p normally help to suppress recombination between divergent sequences. I have shown that *mlh1* deficient strains show increased homeologous recombination rates similar to *Δpms1* (see Chapter 4).

A mutation in the C-terminus of *MLH1* results in increased homeologous recombination in an ectopic recombination system (Francis Fabre, personal communication). Since M86p requires this portion of Mlh1p for interaction, I reasoned that M86 might have a role in regulating homeologous or homologous recombination. I found a 7-fold increase in homologous recombination (100% identical substrate) of the *Δm86* strain compared to wild type (Table 3-2). However, there is no increase in homeologous recombination using the 91% identical substrate in the M86p deficient strain.

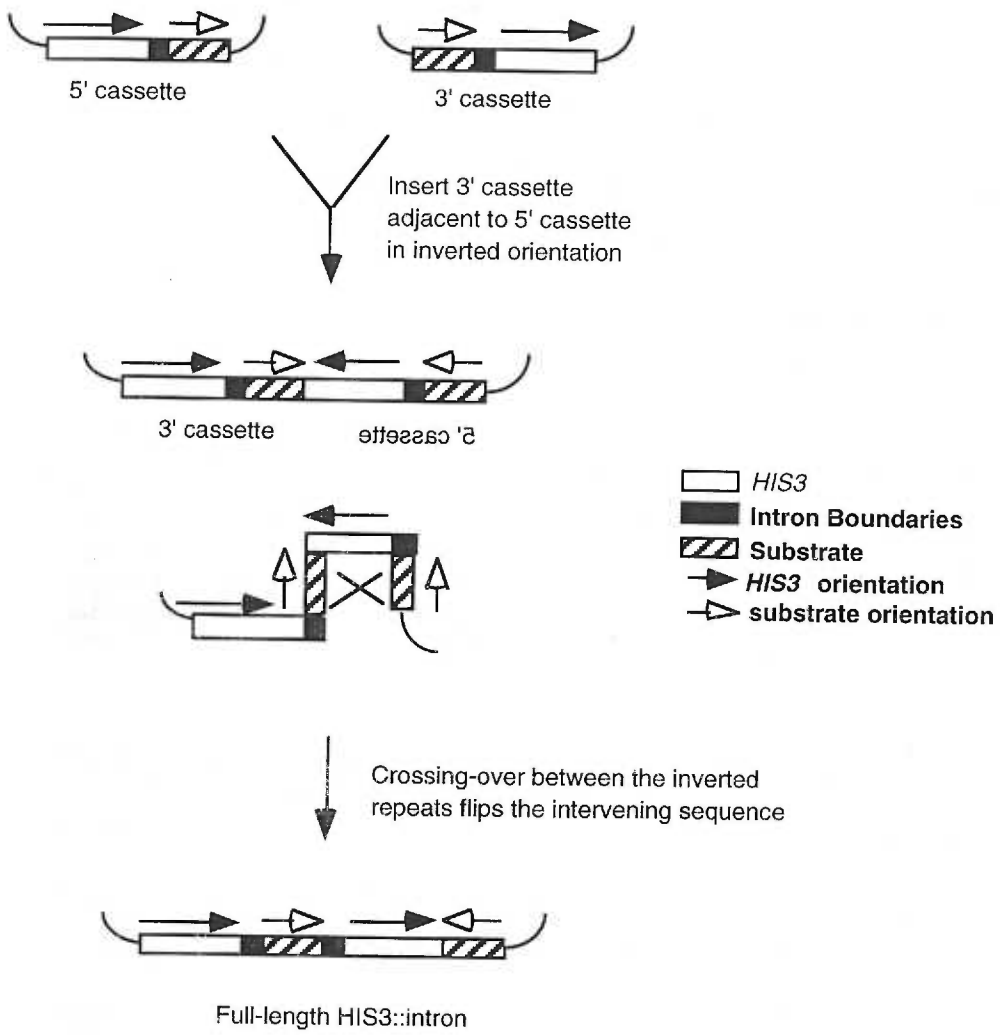


Figure 3-4. Mitotic Intrachromosomal Homeologous Recombination System. A 5′ cassette contains 5′ sequence of the *HIS3* selectable marker, and a 3′ cassette contains 3′ sequence of *HIS3*. Recombination between the substrates, followed by splicing out the substrate between the introns, results in full-length *HIS3*. Figure adapted from Datta et al, 1996.

Strain	% homology	Recombination Rate
wild type	100%	2.4×10^{-6} (1)
Δm86	100%	2.3×10^{-5} (9.6)
wild type	91%	2.2×10^{-8} (1)
Δm86	91%	4.4×10^{-8} (2)

Table 3-2. Recombination rates in *Δm86* strains.
Recombination rates were determined from a minimum of 17 individual colonies using the recombination assay described in materials and methods. 100% and 91% homology was performed in SJR381 and SJR486 backgrounds, respectively. Numbers in parenthesis are fold differences compared to wild type.

As a collaboration, Francis Fabre and Eric Coîc analyzed the *m86* mutant using an allelic and ectopic recombination system that measures both homologous and homeologous recombination. In their system, they found a 3-fold increase in both allelic and ectopic homologous recombination (Table 3-3 and Table 3-4), but no effect on homeologous recombination. Therefore, defects in *M86* results in general mitotic hyper-recombination, but not in elevated mitotic homeologous recombination.

UV dose (J/m^2)	0	10	20	30
$ARG^+/10^5$ survivors[a]				
homo WT	0	18 (1)	35 (1)	71 (1)
homeo WT	0	3 (1)	6 (1)	6 (1)
homo *Δm86*	0	51 (2.8)	119 (3.4)	233 (3.3)
homeo *Δm86*	0	6 (2)	8 (1.3)	15 (2.5)
$ARG^+/10^6$ survivors[b]				
homo WT	0	27 (1)	38 (1)	86 (1)
homeo WT	0	1 (1)	2 (1)	2 (1)
homo *Δm86*	0	56 (2.1)	130 (3.4)	208 (2.4)
homeo *Δm86*	0	1 (1)	3 (1.5)	6 (3)

Table 3-2. Allelic and ectopic UV-induced recombination.
(a) Allelic arg4RV/arg4Bg homologous and homeologous UV-induced recombination in diploid. (b) Ectopic arg4RV/arg4Bg homologous and homeologous UV-induced recombination in haploids (III). Numbers in parenthesis represent fold differences relative to wild type. Data provided by Eric Coîc and Francis Fabre.

	$ARG^+/10^7$ cell divisions
Homo WT	9 (1)
Homeo WT	2.8 (1)
Homo *Δm86*	34 (3.8)
Homeo *Δm86*	5.8 (2.1)

Table 3-3. Allelic spontaneous homologous and homeologous spontaneous recombination in diploids.
Numbers in parenthesis represent folds relative to wild type. Data provided by Eric Coîc and Francis Fabre.

M86p overexpression

Deletion of the CTH domain of Mlh1p results in a mutator phenotype and decreases the ability of Mlh1p and M86p to interact in the two-hybrid system. I hypothesized that if M86p has a role in mutation avoidance, overexpressing M86p might abolish the mutator effect caused by deletion of the CTH domain by increasing M86p/Mlh1p complexes. However, transforming a plasmid containing the wild-type M86p protein into a strain with the CTH deletion did not suppress the mutator phenotype. One caveat is that overexpression of M86p was not confirmed.

Discussion

M86 is highly conserved throughout phylogeny suggesting a fundamentally important biological function. In yeast, however, M86p does not appear to be required for mutation avoidance or homeologous

recombination. Other functionally redundant proteins would obscure my ability to detect a MMR phenotype. However, analysis of the Saccharomyces genome database (SGD) and Genbank alignments with M86p does not provide any obvious candidates for structural homologs.

M86p shares some identity with replication factor C (RFCp) proteins. *In vitro* studies of the replication of simian virus 40 (SV40) have shown that replication protein A (RPAp) unwinds the origin (reviewed in Bambara, et al., 1997, and Figure 3-5). RPAp initiates the leading strand with an RNA primer,

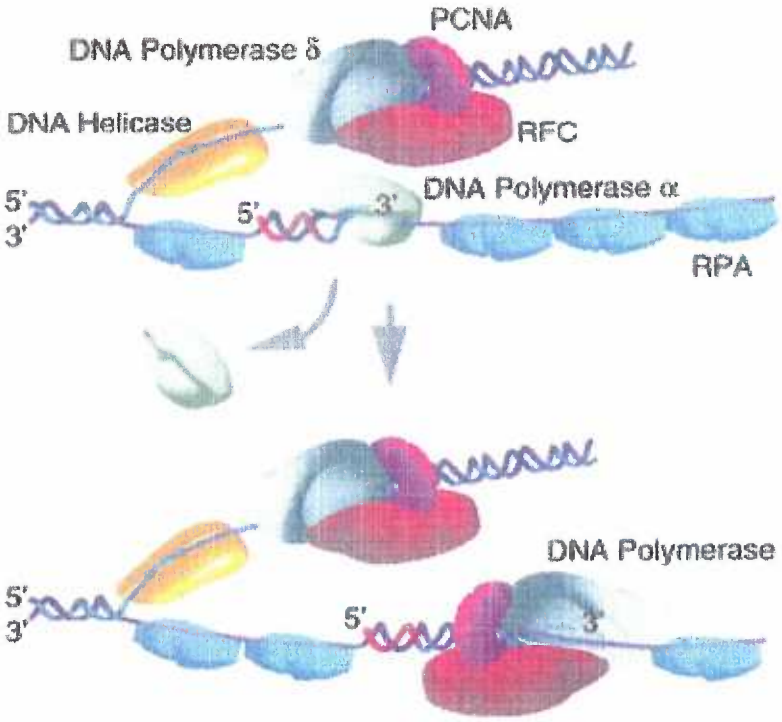


Figure 3-5. Replication of the lagging strand
Figure from Bambara, et al., 1997

generated by the primase subunit of RPAp. DNA pol ∂ adds a stretch of deoxyribonucleotides to the RNA primer. In an ATP-dependent process RFCp dissociates DNA pol ∂ and assembles PCNAp in the region of the primer terminus. The current model is that RFCp transiently opens the PCNAp clamp and allows PCNAp to reclose, recircling the double helix adjacent to the primer (Bambara, et al., 1997). Pol ∂ interacts with PCNAp, which functions as a sliding clamp holding pol ∂ on the primer terminus, resulting in high processivity.

Our lab previously demonstrated that the yeast homolog of PCNAp, Pol30p, interacts with Mlh1p (Umar, et al., 1996). One model is that M86p might associate with a Pol30p/Mlh1p complex. Conceivably, a multimer composed of many proteins, including Pol30p, Mlh1p, and M86p, might bring the binding domain and activation domain of M86p in close enough proximity to activate the reporter genes in the two-hybrid assay. Loss of M86p in the multimer would not compromise MMR activity. In this model, the CTH domain of Mlh1p would be required for interaction with the multimer, since M86p requires the CTH domain of Mlh1p for interaction. However, interaction between Mlh1p and Pol30p in the two-hybrid system is not affected by loss of the CTH domain (Simon and Liskay, unpublished results). Therefore, by this model, interaction between Mlh1p CTH domain would have to occur with some other component of the multimer.

On the other hand, similar to RFCp, M86p might be involved in loading PCNA during repair that is not associated with replication. In the absence of M86p, RFCp might be able to fulfill the loading function, and therefore loss of M86p would not decrease the overall efficiency of repair.

Loss of M86p does result in an increase in mitotic homologous recombination. Hyper-recombination phenotypes have been observed in a

number of mutants including DNA topoisomerases I and II (Christman, et al., 1988), *XRS2*, a DNA repair gene (Ivanov, et al., 1992), and *SGS1*, a homolog of Bloom's and Werner's syndrome genes (Watt, et al., 1996). While all of these proteins are involved in DNA transactions, they are involved at different steps. Therefore, assignment of the normal function of M86p simply based on the hyper-recombination phenotype is difficult.

Mlh1p is necessary for both mutation avoidance and crossing-over (Borts, 1998). Therefore, the interaction between Mlh1p and M86p might reflect a complex that is necessary for crossing-over. Interestingly, while *m86* mutants show a hyper-recombination phenotype, diploids deficient for M86p do not show decreased spore viability, indicating that M86p is not necessary for crossing-over during meiosis. Since Mlh1p has been implicated in both homeologous recombination and crossing-over, it is plausible that it is also involved in other types of recombination, such as ectopic recombination, that is dependent on M86p.

Conclusions

M86p, a member of the SRC family, interacted with Mlh1p in the two-hybrid system. Interestingly, the interaction between M86p and Mlh1p was dependent on the presence of the CTH domain of Mlh1p. The CTH domain of Mlh1p is necessary for mutation avoidance, but is not required for interaction with Pms1p. In addition, overexpression of the CTH domain alone results in a mutator phenotype, suggesting that the CTH domain of Mlh1p interacts with other components necessary for mutation avoidance. I hypothesized that M86p might be required for mutation avoidance. However, M86p deficiency does not result in an increased mutation

frequency at *hom3-10* , *CAN1*, or in dinucleotide repeats. In addition, spore viability and homeologous recombination are normal in the *m86* mutant. Taken together, these results suggest that M86p is not required for normal MMR functions. However, I cannot rule out a role in MMR, since there is a possibility that *S. cerevisiae* possesses a protein that is functionally redundant with M86p. Loss of M86p does result in an increase in mitotic homologous recombination, suggesting M86p plays a normal role in some form of DNA transaction, such as mitotic crossing-over.

CHAPTER 4: CHARACTERIZATION OF *Mlh2p*

Introduction

Mlh2p (accession # YLR035C) is a MutL homolog that was originally identified in the Liskay lab using "degenerate PCR" (Prolla, 1994). Mlh2p shares 26% homology with Mlh1p, 24% homology with Pms1p, and 26% homology with Mlh3p (Figure 4-1), with most of the homology located in the "MMR motifs", and the Mlh1p interaction regions of Pms1p.

Analysis of *mlh2* deficient strains suggest that it is not involved in MMR (Prolla, 1994). Previous studies from our laboratory found that *Δmlh2* strains do not have increased mutations at *CAN1*, *hom3-10*, or in dinucleotide repeats (Prolla, 1994). In addition, loss of *MLH2* did not affect spore viability, suggesting that Mlh2p is not required for processes essential for meiosis, e.g. crossing over (Prolla, 1994).

A recent paper reports that inactivation of *MLH2* leads to increased resistance to the DNA cross-linking drugs cisplatin, carboplatin and doxorubicin (Durant, et al., 1999). Inactivation of other MMR genes, including MLH1, MSH2, MSH3, and MSH6, caused similar drug resistance (Durant, et al, 1999) suggesting a direct role of yeast MMR proteins in potentiating sensitivity to anticancer drugs.

Mlh1p/Mlh2p Interactions

Mlh2p was identified in a two-hybrid genomic library using Mlh1p as a bait. Previous results from two-hybrid screen of the randomly sheared

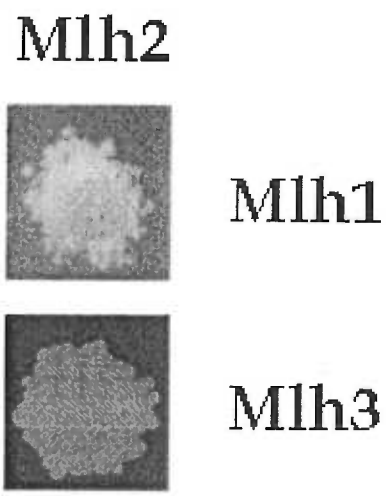
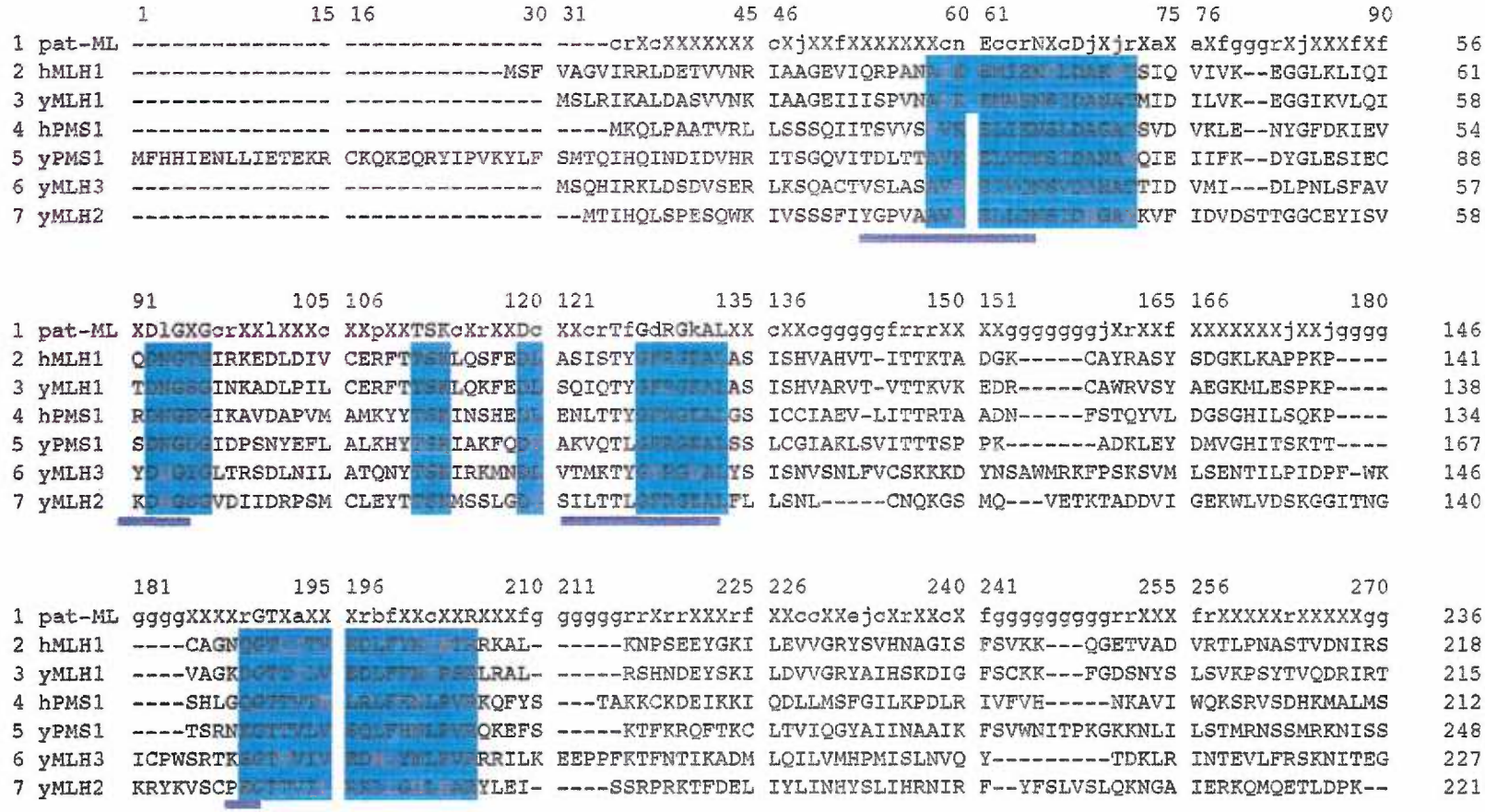
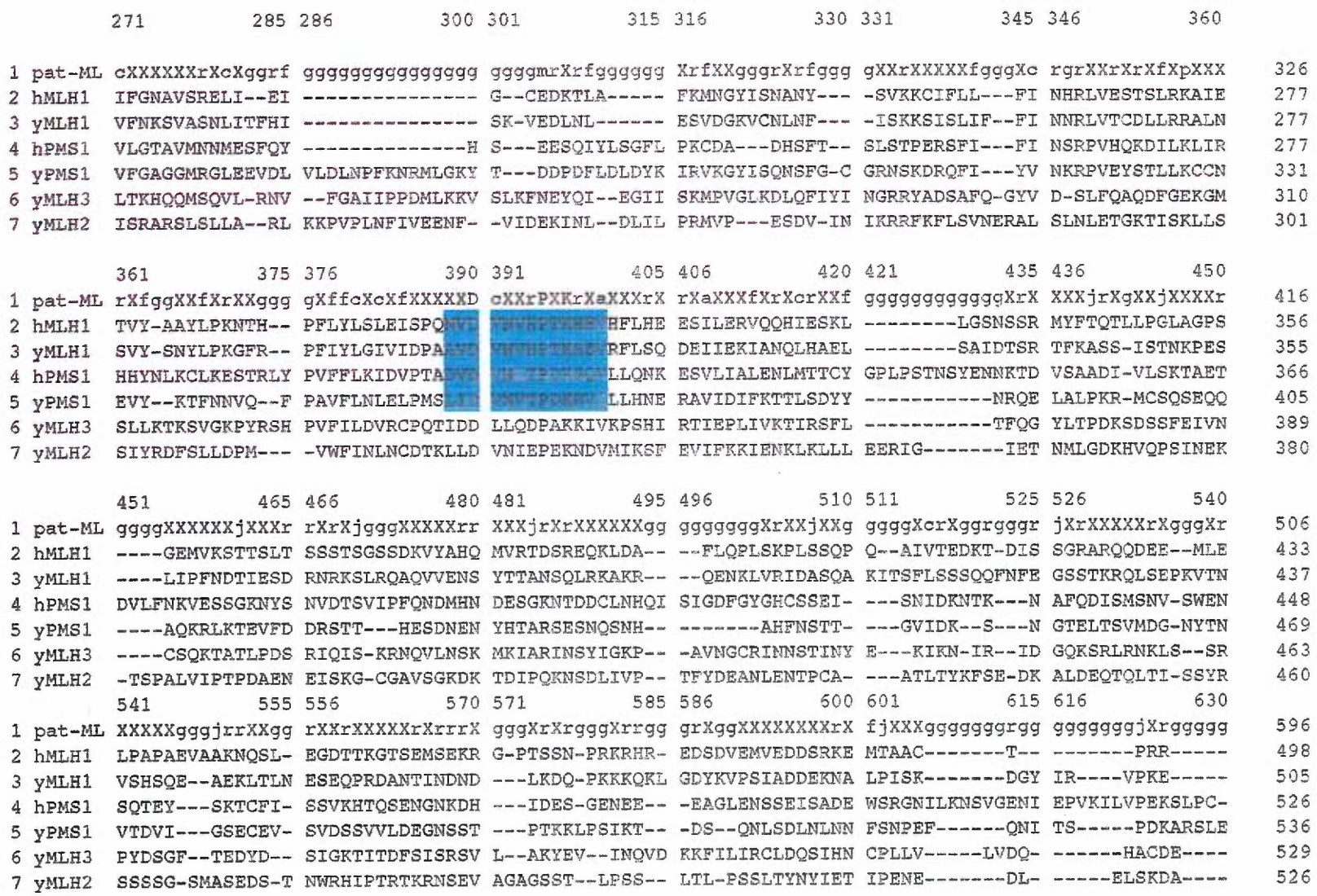


Figure 4-2: Mlh2p Interactions in Two-Hybrid Assay.
Interaction in the two-hybrid system was determined by growth on -THULL as described in Chapter 2. Mlh1 interaction was detected between Mlh1-pBTM and Mlh2-pGAD.CAN (library plasmid) while Mlh3p interaction was detected between Mlh2-pBTM and Mlh3p-pACT (library plasmid).

mating tests had shown that Mlh2p and Mlh1p interacted (J. Simon and Q. Pang, unpublished results). However, I did not detect Mlh2p in multiple two-hybrid screens of the cDNA library using Mlh1p bait, suggesting that *MLH2* was under-represented in the cDNA library.

Mlh2p did not interact with Pms1p, Msh6p, or itself in the two-hybrid mating assay. However, Mlh2p interacted with Mlh1p, Mlh3p (Figure 4-2), and SMT4p, a Mlh1p interacting protein that suppresses a temperature sensitive mutation of MIF2, a putative centromere protein.




271 285 286 300 301 315 316 330 331 345 346 360
1 pat-ML cXXXXXXrXcXggrf gggggggggggggggg ggggmrXrfgggggg XrfXXgggrXrfggg gXXrXXXXXfgggXc rgrXXrXrXfXpXXX 326
2 hMLH1 IFGNAVSRELI--EI ---------- G--CEDKTLA----- FKMNGYISNANY--- -SVKKCIFLL---FI NHRLVESTSLRKAIE 277
3 yMLH1 VFNKSVASNLITFHI ---------- SK-VEDLNL------ ESVDGKVCNLNF--- -ISKKSISLIF--FI NNRLVTCDLLRRALN 277
4 hPMS1 VLGTAVMNNMESFQY ----------H S---EESQIYLSGFL PKCDA---DHSFT-- SLSTPERSFI---FI NSRPVHQKDILKLIR 277
5 yPMS1 VFGAGGMRGLEEVDL VLDLNPFKNRMLGKY T---DDPDFLDLDYK IRVKGYISQNSFG-C GRNSKDRQFI---YV NKRPVEYSTLLKCCN 331
6 yMLH3 LTKHQQMSQVL-RNV --FGAIIPPDMLKKV SLKFNEYQI--EGII SKMPVGLKDLQFIYI NGRRYADSAFQ-GYV D-SLFQAQDFGEKGM 310
7 yMLH2 ISRARSLSLLA--RL KKPVPLNFIVEENF- -VIDEKINL--DLIL PRMVP---ESDV-IN IKRRFKFLSVNERAL SLNLETGKTISKLLS 301
361 375 376 390 391 405 406 420 421 435 436 450
1 pat-ML rXfggXXfXrXXggg gXffcXcXfXXXXXD cXXrPXKrXaXXXrX rXaXXXfXrXcrXXf gggggggggggXrX XXXjrXgXXjXXXXr 416
2 hMLH1 TVY-AAYLPKNTH-- PFLYLSLEISPQ HFLHE ESILERVQQHIESKL --------LGSNSSR MYFTQTLLPGLAGPS 356
3 yMLH1 SVY-SNYLPKGFR-- PFIYLGIVIDPA RFLSQ DEIIEKIANQLHAEL --------SAIDTSR TFKASS-ISTNKPES 355
4 hPMS1 HHYNLKCLKESTRLY PVFFLKIDVPTA LLQNK ESVLIALENLMTTCY GPLPSTNSYENNKTD VSAADI-VLSKTAET 366
5 yPMS1 EVY--KTFNNVQ--F PAVFLNLELPMS LLHNE RAVIDIFKTTLSDYY ----------NRQE LALPKR-MCSQSEQQ 405
6 yMLH3 SLLKTKSVGKPYRSH PVFILDVRCPQTIDD LLQDPAKKIVKPSHI RTIEPLIVKTIRSFL ----------TFQG YLTPDKSDSSFEIVN 389
7 yMLH2 SIYRDFSLLDPM--- -VWFINLNCDTKLLD VNIEPEKNDVMIKSF EVIFKKIENKLKLLL EERIG-------IET NMLGDKHVQPSINEK 380
451 465 466 480 481 495 496 510 511 525 526 540
1 pat-ML ggggXXXXXXjXXXr rXrXjgggXXXXXrr XXXjrXrXXXXXXgg gggggggXrXXjXXg ggggXcrXggrgggr jXrXXXXXrXgggXr 506
2 hMLH1 ----GEMVKSTTSLT SSSTSGSSDKVYAHQ MVRTDSREQKLDA-- --FLQPLSKPLSSQP Q--AIVTEDKT-DIS SGRARQQDEE--MLE 433
3 yMLH1 ----LIPFNDTIESD RNRKSLRQAQVVENS YTTANSQLRKAKR-- --QENKLVRIDASQA KITSFLSSSQQFNFE GSSTKRQLSEPKVTN 437
4 hPMS1 DVLFNKVESSGKNYS NVDTSVIPFQNDMHN DESGKNTDDCLNHQI SIGDFGYGHCSSEI- ---SNIDKNTK---N AFQDISMSNV-SWEN 448
5 yPMS1 ----AQKRLKTEVFD DRSTT---HESDNEN YHTARSESNQSNH-- -------AHFNSTT- ---GVIDK--S---N GTELTSVMDG-NYTN 469
6 yMLH3 ----CSQKTATLPDS RIQIS-KRNQVLNSK MKIARINSYIGKP-- -AVNGCRINNSTINY E---KIKN-IR--ID GQKSRLRNKLS--SR 463
7 yMLH2 -TSPALVIPTPDAEN EISKG-CGAVSGKDK TDIPQKNSDLIVP-- TFYDEANLENTPCA- ---ATLTYKFSE-DK ALDEQTQLTI-SSYR 460
541 555 556 570 571 585 586 600 601 615 616 630
1 pat-ML XXXXXgggjrrXXgg rXXrXXXXXrXrrrX gggXrXrgggXrrgg grXggXXXXXXXXrX fjXXXgggggggrgg gggggggjXrgggggg 596
2 hMLH1 LPAPAEVAAKNQSL- EGDTTKGTSEMSEKR G-PTSSN-PRKRHR- EDSDVEMVEDDSRKE MTAAC-------T-- -------PRR----- 498
3 yMLH1 VSHSQE--AEKLTLN ESEQPRDANTINDND ---LKDQ-PKKKQKL GDYKVPSIADDEKNA LPISK-------DGY IR----VPKE----- 505
4 hPMS1 SQTEY---SKTCFI- SSVKHTQSENGNKDH ---IDES-GENEE-- -EAGLENSSEISADE WSRGNILKNSVGENI EPVKILVPEKSLPC- 526
5 yPMS1 VTDVI---GSECEV- SVDSSVVLDEGNSST ---PTKKLPSIKT-- -DS--QNLSDLNLNN FSNPEF------QNI TS-----PDKARSLE 536
6 yMLH3 PYDSGF--TEDYD-- SIGKTITDFSISRSV L--AKYEV--INQVD KKFILIRCLDQSIHN CPLLV-----LVDQ- ------HACDE---- 529
7 yMLH2 SSSSG-SMASEDS-T NWRHIPTRTKRNSEV AGAGSST--LPSS-- LTL-PSSLTYNYIET IPENE-------DL- -----ELSKDA---- 526

```
2 hMLH1   IL-IYDFANFGVL-R LSEPAPLFDLAMLAL DSPESGWTEEDG--- ----PKEGLAEYIV- EF---LKKKAEMLAD YFSLEIDEEG-----    634
3 yMLH1   IG-LTDFANFGKI-N LQSTNVSDDIVLYNL LS-EFDELNDDA--- ----SKEKIISKIW- DM--------SSMLNE YYSIELVNDGLDNDL    644
4 hPMS1   I--EELWKTLSEEEK LKYEEKATKDLE-RY NSQMKRAIEQES-QM SLKDGRKKIKPTSAW NLAQKHKLK-TSLSN QPKLDELLQS---QI    686
5 yPMS1   IS-KDNYRSLS--DG LTHRKFEDEILEYNL STKNFKEISKNGKQM SSIISKRKSEAQE-- NI---IKNK-DELED FEQGEKYLTL---TV    697
6 yMLH3   YNGDKDYLKM---VL LQHAHDLKDFKKLPM D--LSHFENYTSV-- -----DKLYWWKY-S SC------VPTVFHE ILNSKACRSAVMFGD    678
7 yMLH2   I-PRNSKKKV-TDNY IKKASCTKQGRN-SC I--FPSIPTTTS--- --ILKNEKIVKHDSD NF-------AKETLW LRSRDDATSP-----    663

          811         825 826         840 841         855 856         870 871         885 886         900
1 pat-ML  gggrXXXcXXcgggg gjXXXXXXXfXXXXX gXXc---------- ---------------- ---------------- ----------------    810
2 hMLH1   ---NLIGLPLLIDNY VPPLEGLPIFILRLA -TEVNWDEEKECFES LSKE--CAMFYSIR- -KQYISEESTLSGQ- -QSE-VPGS-----I    708
3 yMLH1   KSVKLKSLPLLLKGY IPSLVKLPFFIYRLG -KEVDWEDEQECLDG ILRE--IALLY-I-- -PDMVPKVDTLDAS- -LSEDEKAQ-----F    720
4 hPMS1   EKRRSQNIKMV---Q IPFSMKNLKINFKKQ -NKVDLEEKDEPCLI HNLRFPDAWLM-TSK TEVMLLNPYRVEEAL LFKRLLENHKLPAEP    771
5 yPMS1   SKNDFKKMEVVGQFN LGFIIVTRKVDNKSD LFIVDQHASDEKYNF ETLQ---AVTV-FKS QKLIIPQPVELS--- VIDELVVLDNLP--V    778
6 yMLH3   ELTRQECIILI--SK LSRCHNPFECAHGRP -SMVPIAELKZ---- ---------------- ---------------- ----------------    716
7 yMLH2   ---SSSLLQALR--- -AHVKKPGHIEATTN EWCLFTKDSPZ---- ---------------- ---------------- ----------------    697
```

Figure 4-1: Alignment of human and yeast MutL homologs.
Highlighted areas represent highly conserved regions. Red indicates where sequence diverges from conserved sequence. Putative ATP-binding motifs are underlined in blue. Alignment performed with PIMA Multiple Sequence `Alignment`.

Homeologous Recombination

To ascertain the function of Mlh2p, I disrupted the MLH2 open reading frame and assayed for MMR phenotypes. As mentioned earlier, loss of Mlh2p does not result in increased mutation rate or affect spore viability (Prolla, 1994). However, Mlh2p might be involved in homeologous recombination, similar to other MMR proteins.

In the mitotic homeologous recombination assay discussed in Chapter 3, loss of Pms1p or Msh2p resulted in increased mitotic recombination of direct repeats that are 91% homologous (Datta, et al., 1996). Recombination of the 91% homologous substrate occurred at approximately the same rate in both the wild type and Δmlh2 strains (Table 4-1), suggesting that the presence of Mlh2p is not necessary for preventing mitotic homeologous recombination. In addition, Δmlh2 strains did not show an effect on allelic or ectopic homologous or homeologous recombination (Francis Fabre, personal communication). *Δmlh1* strains were also analyzed with the 91% homologous substrate, and were found to have a 9-fold increase in homeologous recombination compared to wild type. This increase is consistent with rates observed in the PMS1 deficient strain (Datta, et al., 1996), and implicates Mlh1p in preventing mitotic homeologous recombination.

Strain	% homology	Recombination Rate
Wild type	91%	9.1X10-8 (1)
Δmlh2	91%	7.4X10-8 (0.8)
Δmlh1	91%	8.1X10-7 (8.9)

Table 4-1. Homeologous recombination in *Δmlh2* and *Δmlh1* Strains. Recombination rates were determined from at least 8 independent cultures. Number in parenthesis refers to fold effect compared to wild type.

Discussion

Mlh2p is not required for avoidance of spontaneous mutations, homeologous recombination, or post-meiotic segregation. However, homology to the MutL family suggests some role in DNA transactions. The only function ascribed to Mlh2p is in potentiating cytotoxicity induced by cisplatin, carboplatin and doxorubicin (Durant, et al., 1999). Interestingly, the increased sensitivity to the anticancer agents is observed in all of the MMR mutants analyzed (*MLH1, MSH2, MSH3, MSH6*) with the exception of *PMS1*. Possibly, Mlh1p partners with Mlh2p instead of Pms1p to act on adducts generated by cisplatin, carboplatin and doxorubicin in a manner leading to cell death. Loss of the MMR proteins allows bypass of the lesions and increased cell survival. Another possible role for the Mlh1p/Mlh2p heterodimer is in repairing certain types of DNA damage that could not be observed in my mutation experiments (i.e. certain types of adducts, large loops).

Mlh2p and Mlh3p interacted in the two-hybrid system. This is surprising because *PMS1*, *MLH2*, and *MLH3* are highly conserved in the C-

terminus, which has been shown to be required for Mlh1p/Pms1p interaction (Q. Pang, et al., 1997), and none of the MutLs have been shown to form homodimers. In yeast, Mlh3p forms a heterodimer with Mlh1p and complexes with Msh2p/Msh6p to repair a small percentage of replication errors (Flores-Rozas and Kolodner, 1998). Mlh2p might inhibit Mlh3p activity by forming a Mlh2/Mlh3 heterodimer, thereby allowing the more "efficient" heterodimer, Mlh1p/Pms1p, to form. Alternatively, Mlh2p and Mlh3p might be involved in the repair of other types of DNA damage, such as repair of large IDLs or certain types of DNA adducts.

The interaction between Mlh2p and Smt4p is also interesting, perhaps suggesting a larger complex with Mlh1p, or that Mlh2p can substitute Mlh1p in a complex with Smt4p, to perform whatever function requires Smt4p and Mlh1p.

Conclusions

Mlh2p is not necessary for preventing frameshifts at *hom3-10*, dinucleotide repeats, or mutations within the CAN1 gene (Prolla, 1994). Loss of Mlh2p does not affect spore viability or result in increased homeologous recombination. However, Mlh2p interacts with both Mlh1p and Mlh3p in the two-hybrid assay, suggesting a role for Mlh1p in MMR-related functions.

CHAPTER 5: CHARACTERIZATION OF NTG1 AND NTG2

Introduction

Ntg2p, a structural homolog of *Escherichia coli* Endonuclease III (Figure 5-1), was identified in a two-hybrid screen of a *S. cerevisiae* cDNA library using Mlh1p as bait. Endonuclease III is a DNA N-glycosylase that removes base damage, leaving behind an apurinic/apyrimidinic (AP) site (Demple and Linn, 1980; and Figure 5-2). The EndoIII family has two highly conserved domains: the helix-hairpin-helix (HhH) motif and a 4Fe-4S cluster. Presumably, these domains are necessary for catalytic activity and DNA binding, respectively (Kuo, et al., 1992). EndoIII excises pyrimidine residues damaged by UV resulting in ring saturation, ring fragmentation, or ring contraction (rev. Friedberg, 1995), including thymine glycol and 5,6-dihydrouracil.

Two apparent EndoIII homologs have been identified in *S. cerevisiae*, Ntg1p and Ntg2p, which share 41% identity and 63% similarity with each other (Figure 5-2). Both Ntg1p and Ntg2p cleave DNA substrates containing dihydrouracil, 2,6-diamino-4-hydroxyuracil-5N-methylfomamidopyrimidine (FaPy-7-MeGua) and abasic sites (You, et al., 1998). The roles of Ntg1p and Ntg2p on A·8-oxoguanine (8-oxoG), a residue formed by spontaneous oxidation, is controversial (Eide, et al., 1996; Bruner, et al., 1998; You, et al., 1998). In two reports Ntg1p and Ntg2p did not cleave 8-oxoG (Eide, et al., 1996; You, et al., 1998), while in another study Ntg1p did act on 8-oxoG paired with adenine (Bruner, et al., 1998). These findings are surprising because all other known DNA repair proteins that act on FaPy lesions also act on 8-oxoG. Therefore, Ntg1p and Ntg2p would be unique in acting on FaPy,

but not 8-oxoG residues. In addition, Ntg1p and Ntg2p appear to differ in that Ntg1p removes cytosine photoproducts more efficiently than Ntg2p (Alseth, et al., 1999).

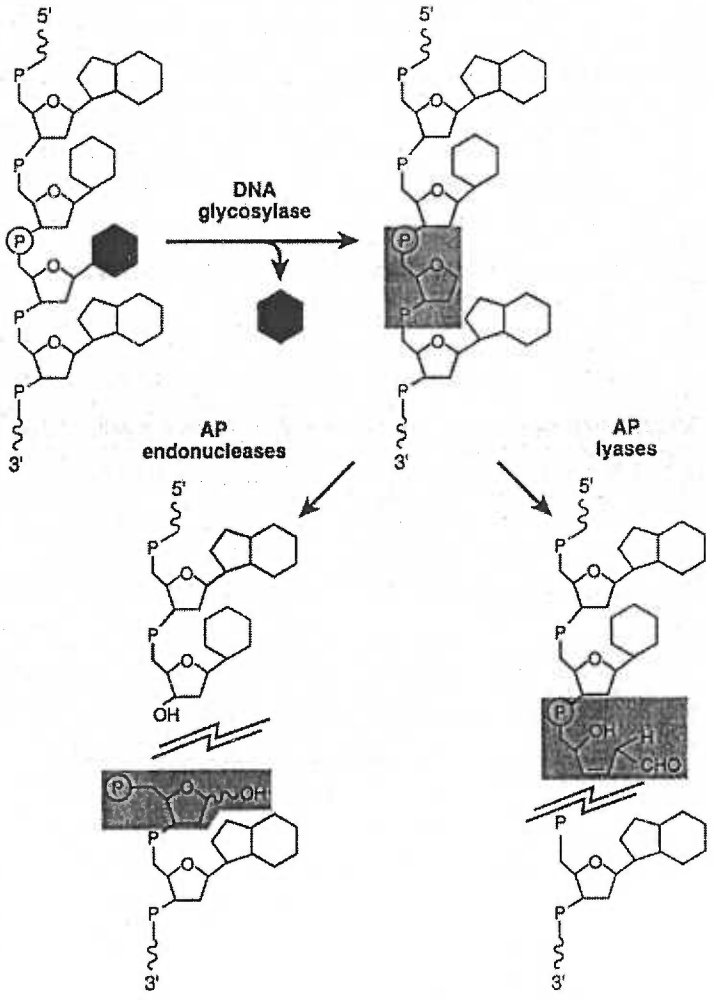


Figure 5-1. Sites of cleavage at an AP site by AP endonuclease and AP lyase. Figure from Friedberg, 1995. Grey shaded areas are the sugar moiety left after removal of the base shown in black.

The *ntg1* and *ntg2* single and double mutants are not sensitive to oxidizing agents, UV, or gamma-rays (Eide, et al., 1996). Results of one study suggest that, relative to wild type, *ntg1* or *ntg2* mutants have an increased spontaneous mutation frequency at *CAN1* (40- to 60-fold), that is further elevated in the *ntg1 ntg2* double mutant, and exposure to hydrogen peroxide increases the rate 10- to 15-fold (Alseth, et al., 1999). However, the increased spontaneous mutation rates reported in this study have not been reproducible, as my research, and that of others (Swanson, et al., 1999), has shown that at least three other strains demonstrated no increase in the spontaneous mutation rate in the *ntg1* or *ntg2* deficient strains at *CAN1*, *hom3-10* or *lys2BglII*. In other studies, a mutant phenotype was observed when the major apurinic endonuclease, Apn1p, was deleted in conjunction with Ntg1p and Ntg2p (Swanson, et al., 1999), suggesting a role for Ntg1p, Ntg2p and Apn1p in the avoidance of spontaneous mutations. One explanation for this phenomenon is that all three proteins recognize a common base excision repair (BER) intermediate, such that Ntg1p, Ntg2p, or Apn1p can compensate for the loss of the other two proteins (Swanson, et al., 1999). Another explanation is that Apn1p provides the AP-lyase activity for a DNA glycosylase that acts on similar residues recognized by Ntg1p and Ntg2p.

The BER proteins, Ntg1p, Ntg2p, and Apn1p, also appear to have overlapping roles with the nucleotide excision repair (NER), recombination, and translesion synthesis (TLS) pathways in the repair of spontaneous mutations (Swanson, et al., 1999 and Figure 6-1). First, disrupting both the BER and NER pathways results in a synergistic increase in mutation and recombination (Swanson, et al., 1999), suggesting that both BER and NER are competing to remove AP sites. Second, recombination is increased in the BER triple mutant (Swanson, et al., 1999). Further, deficiency in both the

recombination (*Δrad52*) and BER pathways results in a synergistic increase in the mutation rate (Swanson, et al., 1999). Third, TLS introduces mutations that can be repaired by the BER proteins (Swanson, et al., 1999). TLS is a mutagenic pathway in which DNA damage can be bypassed by DNA polymerase ζ (Rev3p and Rev7p), allowing the cell to tolerate certain types of adducts, including AP sites (Lawrence and Hinkle, 1996). In the BER mutant loss of TLS decreases the mutation rate to wild type levels, but results in increased recombination (Swanson, et al., 1999). This suggests that the majority of mutations in the BER mutant are due to TLS, and, that in the absence of BER or TLS, a recombination pathway repairs mutations.

Therefore, Ntg1p and Ntg2p are DNA glycosylases involved in preventing both spontaneous and induced mutations in conjunction with Apn1p. In the absence of the BER proteins, Ntg1p, Ntg2p, and Apn1p, mutations are funneled to other repair pathways including recombinational repair, TLS, and NER. Since Ntg2p interacted with Mlh1p in the two-hybrid assay, I wanted to determine if Ntg2p had a role in MMR, or if Mlh1p had a role in Ntg2p-dependent functions.

Two-hybrid Interactions

To determine the domains necessary for Ntg2p/Mlh1p interaction, full length Ntg2p was tested for two-hybrid interaction with fragments of Mlh1p. Ntg2p interaction with full length Mlh1p, the C-terminal Mlh1p fragment C-268, and the CTH Mlh1p mutant (Figure 5-3), resembled Pms1p/Mlh1p interaction. Interaction between Mlh1p and Ntg2p in the presence of Pms1p resulted in decreased levels of β-galactosidase activity, suggesting that Ntg2p and Pms1p might compete for interaction with Mlh1p.

```
   1            15 16           30 31           45 46           60 61           75 76           90
1 NTG2  MREESRSRKRKHIPV DIEEVEVRSKYFKKN ERTVELVKENKINKD LQNYGGVNIDWIKAL KFIEYFEWIESRTCD DPRTWGRPITKEEMI     90
2 NTG1  MQKIS---KYSSMAI LRKRPLVKTETGPES ELLPE--KRTKIKQE EVVPQPVDIDWVKSL PNKQYFEWIVVRNGN VPNRWATPLDPSILV     85
3 ENDO3 --------------- --------------- --------------- --------------- --------------- ---------------      0
4 MUTY  ------------MQA SQFSAQVLDWYDKYG RKTLP--WQIDKTPY KVWLSEVMLQQTQVA TVIPYFERFMARFPT VTDLANAPLDEVLHL     76

        91          105 106         120 121         135 136         150 151         165 166         180
1 NTG2  NDSGAKVPESFLPIY NRVRLMRSKVKTPVD AMGCSMIPVLVSNKC GIPSEKVDP-KNFRL QFLIGTMLSAQTRDE RMAQAALNITEYCLN    179
2 NTG1  TPASTKVPYKFQETY ARMRVLRSKILAPVD IIGGSSIPVTVASKC GISKEQISP-RDYRL QVLLGVMLSSQTKDE VTAMAMLNIMRYCID    174
3 ENDO3 --------------- --------------- MNKAKRLEILTRLRE NNPHPTTELNFSSPF ELLIAVLLSAQATD- -VSVNKATAKLYPVA     58
4 MUTY  ------W--TGLGYY ARARNLHKAAQQVAT LHGG-KFPETFEEVA ALPGVGRST-AGAIL SLSLGKHFPILDGN- --VKRVL-ARCYAVS    152

        181         195 196         210 211         225 226         240 241         255 256         270
1 NTG2  TLKIAEGITLDGLLK IDEPVLANLIRCVSF YTRKANFIKRTAQ-- ----LLVDNFDSDIP YDIEGILSLPGVGPK MGYLTLQKGWGLIAG    263
2 NTG1  ELHSEEGMTLEAVLQ INETKLDELIHSVGF HTRKAKYILSTCK-- ----ILQDQFSSDVP ATINELLGLPGVGPK MAYLTLQKAWGKIEG    258
3 ENDO3 NTP--------AAMLE LGVEGVKTYIKTIGL YNSKAENIIKTCR-- ----ILLEQHNGEVP EDRAALEALPGVGRK TANVVLNTAFGWPT-    134
4 MUTY  GWPGKK--EVENKLW SLSEQVTPAVGVERF NQAMMDLGAMICTRS KPKCSLCPLQNGCIA AANNSWALYPGKKPK QTLPERTGYFLLLQ    239

        271         285 286         300 301         315 316         330 331         345 346         360
1 NTG2  ICVDVHVHRLCKMWN WVDPIKCKTAEHTRK ELQVWLPHSLWYEIN TVLVGFGQLICMAR- --GKRCDLCLANDVC NARNEKLIESSKFHQ    350
2 NTG1  ICVDVHVDRLTKLWK WVDAQKCKTPDQTRT QLQNWLPKGLWTEIN GLLVGFGQIITKSRN -LGDMLQFLPPDDPR SS-LDWDLQSQLYKE    346
3 ENDO3 IAVDTHIFRVC---N RTQFAPGKNVEQVEE KLLKVVPAEFKVDCH HWLILHGRYTCIAR- --KPRCGSCIIEDLC EYKEKVDI-------    211
4 MUTY  HEDEVLLAQRPPSGL WGGLYCFPQFADEES LRQWLAQRQIAADNL TQLTAFRHTFSHFHL DIVPMWLPVSSFTGC MDEGNALWYNLAQPP    329

        361         375 376         390 391         405 406         420 421         435 436         450
1 NTG2  LEDKEDIEKVYSHWL DTVTN----GITTER HKKKZ---------- ------------    381
2 NTG1  IQQN---IMSYPKWV KYLEGKRELNVEAEI NVKHEEKTVEETMVK LENDISVKVEDZ    400
3 ENDO3 --------------- --------------- --------------- ------------    211
4 MUTY  SVGLAAPVERLLQQL RTGAPV---------- --------------- ------------    350
```

Figure 5-2: Alignment of EndoIII homologs. EndoIII and MutY are from E. coli. Yellow highlight is the thymine glycol binding site of EndoIII. Red cysteines are necessary for FeS binding.

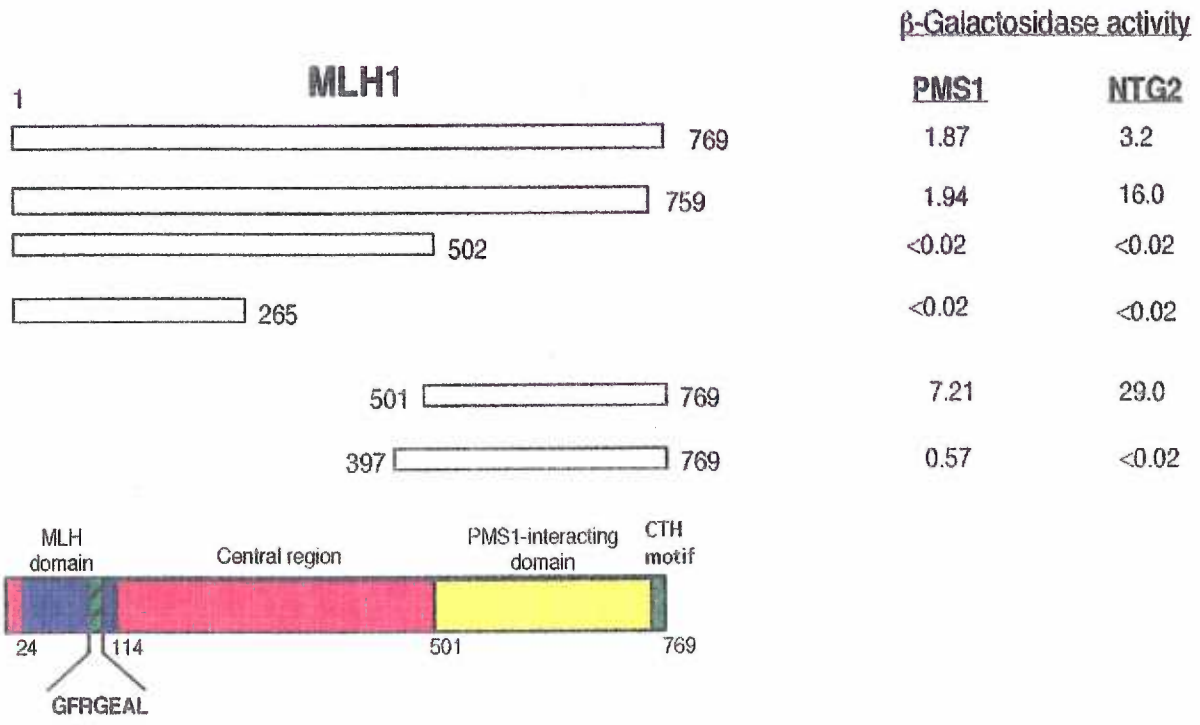


Figure 5-3. Mlh1p and Ntg2p interactions in two-hybrid assay. Interaction in the two-hybrid system was determined by measuring β-galactosidase activity as described in material and methods. Pms1p-Mlh1p values were determined previously by Q. Pang in our laboratory. *MLH1* figure adapted from Pang, et al., 1997.

Analysis of ntg1 and ntg2 mutants

NTG1 and *NTG2* deficiency does not affect spore viability

As mentioned in Chapter 3, spore inviability is a phenotype associated with defects in some MMR genes (Kramer, et al., 1989a; Reenan and Kolodner, 1992b; Prolla, et al., 1994). Both*ntg1* and *ntg1 ntg*2 mutants have 100% spore viability, indicating that these proteins are not required for normal meiotic progression (Figure 5-4).

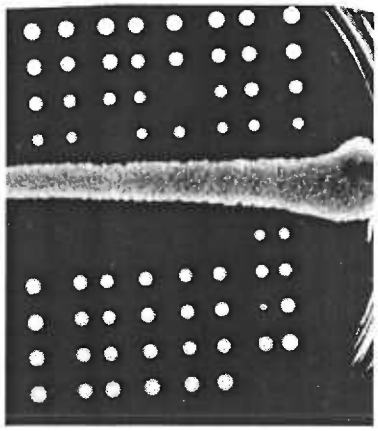


Figure 5-4. Spore viability in *Δntg1Δntg2* strains.
Each column represents a single meiotic event, resulting in four spores. Columns with missing spores were due to technical problems and not inviability.

Strains lacking Ntg1p and Ntg2p do not have a petite phenotype

Ntg1p localizes to the mitochondria, while both Ntg1p and Ntg2p are located in the nucleus (Alseth, et al., 1999). One potential role for Ntg2p is in the repair of mitochondrial mutations. To assess if loss of Ntg1p or Ntg2p resulted in a mitochondrial defect, *ntg1* or *ntg2* mutant strains were analyzed for a petite phenotype. Mitochondrially impaired strains are unable to utilize a non-fermentable carbon source for energy and hence fail to grow on glycerol (Guthrie et al., 1991). *ntg1* and *ntg2* mutants grew normally on YP + glycerol (a non-fermentable carbon source), while a known mitochondrial

mutant strain, KCY135, did not grow, indicating that the Ntg1p and Ntg2p proteins are not essential for mitochondrial function.

Sensitivity to the oxidizing agents menadione and hydrogen peroxide

In vitro assays have suggested that Ntg1p and Ntg2p are involved in processing oxidative base damage (Eide, et al., 1996; Bruner, et al., 1998; You, et al., 1998). However, the sensitivity of *ntg1* and *ntg2* mutants to the oxidizing agents hydrogen peroxide and menadione appears to be strain specific (Alseth, et al., 1999). The insensitivity in some strains suggests that Ntg1p/Ntg2p independent pathways are involved in tolerating and/or processing oxidative DNA damage normally dealt with by Ntg1p and Ntg2p, such as Apn1p. To assess whether MMR is another pathway that acts on oxidative damage, the *mlh1* mutation, in combination with *ntg1/ntg2/apn1* mutations, was assessed for survival following exposure to menadione or hydrogen peroxide. In two different strain backgrounds the *mlh1*, *ntg1 ntg2*, and *ntg1 ntg2 apn1* mutants were not sensitive to hydrogen peroxide or menadione (Figure 5-5). Additionally, the *ntg1 ntg2 apn1 mlh1* quadruple mutant was not sensitive to the oxidizing agents (Figure 5-5), suggesting that loss of MMR does not result in increased sensitivity to oxidizing agents even in the absence of all three BER proteins, Ntg1p, Ntg2p and Apn1p.

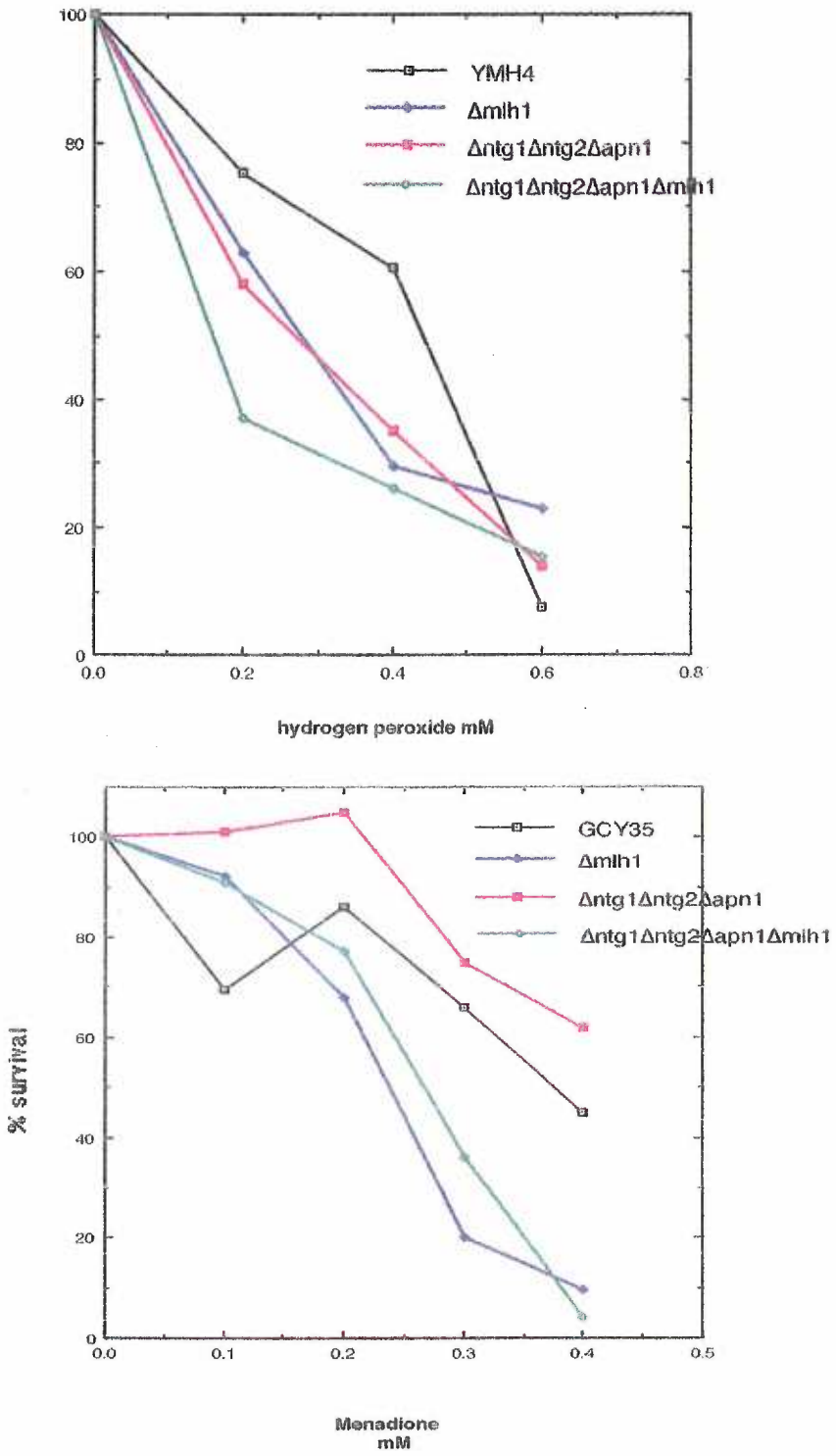


Figure 5-5. Sensitivity to hydrogen peroxide and menadione

Spontaneous Mutation Analysis

To determine whether *NTG2* is involved in MMR, I disrupted the NTG2 ORF and measured increased spontaneous mutation using three assays: *hom3-10*, a reversion assay which measures -1 frameshifts within a mononucleotide repeat, *CAN1*, a forward mutation assay which measures both frameshifts and base changes, and the *CYC1* allele, which reports specific base substitutions. The *ntg2* single mutant did not show an increased spontaneous mutation rate in any of the assays. However, while my studies were in progress a report appeared showing that yeast cells lacking Ntg1p, Ntg2p, and Apn1p showed a spontaneous mutator effect in the *lys2ΔBgl* frameshift and *CAN1* assays (Swanson, et al., 1999). Therefore, I constructed and analyzed *apn1*, *ntg1 ntg2*, and *ntg1 ntg2 apn1* strains for mutator effects (Table 5-1 and 5-2). The *ntg1 ntg2 apn1* triple mutant, but not the single or double mutants, showed a 20-fold increase over wild type in *hom3-10* reversion, compared to a 1500-fold effect for a *Δmlh1* strain. Sequencing of the relevant region of *HOM3* revealed that all *HOM3*$^+$ revertants had the wild type sequence, indicating that Ntg1p/Ntg2p/Apn1p dependent pathways have a role in mutation avoidance at mononucleotide runs. Contrary to previous results performed with the *CAN1* assay (Swanson, et al., 1999), I found that the *apn1* mutation rate was 13-fold higher than wild type GCY35, and the rate was not increased further in the *ntg1 ntg2 apn1* triple mutant. Swanson et al. (Swanson, et al., 1999) found a mutator effect at *CAN1* only in a *ntg1 ntg2 apn1* triple mutant. The different results could reflect strain differences, possibly resulting from differing levels of Apn2p, an AP-endonuclease that repairs base damage in the absence of Apn1p (Johnson, et al., 1998).

STRAIN	RATE X10-8 *hom3-10*	FOLD		RATE X10-8 *CAN1*	FOLD
GCY35	1.21	1		8.26	1
Δapn1	2.12	1.75		110	13.3
Δntg2Δntg1	0.78	0.6		12.5	1.5
Δntg2Δntg1Δapn1	24.7	20.4		115.7	14
Δmlh1	1920	1600		1050	127
Δntg2Δntg1Δmlh1	718	593		630	76
Δapn1Δmlh1	1500	1250		1000	121
Δntg2Δntg1Δapn1Δmlh1	1310	1082		747	90

Table 5-1. Mutation rates in GCY35 strains.
Mutation rates were determined from at least 8 independent cultures and calculated using Chipmunk Basic Rate (Nicholson Jr., 1997). Fold is relative to wild type GCY35.

Next, I used the Hampsey tester system to assess the types of base changes that occur in the *ntg1*, *ntg2*, and *apn1* mutants. The Hampsey system allows for reporting of the six possible base-substitutions (Hampsey, 1991). Each strain has a base pair substitution in the nuclear *CYC1* gene, which encodes iso-1-cytochrome C. Only a specific base change will allow the yeast to grow on media containing a non-fermentable carbon source. Strains deficient in *ntg1*, *ntg2*, and/or *apn1* were analyzed in the strains that report G:C-->T:A and A:T-->C:G transversions, since these are base changes that commonly result from oxidative damage (Wang, et al., 1998) (Table 5-3). G:C-->T:A and A:T-->C:G mutation rates were increased in the *ntg1 ntg2* double mutant 5.8-fold, and 6.1-fold respectively compared to wild type (Table 5-3 and 5-4). In the *apn1* mutant G:C-->T:A rates were increased 4.3-fold (Table 5-3).

STRAIN	Mutation Rate[a] X10^{-9}	FOLD[b]
W303	3.07	1
Δntg2	9.79	3.2
Δapn1	12.2	4
Δntg2Δntg1	6.29	2
Δntg2Δntg1Δapn1	240	78
Δmlh1	22000	7882
Δmlh1Δntg2	14200	4625
Δmlh1Δntg1	17100	5700
Δmlh1Δntg2Δntg1	12800	4267
Δmlh1Δapn1	31300	10433
Δmlh1Δntg2Δntg1Δapn1	12933	4311
Δrev3Δntg1Δntg2Δapn1	192	63
Δrev3Δmlh1	30200	10067
Δrev3Δapn1Δmlh1	15000	5000
Δrev3Δapn1Δntg2Δntg1Δmlh1	10600	3533
Δrad1	19.2	6
Δrad1Δntg1Δntg2Δapn1	79.5	27
Δrad1Δntg1Δntg2Δapn1Δmlh1	28900	9414
Δrev1Δmlh1	45500	15166

Table 5-2. *hom3-10* reversion rates in W303 strains.
(a) Mutation rates were determined using at least 8 independent transformants. (b) Fold is relative to wild-type.

Surprisingly, in the A:T-->C:G reporter strain the *apn1* mutant was 102-fold higher then wild type (Table 5-4) suggesting that Apn1p is responsible for preventing a large proportion of A:T-->C:G events. In the *ntg1 ntg2 apn1* triple mutant G:C-->T:A mutation rates resulted in an additive increase

compared to the *ntg1 ntg2* and *apn1* mutants, 10.5-fold higher then wild type, suggesting that Ntg1p/Ntg2p and Apn1p have separate pathways for preventing the G:C-->T:A transversions (Table 5-3).

STRAIN G:C --> T:A	MUTATION RATES[a]	AVERAGE RATE	FOLD[b]
YMH4	$6.5X10^{-10}$ $7.87X10^{-10}$ $7.09X10^{-10}$	$7.2X10^{-10}$	1
4Δntg1	$3.8X10^{-10}$	$3.8X10^{-10}$	0.5
	$4.15X10^{-9}$	$4.15X10^{-9}$	5.8
4Δsod1	$5.97X10^{-9}$	$5.97X10^{-9}$	8.3
4Δapn1	$3.08X10^{-9}$	$3.08X10^{-9}$	4.3
4Δntg2Δntg1Δapn1	$6.69X10^{-10}$ $1.42X10^{-8}$	$7.43X10^{-9}$	10.3

Table 5-3. Rate of G•C-->T•A events in YMH4 strains.
(a) Individual numbers represent independent experiments using 5 transformants. (b) Fold is relative to wild type. Rate was calculated using Chipmunk Basic Rate (Nicholson Jr., 1997).

STRAIN A:T --> C:G	MUTATION RATES[a]	AVERAGE RATE	FOLD[b]
YMH6	1.59X10-10 1.27X10-10 8.63X10-10	2.2X10-10	1
6Δntg1	5.92X10-10		2.7
6Δntg2Δntg1	1.35X10-9		6.1
6Δsod1	1.31X10-9		6
6Δapn1	2.24X10-8		102

Table 5-4. Rate of A•T-->C•G events in YMH6 strains.
a) Individual numbers represent independent experiments using 5 transformants. (b) Fold is relative to wild type. Rate was calculated using Chipmunk Basic Rate (Nicholson Jr., 1997).

Ntg1/Ntg2/Apn1 *does not synergize with* Mlh1

If the BER pathway and MMR compete for the same substrates, mutation rates higher than additive (synergistic) should occur. In the *mlh1* mutant *hom3-10* reversion rates are increased 1600-fold. However, in the *ntg1 ntg2 apn1 mlh1* quadruple mutant mutation rates are slightly lower then the *mlh1* mutant alone in both GCY35 and W303 backgrounds. The *ntg1 ntg2 mlh1* is also 4-fold lower than the *mlh1* at *hom3-10* in GCY35. This may be insignificant, since the mutation rates are high, but it could also suggest that the presence of Apn1p or Ntg1p/Ntg2p activities provide substrates for Mlh1p. A similar pattern was seen in the *CAN1* forward mutation assay. I found that the *ntg1 ntg2 apn1 mlh1* strains consistently showed a slow growth phenotype, indicating that loss of all four proteins is inhibiting growth.

Since the mutator effect in the *Δmlh1* strains is relatively high compared to the *ntg1 ntg2 apn1* triple mutant, I used *mlh1* point mutants to decrease the overall mutation rate. Hypothetically, a lower mutation rate could enable observation of an additive or multiplicative effect in the *ntg1 ntg2 apn1 mlh1* mutant. Two different *mlh1* point mutations were used, a mutation in the putative ATPase domain (*mlh1*F97A) and in the N-terminus (*mlh1*A41G). The *mlh1A41G* mutation rate is very low in W303 compared to *mlh1* null strains, 21-fold versus 1600-fold at *hom3-10*, while the *mlh1F97A* mutation is about 800-fold (Table 5-6). The *mlh1F97A* and *mlh1A41G* mutations were introduced on a plasmid in the *ntg1 ntg2 apn1 mlh1*

quadruple mutant. The *mlh1A41G* and *mlh1F97A* mutations in the *ntg1 ntg2 apn1* background resulted in a mutation rate increase of 2-fold and 2.5 fold, respectively. The small increase could suggest that Mlh1p and Ntg1p, Ntg2p, and Apn1p are involved in repairing frameshifts by different, non-competing pathways.

I also attempted to look at the combination of MMR and BER mutants using the Hampsey assay, but found that they could not be determined in MMR deficient strains in haploids since the high mutation rates do not yield reproducible results. The irreproducibility is thought to result from second site mutations in other proteins in the cytochrome-C pathway, decreasing the [a]bil[i]ty to report base changes (Hampsey, personal communication and our own findings (10-10 and 10-7)).

STRAIN	Rate X 10-7[a]	Fold[b]
GCY35	0.12	1
Δmlh1	192	1600
Δntg1Δntg2Δapn1	2.47	20.4
mlh1R97A	74.2	613
Δntg1 Δntg2 Δapn1 mlh1R97A	238	1966
Δmlh1A41G	2.5	21
Δntg1 Δntg2 Δapn1 mlh1A41G	4.5	37

Table 5-6. *hom3-10* mutation rates in GCY35 *mlh1* point mutants
(a) Mutation rates determined from 5 independent cultures using ChipmunkBasic Rate (Nicholson Jr., 1997) (b) Fold is relative to wild-type GCY35. Mutations were introduced on plasmids into a *Δmlh1* background.

Mutation Rates are not decreased in a *rev3* strain

Loss of TLS by *rev3* mutation in the BER$^-$ strain (*Δntg1Δntg2Δapn1*) reduced the mutation rate at *lys2ΔBgl* and *CAN1* to wild type levels, suggesting that the majority of mutations in the BER mutant were produced by TLS (Swanson, et al., 1999). To examine whether TLS was responsible for a fraction of mutations in the MMR deficient strain, I introduced the *rev3* mutation into the *mlh1* and *ntg1 ntg2 apn1 mlh1* strains. Mutation rates at *hom3-10* were not greatly decreased by the *rev3* mutation in either the *mlh1* mutant or the *ntg1 ntg2 apn1 mlh1* mutant (Table 5-2), indicating that the majority of mutations in the *mlh1* or *ntg1 ntg2 apn1 mlh1* strains were not arising from TLS.

The mutator phenotype associated with overexpression of Mlh1 is not suppressed in the *Δntg1Δntg2Δapn1* mutant

Overexpression of Mlh1p in wild type cells results in a 122-fold increase in *hom3-10* reversion rate, presumably by disrupting repair complexes by affecting the stoichiometry of Mlh1p complexes. In *Drosphila*, dosage effects can be suppressed by mutations in other genes (Ashburner, 1989; Bantignies, 1999). If the presence of Ntg1p, Ntg2p, and Apn1p are necessary to form the repair complex, then overexpression of Mlh1p in the *ntg1 ntg2 apn1* triple mutant should suppress the Mlh1p overexpression phenotype. However, I found that the mutation rate was the same in both wild type and *ntg1 ntg2 apn1* cells overexpressing Mlh1p, indicating that the dominant mutator phenotype resulting from Mlh1p overexpression is not dependent on the presence of Ntg1p, Ntg2p, or Apn1p (Table 5-7).

STRAIN	hom3-10 Reversion Rate	Fold
W303	3.1X10-9	1
W303+ MLH1-pJAS	3.77X10-6	122
W303*Δntg1Δntg2Δapn1*+ MLH1-pJAS	1.8X10-6	58

Table 5-7. Overexpression of Mlh1p in BER mutant.
At least 7 independent cultures were tested. Mutation rate was determined by method of the mean using Chipmunk Basic Rate (Nicholson Jr., 1997). Fold is relative to wild type without plasmid.

Induction of mutations by hydrogen peroxide and menadione

To determine whether BER and MMR cooperate in preventing mutations induced by oxidative DNA damage, strains deficient in various combinations of the BER proteins and/or MMR proteins were tested for hydrogen peroxide or menadione induced reversion at either *hom3-10, CAN1,* or *CYC1.*

Treatment of a wild type strain (W303 and GCY35) with 4mM hydrogen peroxide resulted in a 50-fold increase in the reversion frequency at *hom3-10* (See Chapter 6, Figure 6-2). Sequence analysis indicates that the primary mutational event was the expected frameshift from 7 to 6 A:T base pairs. The *ntg1 ntg2 apn1* mutant showed a 3-fold increased mutation frequency compared to wild type strains treated with hydrogen peroxide (Chapter 6: Figure 6-6) suggesting that the presence of the "BER proteins" helps to prevent frameshifts in response to oxidative damage (See Chapter 6).

Induced mutation in response to hydrogen peroxide was also analyzed using the YMH4 haploid strain that reports G->T transversions, a signature of oxidative damage (Wang, 1998). In the repair proficient strain, a 170-fold induction was observed with hydrogen peroxide treatment. In the BER mutant, the spontaneous mutation rate is 10-fold higher than wild type. Induced mutagenesis in the BER mutant at the lower doses is almost 10-fold higher then wild type (Figure 5-6), but the same as wild type at the higher doses. This suggests that the BER proteins protect against G->T transversion events in response to low doses of hydrogen peroxide, but provide little protection at higher doses.

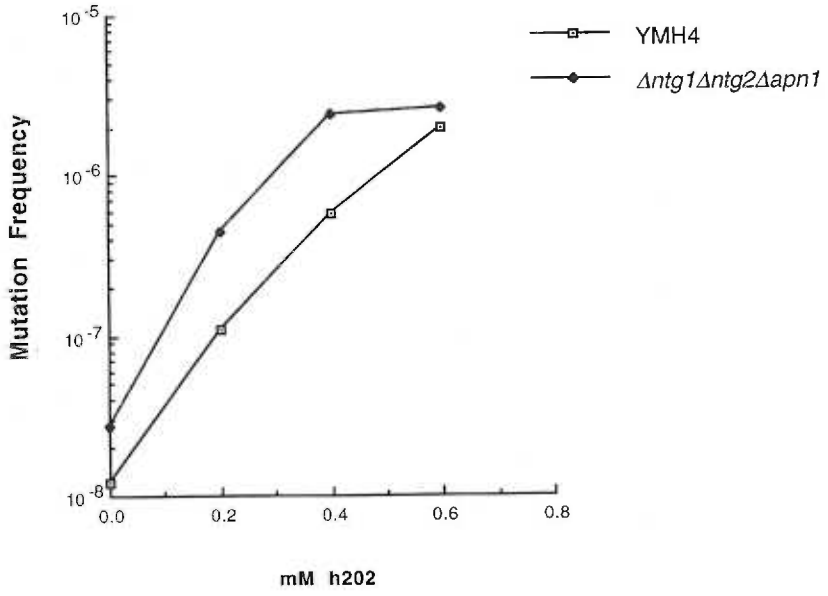


Figure 5-6. Induction of G·C --> T·A transversions with hydrogen peroxide in wild type and BER mutant. Represents the average of two experiments.

One of the difficulties with the Hampsey assay is that second site mutations in the cytochrome-C oxidase biosynthesis pathway can mask revertants and report a lower mutation frequency (Hampsey, 1991). This is particularly evident in the MMR deficient strains where fluctuations of a 1000-fold can occur between different experiments (7×10^{-10} vs. 2.4×10^{-7} findings). Therefore, while I attempted to analyze the *ntg1 ntg2 apn1 mlh1* quadruple mutant to assess the role of MMR proteins in oxidation mutagenesis, I found that it was difficult to obtain a consistent mutation frequency, and this was exacerbated when the quadruple mutant was treated with hydrogen peroxide.

Discussion

The N-glycosylase/AP-lyase, Ntg2p, interacted with Mlh1p in a two-hybrid screen of a cDNA library. This was particularly interesting to me for two reasons: 1) MMR proteins are involved in the repair of oxidative damage and 2) DNA glycosylases are involved in base excision repair of numerous adducts arising from oxidative damage (Friedberg, 1995).

Removal of the BER proteins Ntg1p, Ntg2p, and Apn1p resulted in increased mutation rates at *lys2ΔBgl* and *CAN1* (Swanson, et al., 1999). In addition, I have found that the BER triple mutant *ntg1 ntg2 apn1* also has an increased mutation rate at *hom3-10*. This is surprising, because *hom3-10* reversion involves reverting a mononucleotide run, in which instability is often associated with DNA polymerase replication errors (reviewed in Kunkel, 1992 and discussed in Chapter 6)

An interaction between Mlh1p and Ntg2p, if significant, raises the possibility that the BER proteins play a role in MMR, or that Mlh1 has a role

in BER. However, the combined MMR and BER mutants had a slightly lower mutation rate indicating that the genetic dissection of these pathways would be difficult. In contrast to the data obtained from the null *mlh1* strains, the *mlh1* point mutants, combined with deficiency in BER, resulted in a greater then additive increase in mutation rates suggesting that the BER proteins repair a very small fraction of the frameshifts, while MMR repairs the majority of frameshifts at *hom3-10*. In either case, the contribution of Ntg1p, Ntg2p, and Apn1p to MMR is small. However, redundant BER proteins, such as Apn2p, might hinder the characterization of the BER proteins' role in MMR.

Another explanation for the Mlhp1/Ntg2p interaction is that the two proteins are part of a complex that repairs base damage resulting from oxidation. A role for MMR in repairing damaged bases resulting from oxidizing agents comes from several recent studies. First, reversion rates of some base changes in Msh2p deficient strains are dramatically decreased in an anaerobic environment (Earley and Crouse, 1998). In addition, the MSH2p-MSH6p complex can bind to adenine or cytosine misincorporated opposite 8-oxoguanine (Ni et al, submitted, 1999). The role for MMR in the repair of oxidative damage is further extended by the finding that combining mutations in *MSH2* or *MSH6* with *OGG1*, a protein involved in the removal of ^{O}G:C, results in a synergistic increase of GC-->TA transversions (Ni et al, 1999).

Another link between MMR and oxidative damage is in TCR. TCR of oxidative damage, particularly thymine glycols, occurs in yeast and human cells (Leadon and Lawrence, 1992; Leadon and Cooper, 1993). Yeast strains lacking Msh2p, or both Mlh1p and Pms1p, are deficient in the removal of thymine glycols from the transcribed strand (Leadon and Avrutskaya, 1998).

The MMR proteins might interact with the BER proteins to signal which strand is being transcribed, thereby directing which adducts are preferentially repaired. A signaling function for the MMR proteins is supported from studies that found that global thymine glycol repair was not decreased in MMR deficient strains (Leadon and Avrutskaya, 1998).

Evidence also suggests that the BER proteins, Ntg1p, Ntg2p, and Apn1p are involved in protecting against oxidative damage. First, Ntg1 and Ntg2 process certain adducts associated with oxidative damage *in vitro*, including thymine glycols and dihydrouracil (Eide, et al., 1996; You, et al., 1998). Secondly, I have found that the BER proteins are protective against frameshifts at *hom3-10* and G:C→T:A transversions in response to hydrogen peroxide. MMR proteins might work with the BER proteins to prevent and/or repair oxidatively damaged DNA. Perhaps the MMR proteins recognize the mispair opposite a damaged base and signal the BER proteins, which then repairs the adduct. By this model, loss of the BER proteins shuttles repair into NER, TLS, or recombination pathways resulting in increased frameshifts.

Another possibility is that the interaction between Ntg2p and Mlh1p does not occur *in vivo*, and is an artifact of the two-hybrid system. Ntg2p associated with the Pms1p interacting portion of Mlh1p. In general, most of the proteins identified in the two-hybrid screen tended to interact with the Pms1p interacting portion of Mlh1p, suggesting that either Mlh1p forms most of its complexes via this region, or it is somewhat "sticky". However, we identified only seventeen proteins that interact with Mlh1p, including Pms1p, Pol30p, Mlh2p and Mlh3p, suggesting that Mlh1p is somewhat discriminatory in the two-hybrid assay.

Conclusions

Ntg2p interacted with Mlh1p in the two-hybrid system requiring the C-terminal Pms1p-interacting domain of Mlh1p.

In the absence of Ntg1p, Ntg2p and Apn1p there was an increase in *hom3-10* reversions and G:C-->T:A transversions. Treatment of the *ntg1 ntg2 apn1* strain with hydrogen peroxide induced mutants above the induced wild type levels, suggesting that BER has a protective effect against oxidative damage. The mutation rate in the *ntg1 ntg2 apn1 mlh1* mutant was similar to the *Δmlh1* strain at *hom3-10* and *CAN1* excluding a multiplicative or synergistic effect. The mutation rate was not significantly decreased in the *ntg1 ntg2 apn1 mlh1* mutant upon introduction of the *rev3* mutation.

In addition, I found that loss of Ntg1p or Ntg2p does not affect spore viability, result in a petite phenotype, or affect sensitivity to oxidizing agents.

Future of Two-Hybrid Studies with MMR Proteins

Using Mlh1p in the two-hybrid assay has allowed identification of a number of interesting proteins, including some involved in MMR, such as Pol30p, Exo1p, and Mlh3p. While Mlh2p, M86p, and Ntg2p are not required for mutation avoidance associated with MMR, they may point to other functions involving Mlh1p, such as repairing base damage or recombination. Future screens with Mlh1p of different libraries, such as meiotically derived libraries, may yield other interesting partners.

Currently a Ph.D. student in our lab, Phuoc Tran, has designed baits based on crystallography information of the structure of MutL in *E. coli*. In *E. coli* , MutL forms a homodimer through its C-terminus, and, upon binding ATP, dimerizes at the N-terminus, providing an interaction surface (Ban and Yang, 1998). A bait composed of Mlh1p and Pms1p fragments that dimerize at the N-terminus will be used to screen for proteins that interact with the dimerized N-termini of Mlh1p and Pms1p. Baits of smaller fragments of Mlh1p and Pms1p, as well as full-length proteins fused at the C-terminus with the LexA DNA binding domain, might also yield other proteins involved in MMR.

CHAPTER 6: MICROSATELLITE INSTABILITY IN RESPONSE TO DNA DAMAGING AGENTS

Introduction

The human genome contains over 50,000 simple repeat sequences, called microsatellites, that show instability resulting in frequent deletions and additions (Loeb, 1998). MSI is associated with human diseases, including the fragile X syndrome where expansions of a trinucleotide tract is observed, and in HNPCC and sporadic cancers (reviewed in Karran, 1996; Sia, et al., 1997). The accepted model for frameshifts in microsatellites invokes slippage by polymerases during replication, an idea supported by the findings that certain yeast polymerase mutants demonstrate MSI (Schaaper and Dunn, 1987). Two known pathways are responsible for repairing replication associated frameshifts: the associated exonuclease activity of polymerase and MMR. MSI is seen in over 90% of tumors from HNPCC patients harboring MMR gene mutations (Bishop and Hall, 1994; Kinzler and Vogelstein, 1996). However, MSI is observed in a number of sporadic cancers that have been shown to lack mutations in the known MMR genes, implicating other sources of MSI in these tumors (Reviewed in Loeb, 1998). One possible source for MSI is spontaneous or induced DNA damage that can result from DNA damaging agents. This idea is supported by the finding in yeast that frameshifts occur in mononucleotide runs in response to ultraviolet light (Lawrence and Christensen, 1976; Lawrence and Christensen, 1979; Lawrence, et al., 1984), and, in *E. coli*, mononucleotide and dinucleotide repeat instability can result from treatment with hydrogen peroxide (Jackson, et al., 1998).

Multiple pathways are involved in processing spontaneous and induced base damage, including base excision repair (BER), nucleotide excision repair (NER), "error-prone" translesion synthesis (TLS), and recombinational repair (Lawrence and Hinkle, 1996; Alseth, et al., 1999; Swanson, et al., 1999). One model, which I refer to as the "sink" model, suggests that all four pathways, BER, NER, TLS, and recombinational repair, repair or "tolerate" spontaneous DNA damage, and in the absence of one pathway the damage is "drained" into the other pathways (Swanson, et al., 1999 and Figure 6-1). Three BER proteins, Ntg1p, Ntg2p, and Apn1p, recognize adducts associated with oxidative damage and have recently been shown to be required for the repair of both spontaneous (Alseth, et al., 1999; Swanson, et al., 1999 and this work) and induced oxidative DNA damage (Alseth, et al., 1999 and this work). Rad1p, a NER protein involved in cleaving pyrimidine dimers in conjunction with Rad10p (Davies, et al., 1995), also repairs spontaneous damage (Swanson, et al., 1999). In the absence of BER and NER, base damage is "drained" into the Rad52p pathway (Swanson, et al., 1999), which is normally involved in the repair of DNA double-strand breaks (DSBs) through homologous recombination (Reviewed in Petes, et al., 1991). In BER^- or NER^- backgrounds spontaneous mutation is restored to wild type levels in the absence of the TLS protein, Rev3p, indicating that the majority of the mutations in the BER^- or NER^- strains are arising as a by-product of TLS (Swanson, et al., 1999).

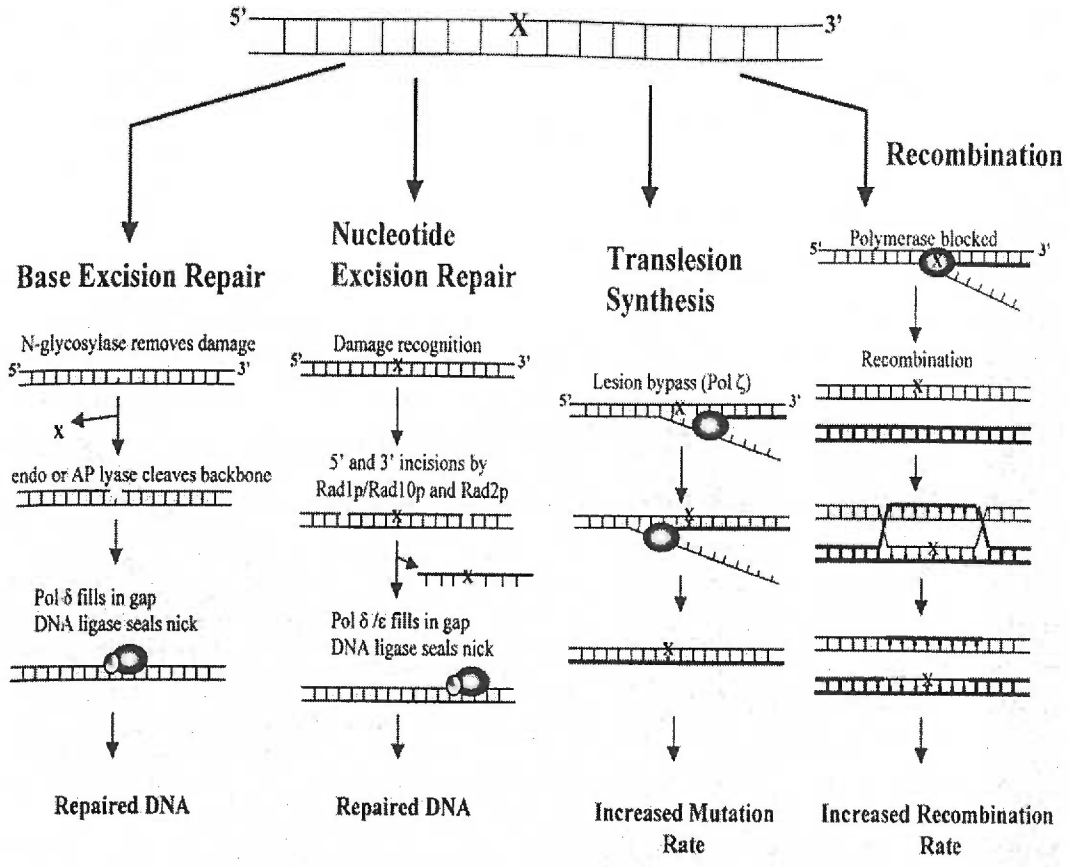


Figure 6-1. The "Sink" Model.
Figure from Swanson, et al., 1999.

To dissect the pathways influencing induced MSI, I examined the reversion frequency at *hom3-10* in a number of yeast DNA repair mutants. I found that, in wild type cells, *hom3-10* reversion was induced by treatment with both hydrogen peroxide and UV light. Furthermore, my results suggest that instability in response to hydrogen peroxide is dependent upon BER and Rad1p whereas TLS and homologous recombination pathways have a role in UV-induced MSI.

UV and hydrogen peroxide induced mutation

Induction of *hom3-10* reversion in wild type cells

Consistent with other studies of mononucleotide runs (Lawrence and Christensen, 1979; Lawrence, et al., 1984), *hom3-10* reversion in wild type strains was induced with UV light, approximately 90-fold (Figure 6-2). In addition, treatment of wild type strains with hydrogen peroxide resulted in a 100-fold induction of *hom3-10* reversion (Figure 6-3). Sequence analysis of 10 revertants revealed the expected reversion event of 7 to 6 A/T base pairs, indicating that both hydrogen peroxide and UV light can induce instability at mononucleotide repeats.

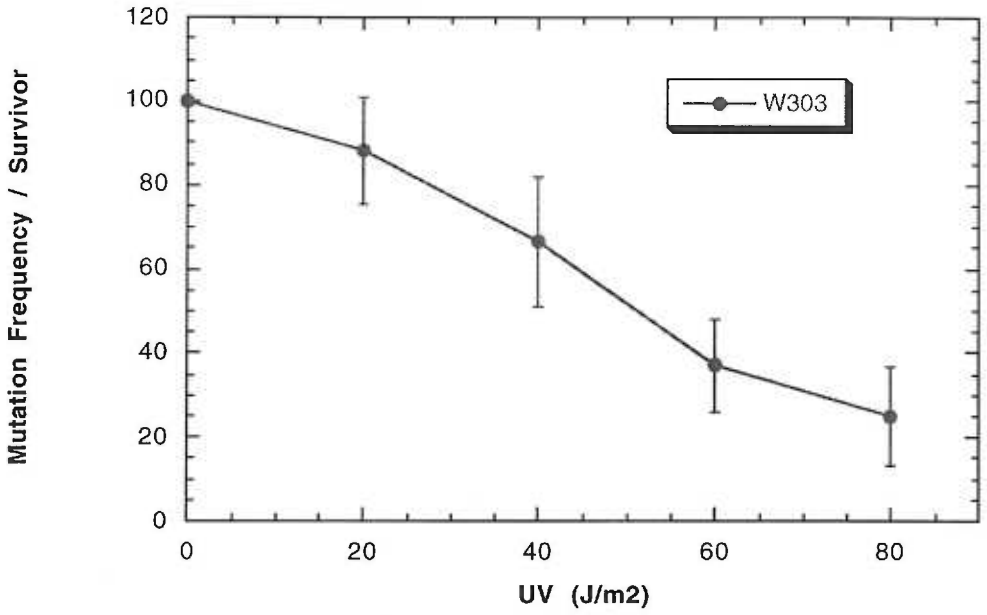
A role for Rev3 and Rev1 in UV light induced DNA damage

TLS is a mutagenic pathway involving polymerase ζ (zeta), encoded by REV3 and REV7, and the deoxycytidyl transferase, Rev1p, which allows replication past a lesion (reviewed in Lawrence and Hinkle, 1996). Strains deficient for Rev3p or Rev1p were treated with UV light to determine the effect of TLS on UV light induction of *hom3-10* reversion by UV light. I found that induced mutation was decreased 2-4 fold (Figure 6-4). In other studies of mononucleotide repeats involving A/T base pairs, induction by UV light was shown to be *REV3*-dependent, but *REV1*-independent (Lawrence and Christensen, 1979). However, UV induced *hom3-10* reversion is only slightly dependent on both Rev3p and Rev1p. Interestingly, only half of the mutations appear to result from the *REV3/REV1* dependent TLS pathway, indicating that other sources are contributing to *hom3-10* mutations.

UV-light mutagenesis is dependent on Rad52p

In a *rad52Δ* strain, *hom3-10* reversion was induced 7300-fold at the highest UV dose then the spontaneous mutation frequency, and almost 90-fold higher than wild type at the equivalent doses of UV light (Figure 6-5). The high levels of induction implicate recombinational repair in tolerating DNA damage in response to UV light. Although *rad52Δ* strains show increased sensitivity to hydrogen peroxide, Rad52p does not appear to have a role in oxidative induced mutagenesis since *rad52Δ* strains demonstrated a similar mutation frequency at *hom3-10* compared to wild type cells when treated with hydrogen peroxide.

A.



B.

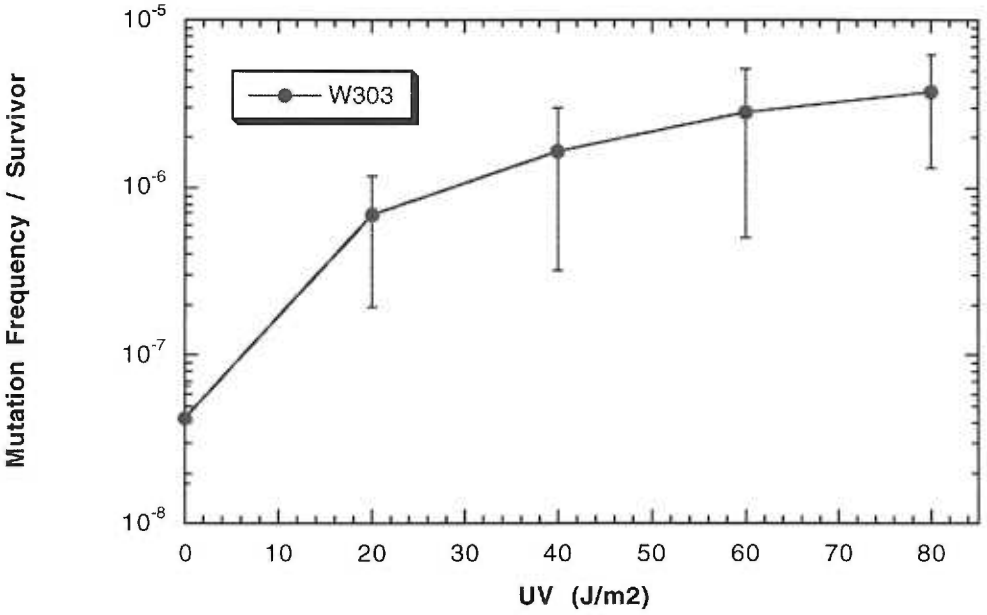
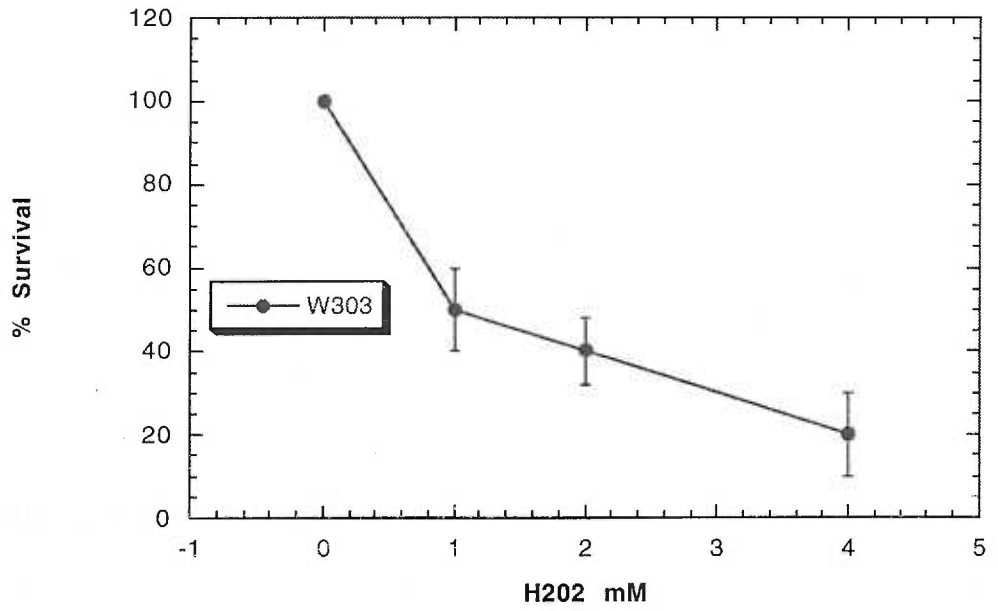


Figure 6-2. UV Survival and *hom3-10* Reversion in W303. (A) Survival and (B) *hom3-10* reversion frequency corrected for cell survival. Error bars represent plus or minus one standard deviation.

A.



B.

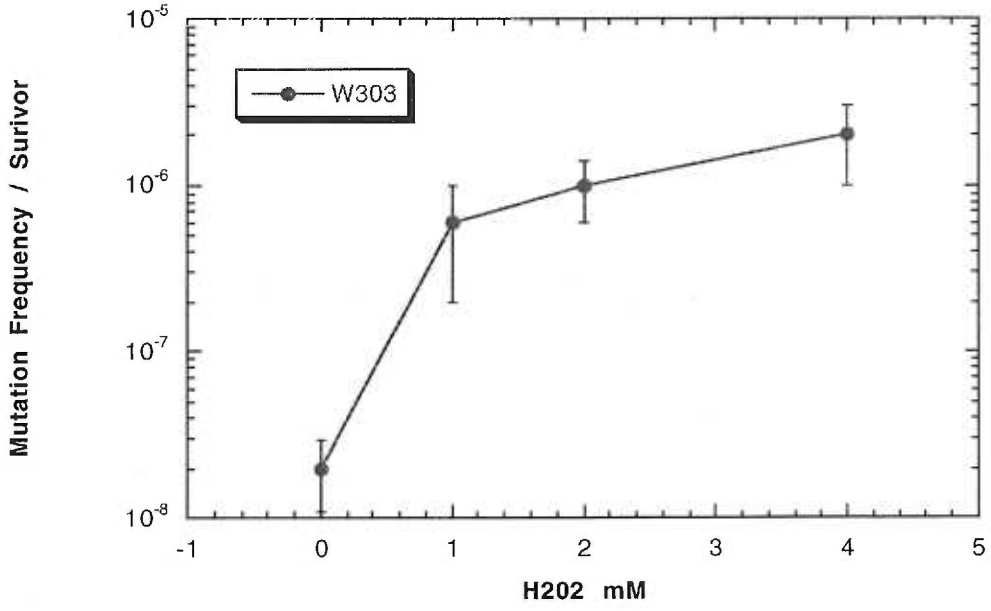
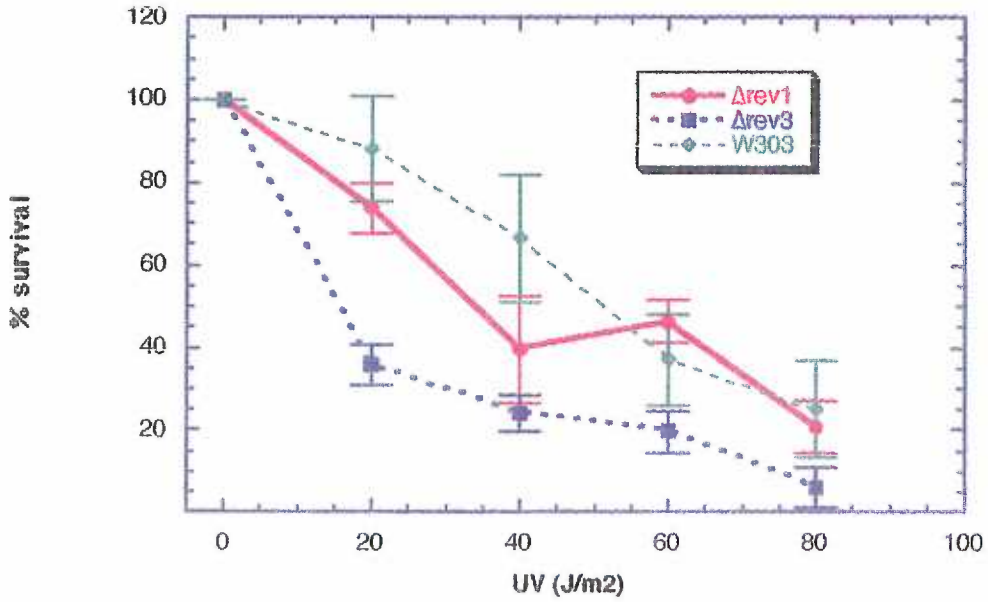


Figure 6-3: Hydrogen Peroxide Survival and *hom3-10* Reversion in W303. (A) Survival and (B) *hom3-10* reversion frequency corrected for survival. Error bars represent plus and minus one standard deviation.

A.



B.

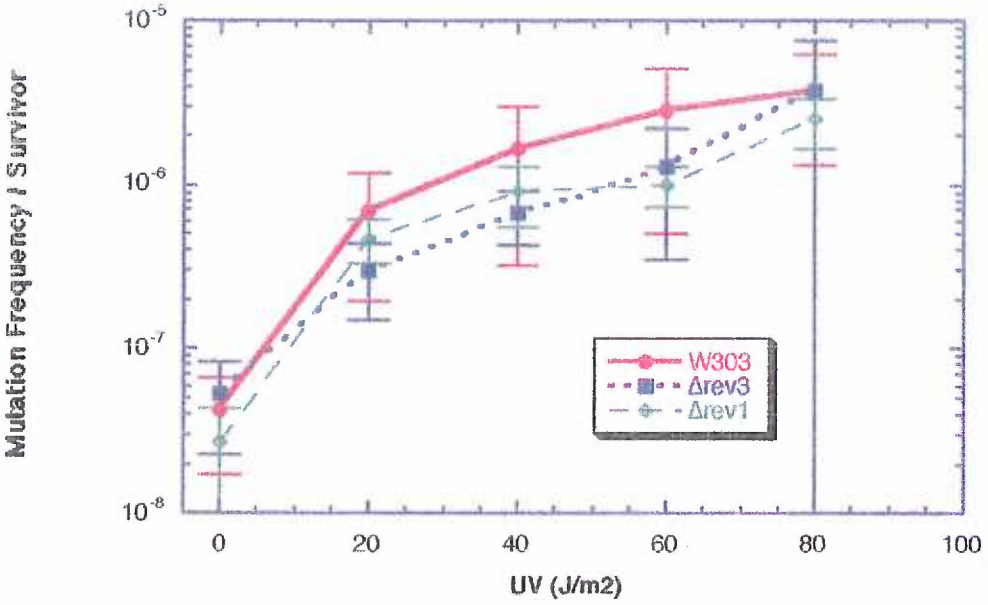
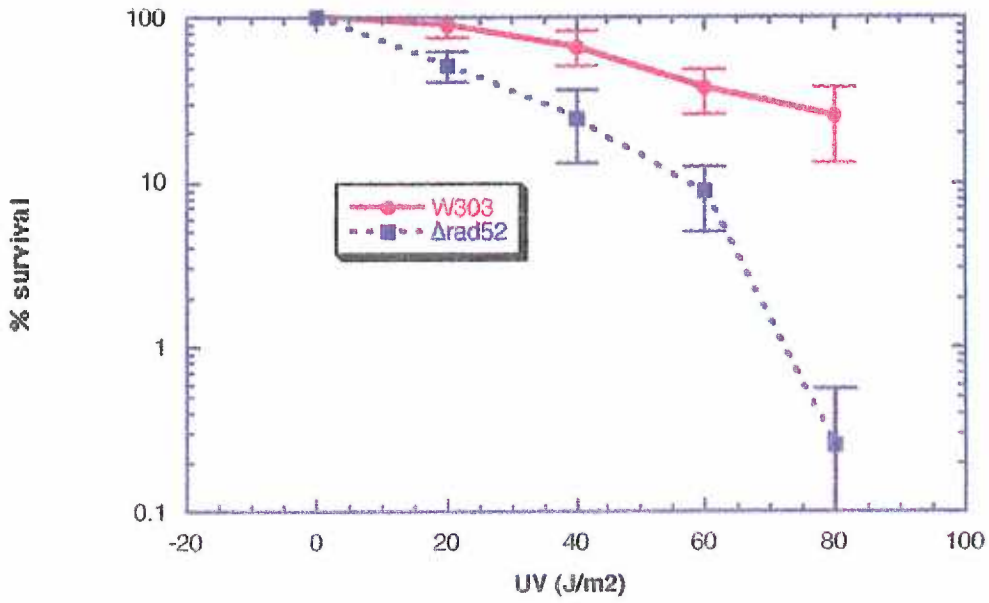


Figure 6-4. UV Induction and Survival in* rev3 *and* rev1 *Mutants.
(A) Survival and (B) *hom3-10* reversion frequency corrected for survival. Error bars represent plus or minus one standard deviation.

A.



B.

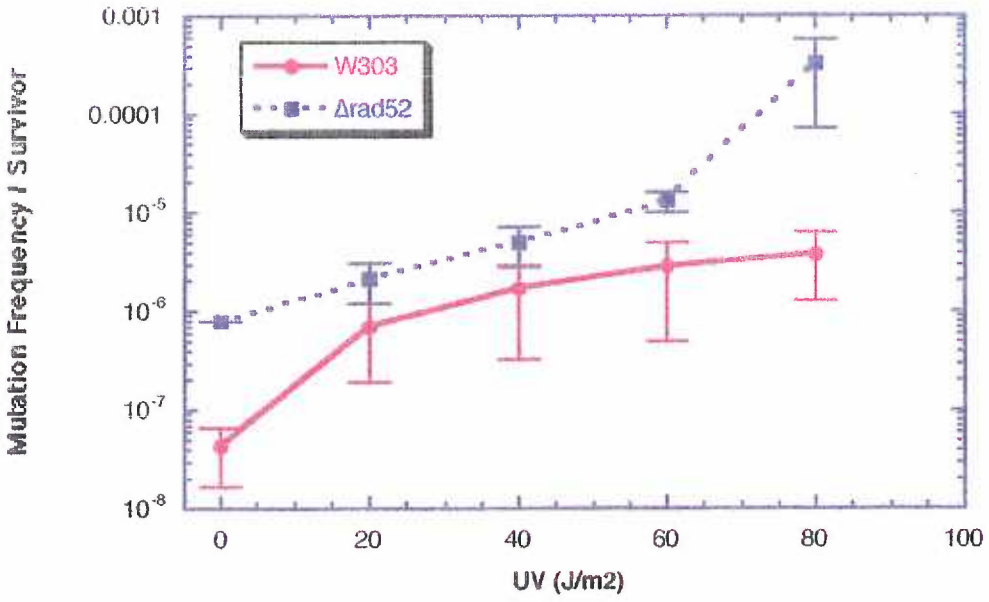


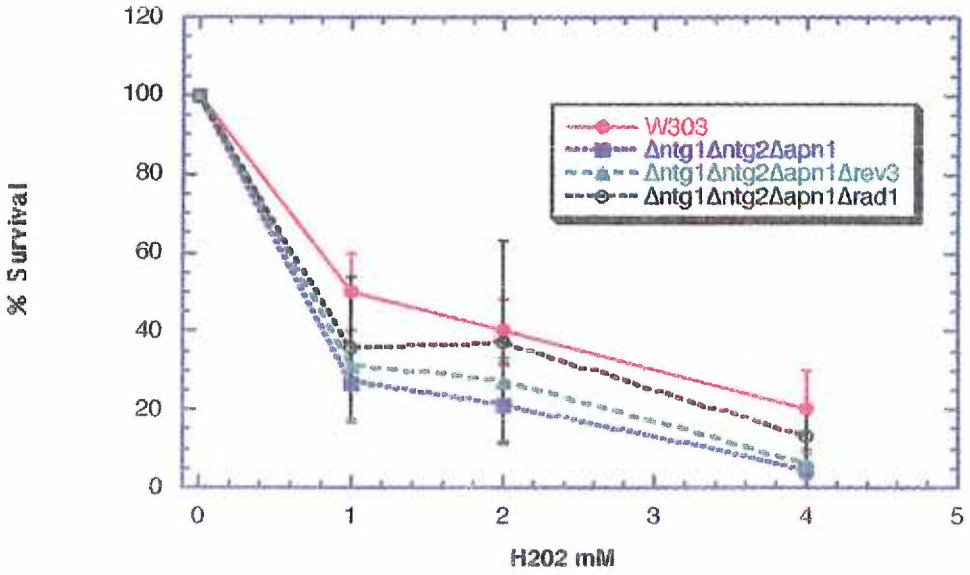
Figure 6-5: UV Survival and Induction in **rad52** *Mutants.*
(A) Survival and (B) *hom3-10* reversion frequency corrected for survival. Error bars represent plus and minus one standard deviation.

BER proteins have a role in induced oxidative DNA damage

Strains deficient in Ntg1p, Ntg2p, and Apn1p were treated with hydrogen peroxide and UV light. At higher doses of hydrogen peroxide, the mutation frequency was 14-fold higher than wild type levels suggesting a protective role of the BER proteins against oxidatively-induced mutagenesis (Figure 6-6). Introduction of the *rev3* mutation into the *ntg1 ntg2 apn1* strain did not decrease the mutation frequency, indicating that the mutations were not resulting from TLS. However, the *ntg1 ntg2 apn1 rad1* mutant showed an induced mutation frequency resembling wild type levels at all doses, indicating that a *RAD1*-dependent pathway is responsible for the 10-fold greater induction (Figure 6-6).

The *ntg1 ntg2 apn1* mutant induction levels at *hom3-10* in response to UV light were similar to wild type, indicating that loss of these proteins does not influence UV-induced mutagenesis (Figure 6-9).

A.



B.

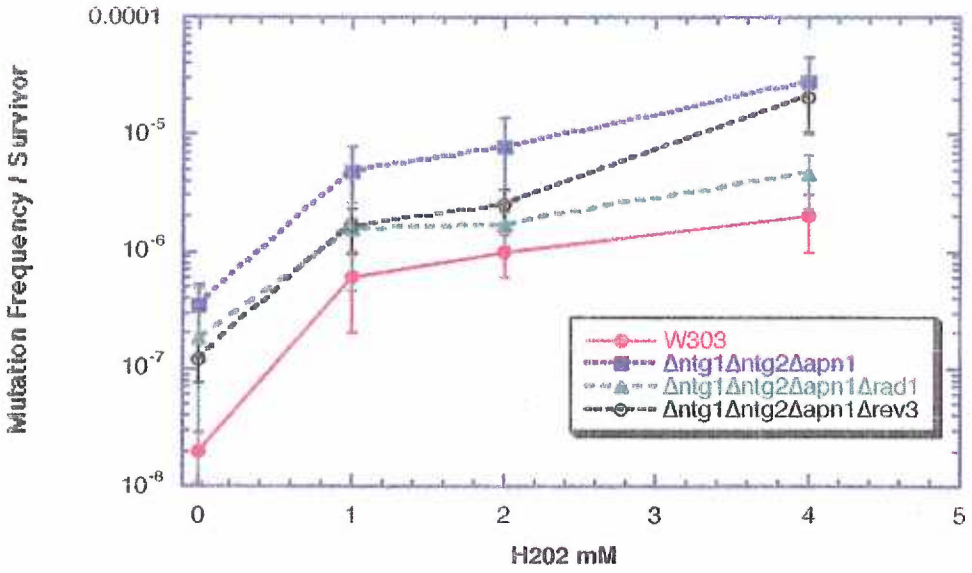


Figure 6-6. Hydrogen Peroxide Induction and Survival in BER Mutants. (A) Survival and (B) *hom3-10* reversion frequency corrected for kill. Error bars represent plus and minus one standard deviation.

Rad1p deficiency results in a decrease of hydrogen peroxide induced *hom3-10* reversion

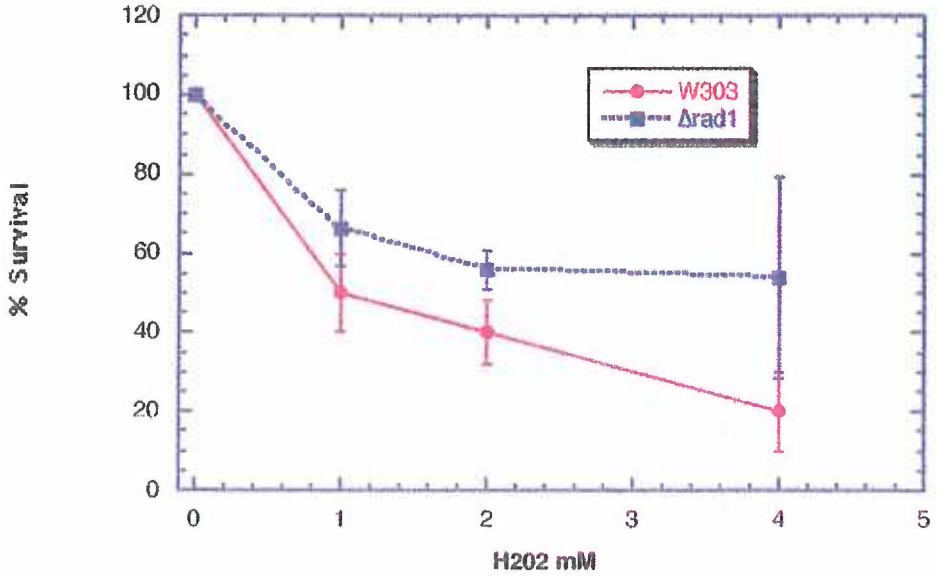
Surprisingly, treatment with hydrogen peroxide in the *rad1* mutants did not significantly induce *hom3-10* reversion (Figure 6-7), suggesting that instability at *hom3-10* in response to oxidative damage is dependent upon the presence of Rad1p.

Since *rad1* mutants are particularly sensitive to UV light (Figure 6-8), I was unable to compare levels of UV-induced *hom3-10* reversion to wild type strains.

MMR and Induced Mutagenesis

I was unable to study the effects of MMR on induced mutagenesis, because the spontaneous mutation rates of MMR mutants surpasses the induced mutation rates (Figure 6-10). I found that in an *mlh1* deficient strain that treatment with hydrogen peroxide did not result in a higher mutation frequency (Figure 6-10).

A.



B.

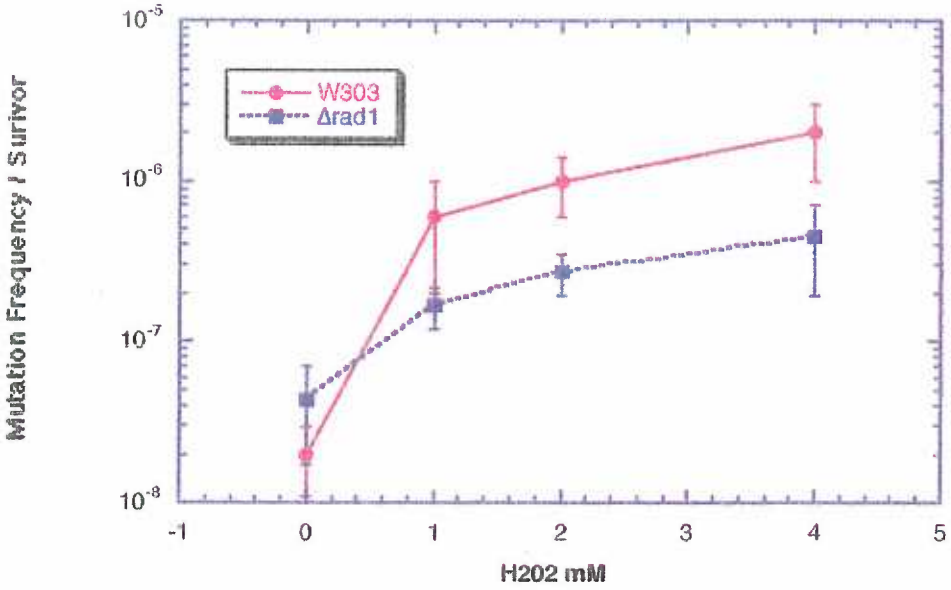


Figure 6-7. Hydrogen Peroxide Induction and Survival of *rad1* Mutants. (A) Survival and (B) hom3-10 reversion frequency corrected for survival. Error bars represent plus and minus one standard deviation.

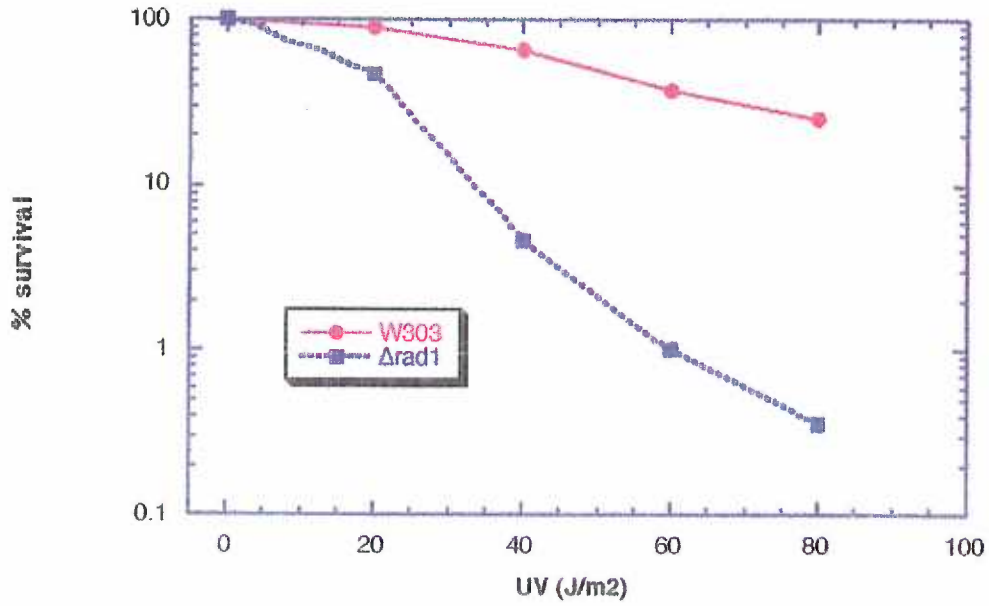


Figure 6-8. UV Survival of **rad1Δ.**

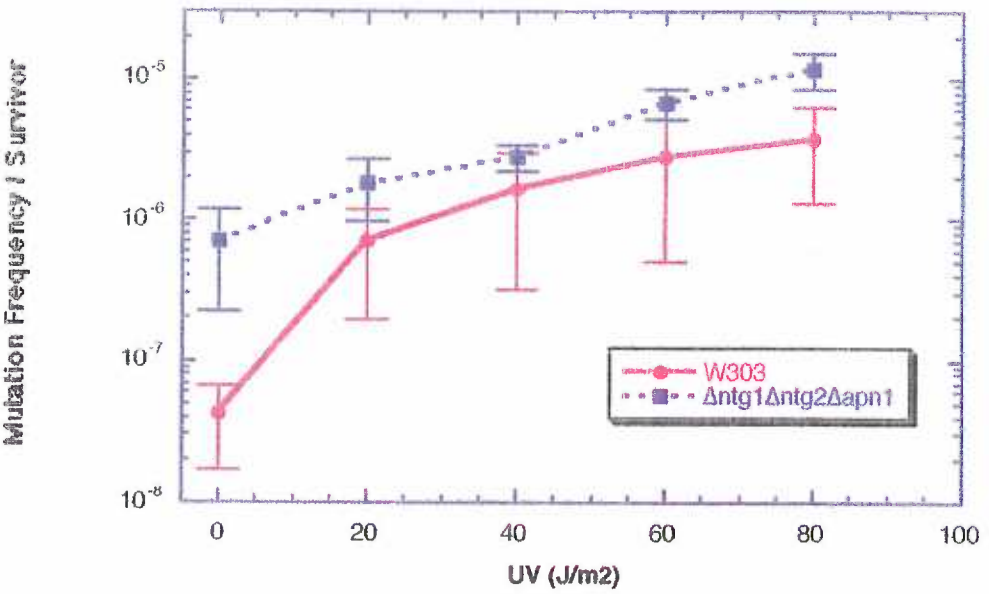


Figure 6-9. Hydrogen Peroxide Induction and Survival of BER Mutant. *hom3-10* reversion frequency corrected for survival. Error bars represent plus and minus one standard deviation.

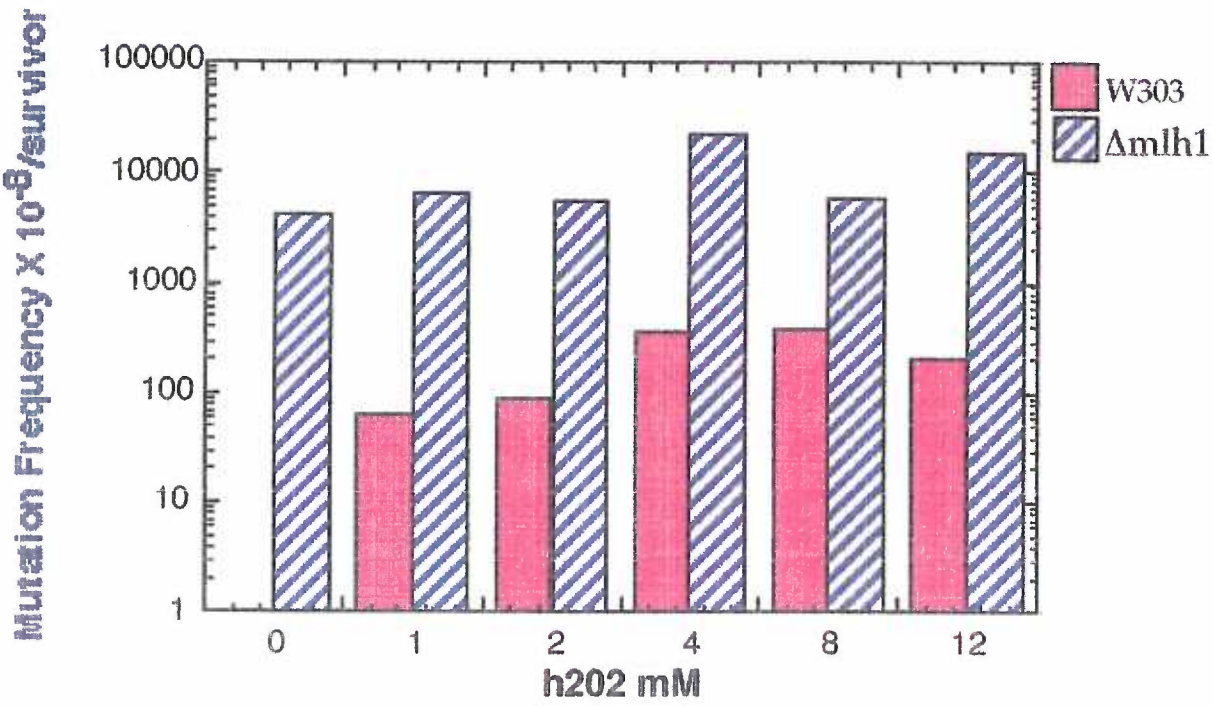


Figure 6-10. Hydrogen Peroxide Induced Mutagenesis at hom3-10 in Δmlh1 Strains.
Mutation frequency determined from 4 different transformants. Mutation frequency was determined after correcting for cell survival

Discussion

My studies have shown that multiple repair pathways are involved in induction of *hom3-10* reversion by the DNA damaging agents hydrogen peroxide and UV light. One explanation for the induction of frameshifts by DNA damaging agents comes from *E. coli* studies that demonstrate that the PolIII holoenzyme can bypass a lesion, but leaves a frameshift behind in the absence of "error-prone" repair (Tomer, et al., 1998). If lesion bypass resulting in frameshifts occurs in yeast is unknown, but recent studies in mammals have shown that polδ can bypass AP sites without a frameshift (Daube SS, Tomer G., and Livneh Z., Biochemistry, in press), indicating that

the frameshift mechanism is not conserved in higher eukaryotes. Another explanation is that "repair" pathways are mutagenic due to repair polymerases slipping in mononucleotide repeats during resynthesis. However, while MSI is common with both UV and oxidative damage, the frameshift or adducts are influenced by different repair pathways, suggesting that there might be multiple causes for frameshifts that are dependent on the type of lesion. UV light can induce pyrimidine dimers, thymine glycols, DNA cross-links and strand breaks, while oxidative damage can result in over 100 different adducts including 8-hydroxyguanine, thymine glycol, and thymidine dihydrodimer (reviewed in Friedberg, 1995).

A proportion of MSI in response to UV light is dependent on the presence of the TLS proteins, Rev1p (this study) and Rev3p (Lawrence and Christensen, 1979, and this study). Rev3p is a subunit of polymerase ζ that allows replication bypass of UV and gamma-induced lesions, which can result in frameshifts (reviewed in Lawrence and Hinkle, 1996). Since Rev1p is a deoxycytidyl transferase, which inserts a cytosine residue opposite damaged DNA (Nelson, et al., 1996), it is unclear how the presence of Rev1p could promote frameshifts. One explanation is that Rev1p might enhance the activity of polζ.

Loss of the Rad52-dependent recombinational pathway resulted in induction of *hom3-10* instability in response to UV light above wild type levels, implicating recombinational repair in preventing some frameshifts in response to UV damage. According to the "sink" model, where BER, NER, TLS, and recombination represent different "drains", stopping flow down the

recombination pathway will direct DNA damage to the other pathways, including the error-prone TLS pathway, resulting in increased mutation. Another source for mutations in the *rad52* mutant could be the error prone single-strand annealing and non-recombinational end-joining pathways, that might deal with UV lesions through recombination in the absence of Rad52p (reviewed in Petes, et al., 1991; Ivanov, et al., 1996). Interestingly, one study found that disruption of *RAD52* resulted in a decreased frequency of UV-induced substitutions, particularly G·C→T·A transitions, a mutation that is often associated with TLS past UV photoproducts (Armstrong, et al., 1994).

In the absence of the BER proteins, Ntg1p, Ntg2p, and Apn1p there is an increase in *hom3-10* revertants upon treatment with hydrogen peroxide, suggesting that Ntg1p/Ntg2p/Apn1p protect the genome from a proportion of the frameshifts caused by free radicals. If induced MSI is due to replication bypass, then perhaps repair of the oxidative damage by Ntg1p, Ntg2p, and Apn1p before replication could protect against frameshifts.

The presence of Rad1p potentiates *hom3-10* reversion in response to oxidizing agents. Is this due to NER or other Rad1-dependent functions? In NER the Rad1p/10p complex catalyzes the 5' incision to the lesion, while Rad2p catalyzes the 3' incision. If the lack of induction in the *rad1* mutant is due to loss of NER, this implicates NER in promoting instability at *hom3-10* in response to oxidative damage. One explanation for the induction is that NER is responsible for repairing the majority of damage caused by oxidative damage, but the repair results in frameshifts, perhaps by DNA polymerase during resynthesis. However, the Rad1p/10p proteins are also involved in

other DNA transactions including recombination (*RAD52*-independent) (reviewed in Friedberg, 1995). Possibly, a Rad1p-dependent error prone recombination pathway generates frameshifts in oxidatively damaged DNA. Additional strains defective in NER will need to be examined to determine if the induction is NER-dependent or resulting from another Rad1p pathway.

The ability of DNA damaging agents to induce MSI is especially interesting in terms of tumor development. In particular, free radicals result from normal cellular metabolism, and are a constant threat to the genome. Without repair of oxidative damage, MSI can ensue, alter oncogenes and tumor suppressors, resulting in malignancy. Understanding the pathways influencing induced MSI could implicate certain candidate genes in which mutations can result in tumor development.

Conclusions

hom3-10 reversion is induced by treatment with hydrogen peroxide and UV light in wild type yeast strains. In the absence of the TLS proteins, Rev1p and Rev3p, *hom3-10* reversion frequency is decreased in response to UV light. In addition, loss of Rad52p results in a significant increase in UV light induced mutagenesis at *hom3-10*.

Increased reversion of *hom3-10* in response to hydrogen peroxide is seen in the BER mutant, *ntg1 ntg2 apn1*. Further, *rad1* deficient strains do not show induction of *hom3-10* reversion in response to hydrogen peroxide. Therefore, BER and Rad1p play a role in oxidatively induced MSI, while TLS and recombinational repair are involved in UV induced MSI.

Concluding Discussion

My initial goal was to use Mlh1p to identify other "players" involved in MMR. However, DNA repair encompasses a network of proteins that "communicate" with each other. This is very evident from the studies by Swanson et al, where at least four different repair pathways are responsible for repairing DNA damage (Swanson et al, 1999). My studies also implicate other repair networks, including MMR "communicating" with BER (Ntg2p) and recombinational pathways (M86p).

However, the networking appears to be specific to the type of lesion, as shown by my studies of MSI in response to DNA damaging agents. BER *(NTG1/NTG2/APN1)*, NER *(RAD1)*, TLS *(REV3)* and recombination *(RAD52)* are all involved in the repair of spontaneous mutations at *lys2BglII* and *CAN1* (Swanson et al, 1999). However, while induction of *hom3-10* reversion by hydrogen peroxide is affected by mutations in *rad1* and *ntg1 ntg2 apn1*, induction of *hom3-10 reversion* by UV is dependent on Rad52p and Rev3p. Therefore, the "communication" between the DNA repair pathways is dependent on the type of DNA damage that needs to be repaired.

MSI has been associated with defects in MMR or DNA polymerases, however, my studies of induced MSI have shown that MSI can result from unexpected sources, including oxidative or UV damage. Future studies will include further defining what repair pathways are involved in induced MSI, including single-strand annealing and NER.

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APPENDIX A: YEAST STRAINS

Strain	Genotype	Reference
W303a (579-10A)	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+	Hannah Klein
W303α (580-10D)	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+	Hannah Klein
ELS100	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10	This study
ELS101	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG-URA3-hisG	This study
ELS102	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG-URA3-hisG	This study
ELS103	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG-URA3-hisG mlh1::Leu2	This study
ELS104	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG apn1::hisG URA3 hisG	This study
ELS105	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG msh2Δ::hisG-URA3-hisG	This study
ELS106	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG apn1::hisG URA3 hisG mlh1Δ::LEU2	This study
ELS107	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG apn1Δ::hisG mlh1Δ::LEU2 rev3Δ::hisG-URA3-hisG	This study
ELS108	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg1Δ::hisG-URA3-hisG	This study
ELS109	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg1Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study

ELS110	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS111	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 apn1Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS112	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG apn1Δ::hisG rev3Δ::hisG-URA3-hisG	This study
ELS113	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 rev3Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS114	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 apn1Δ::hisG rev3Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS115	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 rad1Δ::LEU2	This study
ELS116	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG apn1Δ::hisG URA3 hisG rad1Δ::LEU2	This study
ELS117	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 ntg2Δ::hisG ntg1Δ::hisG apn1::hisG mlh1Δ::URA3 rad1::LEU2	This study
ELS118	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 rev1Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
YHK615-1Ba	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad1Δ::LEU2	H. Klein
YHK615-1Aα	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad1Δ::leu2	H. Klein
YHK614-10Bα	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad52Δ::TRP1	H. Klein
YHK580-5Ba	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad52Δ::TRP1	H. Klein
C17-6B	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad30Δ::HIS3 rev3Δ::hisG	Genetics 147:1557, Woodgate
C17-2B	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rev3Δ::hisG	Genetics 147:1557, Woodgate

C22-4C	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad5Δ::HIS3	Genetics 147:1557, Woodgate
C22-8A	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad30Δ::HIS3 rad5Δ::HIS3	Genetics 147:1557, Woodgate
ELS119	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad30Δ::HIS3 rad5 Δ::HIS3 hom3-10	This study
ELS120	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad30Δ::HIS3 hom3-10	This study
ELS121	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad30Δ::HIS3 rev3Δ::hisG hom3-10	This study
ELS122	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rev3Δ::hisG hom3-10	This study
ELS123	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad5Δ::HIS3 hom3-10	This study
ELS124	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ hom3-10 rev1Δ::hisG-URA3-hisG	This study
ELS125	MATα leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad52Δ::TRP1 hom3-10	This study
ELS126	MATa leu2-3, 112 his3-11,15 ade2-1 ura3-1, trp 1-1 can1-100 RAD 5+ rad52Δ::TRP1 hom3-10	This study
KCY135	MATa leu2-3, 112 his3-11,15 ura3-1 trp1-1 ade2-1 can1-100 chromoXV gene::HIS3	H. Klein
GCY35	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4	G. Crouse
ELS201	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 apn1Δ::hisG-URA3-hisG	This study
ELS202	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 ntg1Δ::hisG-URA3-hisG ntg2Δ::hisG	This study

ELS203	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 ntg1Δ::hisG ntg2Δ::hisG apn1Δ::hisG-URA3-hisG	This study
ELS204	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 mlh1Δ::URA3	This study
ELS205	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 ntg1Δ::hisG ntg2Δ::hisG mlh1Δ::URA3	This study
ELS206	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 apn1Δ::hisG mlh1Δ::URA3	This study
ELS207	MATa ura3-52 trp1 hom3-10 ade2 met13 his3-kpn met4 ntg1Δ::hisG ntg2Δ::hisG apn1Δ::hisG mlh1Δ::URA3	This study
YMH4	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA)	Hampsey, Genetics 128: 59-67
YMH6	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG)	Hampsey, Genetics 128: 59-67
YMH7	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TAC AT - GC)	Hampsey, Genetics 128: 59-67
ELS301	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) ntg1Δ::hisG-URA3-hisG	This study
ELS302	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) ntg1Δ::hisG-URA3-hisG ntg2Δ::hisG	This study
ELS303	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) mlh1Δ::LEU2	This study
ELS304	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) ntg1Δ::hisG-URA3-hisG ntg2Δ::hisG mlh1Δ::LEU2	This study
ELS305	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) sod1Δ::hisG-URA3-hisG	This study

ELS306	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) msh2Δ::hisG-URA3-hisG	This study
ELS307	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) apn1Δ::hisG-URA3-hisG	This study
ELS308	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) ntg1Δ::hisG ntg2Δ::hisG apn1Δ::hisG-URA3-hisG	This study
ELS309	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA GGC) GC – TA) ntg1Δ::hisG ntg2Δ::hisG apn1Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS401	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) ntg1Δ::hisG-URA3-hisG	This study
ELS402	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) ntg1Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS403	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) ntg1Δ::hisG ntg2Δ::hisG-URA3-hisG	This study
ELS404	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) mlh1Δ::LEU2	This study
ELS405	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) ntg1Δ::hisG ntg2Δ::hisG-URA3-hisG mlh1Δ::LEU2	This study
ELS406	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) sod1Δ::hisG-URA3-hisG	This study
ELS407	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) msh2Δ::hisG-URA3-hisG	This study
ELS408	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) apn1Δ::hisG-URA3-hisG	This study
ELS409	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TTC AT - CG) ntg2Δ::hisG-URA3-hisG mlh1Δ::LEU2	
ELS501	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TAC AT - GC) mlh1Δ::LEU2	This study

ELS502	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TAC GC) ntg1Δ::hisG-URA3-hisG mlh1Δ::LEU2	AT -	This study
ELS503	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TAC GC) ntg1Δ::hisG ntg2Δ::hisG-URA3-hisG mlh1Δ::LEU2	AT -	This study
ELS504	MATa cycl-706::CYH2 cyc7-63 ura3-52 leu2-3,112 cyh2 (codon 21-22 CAA TAC GC) sod1Δ::hisG-URA3-hisG	AT -	This study
SJR381	MATα ade2-101 his3Δ200 URA3 100% c_2 repeats lys2ΔRV::hisG leu2R		Mol. and Cell. Biology 16(3) :1085-1093 Datta, Adjiri, New, Crouse, Jinks-Robertson
ELS601	MATα ade2-101 his3Δ200 URA3 100% c_2 repeats lys2ΔRV::hisG leu2R m86Δ::LEU2		This study
ELS602	MATα ade2-101 his3Δ200 URA3 100% c_2 repeats lys2ΔRV::hisG leu2R mlh1Δ::LEU2		This study
SJR486	MATα ade2-101 his3Δ200 URA3 92% c_2 repeats lys2ΔRV::hisG leu2-R		Mol. and Cell. Biology 16(3) :1085-1093 Datta, Adjiri, New, Crouse, Jinks-Robertson
ELS701	MATα ade2-101 his3Δ200 URA3 92% c_2 repeats lys2ΔRV::hisG leu2-R mlh2Δ::LEU2		This study
ELS702	MATα ade2-101 his3Δ200 URA3 92% c_2 repeats lys2ΔRV::hisG leu2-R m86Δ::LEU2		This study
ELS703	MATα ade2-101 his3Δ200 URA3 92% c_2 repeats lys2ΔRV::hisG leu2-R mlh1Δ::LEU2		This study
BY26	MATa his1		D. Lycan
BY27	MATα his1		D. Lycan

L40	MATa his3Δ200 trp1-901 leu2-3, 112 ade2 lys2::(lexAop)4 - His 3 gal4 gal80 ura3::(lexAop)8 -lac 2 (incomplete genotype)	S. Hollenberg
AMR70	MAT a his3 lys2 trp1 URA3::$(lexAop)_8$-lacZ GAL4 gal80?? (incomplete genotype)	S. Hollenberg

APPENDIX B: OLIGONUCLEOTIDES

PRIMERS	SEQUENCE (5'->3')	PURPOSE[a]
hisG	ACCATGAGCTTCAATACCCTG	TD
MO37 anchor MO37 untargeted	ATGTATTGGGCGCTTTGC GGGAATTCTCTCGTTACCTGG	TD
YAB5 anchor YAB5 untargeted	GTGCCCGTGGTATCGTTAG CAGGTCCTTCTTGGGGTG	TD
M86 anchor M86 untargeted	GGCGTCTTACCCATTTCG ACCATGAGCTTCAATACCCTG	TD
SOD1 Anchor SOD1 Untargeted	GGTCATTAGGGCTTATTCCCG TGGGTAACGTAAAGACGGACG	TD
MO37 forward MO37 reverse	AAGCTGCGAGACAACACC GTAGATACCGACGACGGAAC	P
HOM3 FOR HOM3 REV	CTTTCCTGGTTCAAGCATTG GGATCAGCAGTAAATATACC	A
M86 Probe FOR M86 Probe rev	CAACCCGGTGCCTGGGAG CATGCTTTCTCTTGCGGGGC	P
3'-yMLH2 for 3'-yMLH2 rev SCPTP yMLH2 (rev)	CCTGTTGTCATGCGACTAAA CTGGGTCCATATAAGCCACA ACAATGGATGAGCCCAAGTA CTGGGTCCATATAAGCCACA	P
yMLH2 probe-for yMLH2 probe-rev	GGACCGTGGTTCTGAGTA CGACGACTCATGTGTGTT	P
YAB5 PROBE for YAB5 PROBE rev	AAAATTCAATCTGGCACGGC TGCTCCTGAGTTGAACCCG	P
MO37 PROBE-FOR MO37 PROBE-REV	AAGCTGCGAGACAACACC GTAGATACCGACGACGGAAC	P
MO37-left for MO37-left rev (XbaI)	CCTGATGACGATATAAAG AGACGCTCTAGACCTACTTTCCTCTCTCAT	C
MO37-right for MO37-right rev	AGACGCGGATCCGCATTGGTTGGACACGG T AGACGCGAATTCACTCCAACCCTAAAGGG C	C

MO37 Forward	AGACGCCTCGAGAGATGAGAGAGGAAAGTAGG	A
MO37 Reverse	AGACGCCTCGAGAGCTATTTTTTCTTGTGTCT	
YAB5 Forward	AGACGCCTCGAGAGATGCAAAAGATCAGTAAA	A
YAB5 Reverse	AGACGCCTCGAGAGTTAGTCCTCTACTTTAAC	
YAB5-lt forward	AGACGCCCGCGGTAGAGACAGATCTCACGACAGC	C
YAB5-lt Reverse	AGACGCTCTAGAGCATATTTTTTTTTTTTTTGGG	
YAB5-rt forward	AGACGCGAATTCAAGAAACTATGGTCAAACTGG	C
YAB5-rt reverse	AGACGCGTCGACAAGATCTTACCTGCTGTGCAG	
GAL4F-2522	GGACCAAACTGCGTATAACG	S
GAL4F-2942	CGTTTGGAATCACTACAGGG	S
GAL4R-2673	CGGGGTTTTTCAGTATCTACG	S
pACT forward	CTATTCGATGATGAAGATACCC	A
pACT reverse	GATGCTAAGTATCTAGAGAGCTC	

TD Targeting Detection
P Probe
S Sequencing
A Amplification
C Cloning