NOTE TO USERS

University of Alberta

A novel DNA polymerase delta mutant of *Saccharomyces cerevisiae* with sensitivity to phosphonoacetic acid

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Doctor of Philosophy

in

Molecular Biology and Genetics

Department of Biological Sciences

Edmonton, Alberta

Fall 2004

Your file Votre référence
ISBN: 0-612-95966-X
Our file Notre référence
ISBN: 0-612-95966-X

ACKNOWLEDGEMENTS

First, I would like to sincerely thank my supervisor, Dr. Linda Reha-Krantz, for providing me the opportunity to work on this project and for her continuous advice. I am also most grateful to my supervisory committee members, Drs. Shelagh Campbell and Susan Andrew, for their guidance, suggestions and support in my program.

I appreciate my lab colleagues, Dr. Elisabeth da Silva, Kelly Murphy, Dongling Zhao, Zerrin Ozum, Shelli Stocki, Dr. Subhrangsu Mandal and Laura Tsujikawa, for their help, support and technical expertise. Special thanks to Kelly Murphy for providing experimental data of KY strains and other unpublished data that are related to this study, also for her helpful discussions and teaching me the techniques in yeast biology. I also thank Zhigang Jin for sharing expertise in cell cycle studies and his friendship. Special thanks to Dr. Peter Hurd, who introduced the "Bootstrap" method to me and provided assistance in the statistical calculations.

I wish to thank the Department of Biological Sciences for Graduate Teaching Awards, tuition supplements and overall support. I am grateful to Patricia Murray and Lisa Ostafichuk in the Molecular Biology Service Unit for technical support. Many thanks to others in the Molecular Biology and Genetics group.

I thank Drs. T. Petes, D. Stuart, P. Burgers, B. Andrews and M. Pickard for providing experimental materials.

Finally I would like to express my deepest gratitude to my parents, grandmother and my sister for their everlasting and unconditional love and understanding.

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LIST OF ABBREVIATIONS

aa	Amino acid
ATP	Adenosine Triphosphate
bp	Base pair(s)
BSA	Bovine Serum Albumin
CDC	Cell division cycle
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxynucleoside triphosphate
DSB	Double strand break
DTT	1,4-Dithiothreitol
dsDNA	Double-stranded DNA
DUN	Damage uninducible
EDTA	Ethylenediamine tetraacetic acid
Exo	Exonuclease
5-FOA	5-Fluro-orotic acid
HU	Hydroxyurea
kb	Kilobase
kDa	Kilodalton
L	Leucine
LB	Luria-Bertani medium
Leu	Leucine
M	Methionine
Met	Methionine
MLH	MutL homolog
MMR	Mismatch repair

MMS	Methyl methanesulphonate
MSH	MutS homolog
Mut	Mutator
nt	Nucleotide
PAA	Phosphonoacetic acid
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PMS	Post Meiotic segregation
pol	Polymerase
RAD	Radiation sensitivity
REV	(defective) Reversion
RP-A	Replication protein A
RFC	Replication factor C
RNase	Ribonuclease
RNR	Ribonucleotide reductase
SD	Synthetic dextrose medium
SDS	Sodium Dodecyl Sulfate
SSA	Single stand annealing
ssDNA	Single-stranded DNA
TLS	Trans-lesion synthesis
ts	Temperature sensitive
TE	Tris-EDTA buffer
UV	Ultraviolet
UVC	Ultraviolet-C
YPD	Yeast extract-peptone-dextrose medium

LIST OF SYMBOLS

α	alpha
β	beta
δ	delta
ε	epsilon
γ	gamma
η	eta
ζ	zeta
Δ	deletion
::	insertion

CHAPTER 1 INTRODUCTION

Eukaryotic DNA replication is a complex but well-coordinated and accurate process, in which DNA polymerases play a key role. DNA polymerase (pol) δ, together with DNA pol α and ε, are three essential polymerases for eukaryotic DNA replication. In order to focus on DNA pol δ among all of the other essential DNA polymerases, I engineered a novel *Saccharomyces cerevisiae* mutant strain, in which the DNA pol δ can be inhibited by an antiviral drug, phosphonoacetic acid (PAA). This PAA-sensitive DNA pol δ mutant was used to investigate how replication abnormalities generated by DNA pol δ are corrected in yeast cells.

In this chapter, I briefly review the high fidelity feature of DNA polymerases, why PAA was selected as an inhibitor, the rationale in the mutant construction, our current understanding of DNA replication fork biology, the role of post-replication mismatch repair (MMR) system in ensuring genome integrity, and cellular responses to stalled replication forks. While this review demonstrates that there is considerable knowledge about DNA pol δ, there are still many questions remaining to be answered, which can be addressed with a chemical sensitive DNA pol δ.

1.1 DNA polymerase: an overview

DNA polymerases are the central enzymes in genome replication. DNA polymerases are classified into several families based on their amino acid sequence similarities (Braithwaite and Ito, 1993). Family B (or α-like) is a large group of DNA polymerases, which includes many genome-replicating enzymes, such as eukaryotic DNA pol α, δ and ε, bacteriophage T4 DNA polymerase, herpes simplex virus (HSV) DNA polymerase. Some members of this family, for example, T4 DNA polymerase, have been extensively studied as a model system for DNA polymerase. Although there is only limited sequence conservation, the conserved motifs in Family B DNA polymerases have the same linear

arrangement (Wong *et al.*, 1988; Delarue *et al.*, 1990). Mutational and structural studies indicate functional importance of those motifs.

1.1.1 High fidelity of DNA polymerase

Maintainance of genome integrity from generation to generation requires high fidelity of DNA polymerase function during DNA replication. The genome-replicating DNA polymerases achieve this precision at two levels: (1) high accuracy in nucleotide incorporation during 5'→ 3' polymerization and, (2) 3'→ 5' proofreading exonuclease activity to remove misincorporated nucleotides. Accurate incorporation is achieved by ensuring shape complementarity between the template and incoming nucleotides (Moran *et al.*, 1997). DNA polymerases tend to synthesize DNA with a misincorporation rate of approximately one per 10^4 to 10^5 nucleotides incorporated (Kuchta *et al.*, 1988; Perrino and Loeb, 1989; Wong *et al.*, 1991). When a nucleotide is incorrectly incorporated, most DNA polymerases utilize 3'→ 5' exonuclease activity to correct the error, providing a second chance for correct incorporation. In general, the exonuclease activity increases fidelity in DNA replication by approximately 100-fold or even higher (DiFrancesco *et al.*, 1984; Reha-Krantz *et al.*, 1991; Morrison *et al.*, 1993; Tran *et al.*, 1999). Consequently, inactivation of proofreading causes dramatic increases in mutation rates. In addition to proofreading, the 3'→ 5' exonuclease activity of DNA polymerases may also contribute to genome integrity during other processes, for example, preventing the excessive formation of 5'-flap by strand displacement synthesis during DNA replication and repair synthesis (Jin *et al.*, 2001; Jin *et al.*, 2003).

It has been implicated in mammals that compromised DNA polymerase replication fidelity causes deleterious consequences, although the detailed mechanisms are still unknown. For example, transgenic mice with deficient proofreading of DNA pol δ display high incidence (up to 94%) of epithelial and other types of cancers (Goldsby *et al.*, 2002). In human, DNA pol δ variants have been identified in colorectal tumor cells

(da Costa *et al.*, 1995; Flohr *et al.*, 1999). Some of these variants have mutations in the exonuclease domains, which presumably result in elevated mutation rates and contribute to tumorigenesis. Another example implying DNA polymerase deficiency in cancer development is the XPV (xeroderma pigmentosum variant) patient, who has a deficient human DNA pol η and increased susceptibility in developing skin cancer (Johnson *et al.*, 1999; Masutani *et al.*, 1999).

1.1.2 Coordination between polymerization and proofreading

Coordination between the polymerase and exonuclease activities of DNA polymerases is required in order to achieve high DNA replication fidelity. It has been proposed that the status of the primer-terminus may play a critical role in determining the choice between the polymerization and proofreading activities of DNA polymerase during DNA replication (reviewed by Johnson, 1993). While elongation at the correctly synthesized primer-terminus is fast, the presence of a misincorporation significantly reduces elongation rate, when proofreading is likely to occur.

Structural studies of DNA polymerases with proofreading exonuclease activity show that the polymerase active center and exonuclease active center are spatially distinct (Beese *et al.*, 1993; Wang *et al.*, 1997; Doublie *et al.*, 1998; Franklin *et al.*, 2001). In order for proofreading to occur, the primer terminus must transfer from the polymerase active center to the exonuclease active center and strand separation for at least two to three nucleotides must take place (Cowart *et al.*, 1989; Marquez and Reha-Krantz, 1996; Baker and Reha-Krantz, 1998), which is followed by the hydrolysis reaction. Disturbances in the coordination of these events can result in alterations in replication fidelity. For example, active site "switching" mutants have been isolated in T4 DNA polymerase (Stocki *et al.*, 1995). Those mutants have reduced ability to transfer the primer terminus from polymerase to exonuclease active sites and as a consequence, display lower replication fidelity (mutator phenotype) compared with the wildtype. On

the other hand, mutants with increased replication fidelity (antimutator phenotype) have also been identified for both T4 DNA polymerase and the DNA polymerase III of *Escherichia coli* (Drake *et al.*, 1969; Fijalkowska *et al.*, 1993; Reha-Krantz and Nonay, 1994). As revealed by the *in vitro* assays, the antimutator mutants have shifted balance between polymerization and proofreading activities towards increased proofreading, i.e., they demonstrate excessive 3'→ 5' exonuclease activity compared with the wildtype enzyme (Muzyczka *et al.*, 1972; Reha-Krantz and Nonay, 1994). Although higher fidelity in replication is achieved in those mutants, it also results in disadvantages. The antimutator mutants replicate DNA more slowly and require a higher deoxynucleotide triphosphate (dNTP) concentration to support their genome replication (Beauchamp and Richardson, 1988). These results suggest that elegant mechanisms are required to coordinate the polymerase and exonuclease activities, so that DNA replication is performed in an efficient but also accurate manner.

1.2 Objectives of this study

In this study, the DNA pol δ of budding yeast *S. cerevisiae* was chosen to explore the function of eukaryotic DNA pol δ in genome replication. There are several reasons why *S. cerevisiae* is a good model organism for eukaryotic DNA replication studies: first, it is a genetically tractable organism. Classic genetic studies of *S. cerevisiae* already provide considerable information in genetic background of this organism and generate numerous mutants that can be used for studying DNA replication [for example, the *cdc* (cell division cycle) mutants isolated by L. Hartwell and his colleagues]. In addition, the whole genome sequence of *S. cerevisiae* was also reported (Cherry *et al.*, 1997). More recently, a complete collection of mutant strains with deletion of each non-essential gene was developed (Tong *et al.*, 2001), which provides a means to study DNA replication genome-wide and the interactions between DNA replication and other processes. Second, yeast cells can be genetically modified readily (e.g., inactivation of a non-essential gene,

replacement of an amino acid residue with another, etc.). Third, significant conservation is identified between *S. cerevisiae* and mammalian DNA replication. For example, the *S. cerevisiae* DNA pol δ has 54% and 49% amino acid identity to mouse and human DNA pol δs, respectively (Cullmann *et al.*, 1993). Therefore discoveries found in *S. cerevisiae* DNA replication will also shed light on our understandings in human DNA replication.

In *S. cerevisiae*, three DNA polymerases are required for cell viability: DNA pol α (Johnson *et al.*, 1985), DNA pol δ (Boulet *et al.*, 1989; Sitney *et al.*, 1989) and DNA pol ε (Morrison *et al.*, 1990). One open question regarding DNA pol δ is what is the precise role that DNA pol δ plays during genome replication. One obstacle in answering this question is that currently there is no effective tool by which an essential DNA polymerase can be studied specifically. My objective in this study is to develop a method that can efficiently and specifically inhibit *S. cerevisiae* DNA pol δ, such that DNA pol δ can be investigated independently. To achieve this goal, information gained from previous studies of T4 DNA polymerases in our laboratory was used to guide investigations of *S. cerevisiae* DNA pol δ. The rationale for this guidance is that both T4 DNA polymerase and *S. cerevisiae* DNA pol δ share six regions of sequence homology and are classified into the same family, i.e., Family B (or α-like) DNA polymerases (Wong *et al.*, 1988; Braithwaite and Ito, 1993). Striking sequence similarity is found in Motif A (Figure 1.1), which suggests functional conservation between these two DNA polymerases. Structural studies on the bacteriophage RB69 DNA polymerase (a Family B DNA polymerase) indicate that Motif A is in the polymerase active center (Franklin *et al.*, 2001), implying the functional importance of this motif.

Among T4 DNA polymerase mutants previously characterized, the L412M mutant is particularly interesting. This mutation was first isolated as a second-site suppressor that suppresses the sensitivity to reduced dNTP concentration of some T4 DNA polymerase mutants (Reha-Krantz and Nonay, 1994; Stocki *et al.*, 1995). It has a single amino acid substitution (leucine to methionine) at the 412th position in Motif A. The L412M

substitution confers T4 phage sensitivity to phosphonoacetic acid (PAA) (Reha-Krantz *et al.*, 1993). PAA is an antiviral drug used for treatment of HSV and vaccinia virus infection (Shipkowitz *et al.*, 1973; Sridhar and Condit, 1983) because of its selective inhibition on viral DNA polymerase, i.e., the viral DNA polymerase is over 400 times more sensitive than the host DNA polymerase as shown by *in vitro* studies (Mao *et al.*, 1975). Biochemical studies suggest that PAA inhibits the L412M-T4 DNA polymerase from translocation along the template (Reha-Krantz and Nonay, 1993). Because wildtype yeast cells are not PAA-sensitive (Reha-Krantz, unpublished results), it suggests that the wildtype DNA pol α, δ and ε are not inhibited by PAA, at least at the tested concentrations, which makes it possible to construct a PAA-sensitive DNA pol δ mutant.

Based on the sequence similarity between T4 DNA polymerase and yeast DNA pol δ in Motif A (Figure 1.1), the yeast counterpart of T4 L412M mutant, *pol3-612*, was predicted to be PAA-sensitive. If that is true, then the "PAA and *pol3-612*" system can be used to probe the precise function of DNA pol δ, since PAA specifically inhibits the L612M-DNA pol δ.

In addition to PAA-sensitivity, the L412M T4 mutant also has a moderate mutator phenotype for base substitution (Reha-Krantz and Nonay, 1994). Biochemical analysis indicates that the Leu to Met substitution switches partitioning between polymerase and exonuclease activity towards more polymerase activity (Reha-Krantz and Nonay, 1994), possibly due to its increased ability to bind DNA in the polymerase active center (Fidalgo da Silva *et al.*, 2002).

The L612M substitution in *S. cerevisiae* DNA pol δ was also predicted to disturb the balance between polymerase and exonuclease activity of yeast DNA pol δ and thereby generate a mutator phenotype. Confirmation of these predictions would suggest that these two DNA polymerases are functionally conserved, beyond their similarities in amino acid sequence.

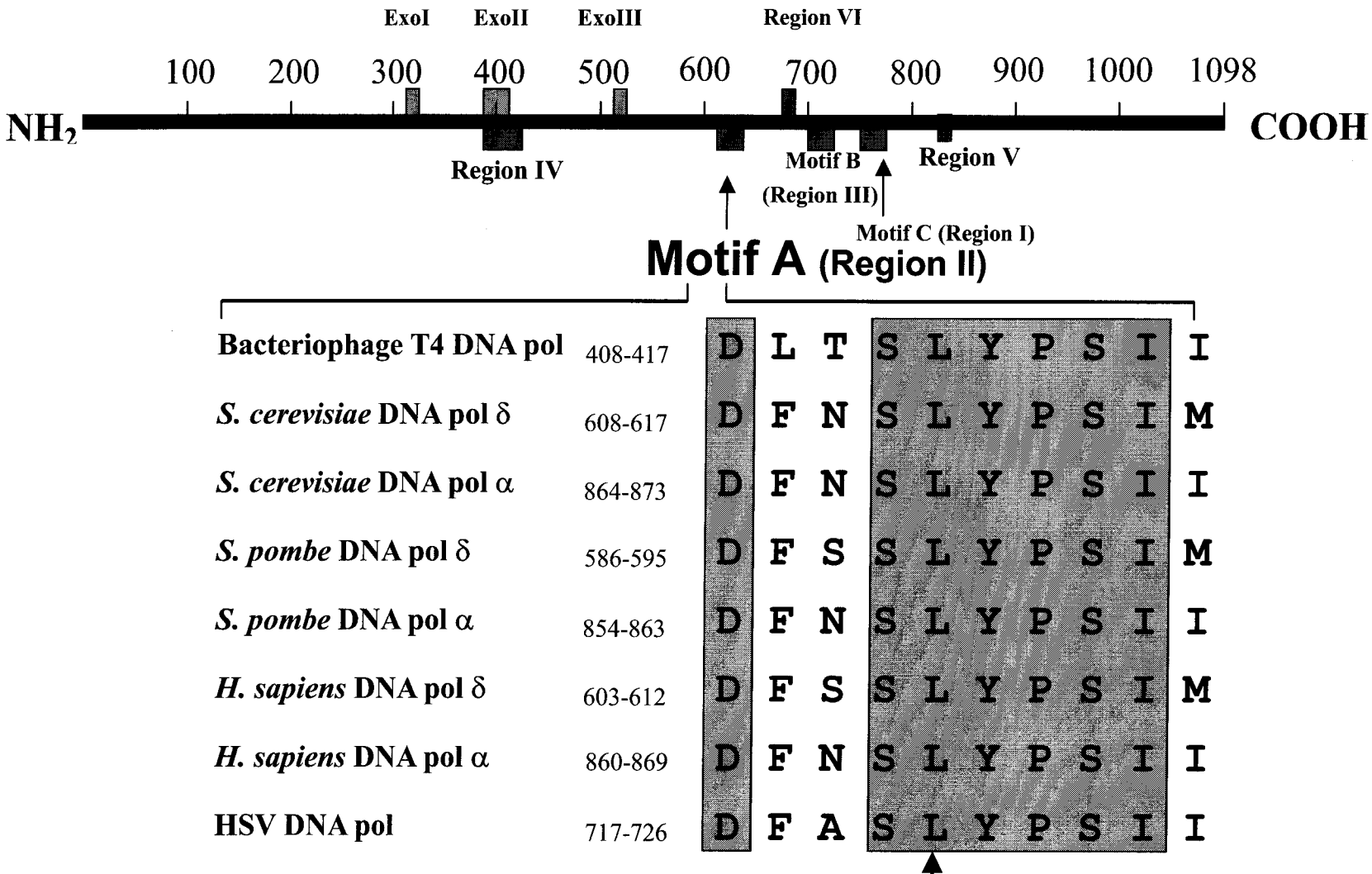

Figure 1.1. Sequence alignment of Motif A in Family B DNA polymerases. The conserved regions (Region I to VI) and exonuclease motifs (Exo I to III) are shown in boxes. Region I, II and III are also named as Motif C, A and B, respectively. Sequence alignment is taken from Braithwaite and Ito (1993). The numbering of the amino acids shown above the bar is based on the sequence of *S. cerevisiae* DNA pol δ.

Creation of a PAA-sensitive *S. cerevisiae* DNA pol δ mutant can also provide a useful strategy for selecting antimutator DNA polymerase mutants in eukaryotes. Previous studies in T4 PAA-sensitive DNA polymerase mutants demonstrated that selection for second-site mutations that suppressed the PAA-sensitivity was a valid strategy to select for antimutator T4 DNA polymerase mutants (Reha-Krantz and Nonay, 1994; Reha-Krantz and Wong, 1996). If there is a functional conservation between T4 DNA polymerase and *S. cerevisiae* DNA pol δ, then selection for second-site mutations that suppress the PAA-sensitivity caused by the L612M substitution may also be useful to isolate antimutator DNA polymerase mutants in eukaryotes. Studies in antimutator DNA polymerases provide insights into the mechanisms by which high replication fidelity is achieved (reviewed by Reha-Krantz, 1995; Reha-Krantz, 1998; Schaaper, 1998).

1.3 DNA replication forks in *S. cerevisiae*

1.3.1 Essential DNA polymerases in budding yeast

Studies in eukaryotic DNA polymerase started from biochemical purification of DNA polymerase α from calf thymus in 1960. In budding yeast, two nuclear DNA polymerases, DNA polymerase I and II, were biochemically purified in 1970's (Wintersberger and Wintersberger, 1970; Wintersberger, 1974), which are now named DNA pol α and pol ε, respectively. The third *S. cerevisiae* DNA polymerase, DNA pol III (now is called DNA pol δ) was purified in 1988 (Bauer *et al.*, 1988). All three DNA polymerases are shown to be biochemically and immunologically distinct (Chang, 1977; Burgers and Bauer, 1988). Using reverse genetics, researchers identified genes encoding DNA polymerases α, δ and ε in *S. cerevisiae* and demonstrated that all three enzymes are essential for viability (Johnson *et al.*, 1985; Boulet *et al.*, 1989; Sitney *et al.*, 1989; Morrison *et al.*, 1990). DNA pol α and δ were discovered in L. Hartwell's temperature-sensitive *cdc* mutants collection that block at specific stage of cell cycle at restrictive temperature (Hartwell *et al.*, 1973), with DNA pol α in the *cdc17* mutant and

DNA pol δ in the *cdc2* mutant (Johnson *et al.*, 1985; Boulet *et al.*, 1989; Sitney *et al.*, 1989).

Intensive studies indicate that *S. cerevisiae* DNA pol α has a four-subunit structure. Unlike DNA pol δ and ε, DNA pol α does not possess 3' → 5' exonuclease activity. It is generally believed that DNA pol α (and its associated primase) is responsible for replication initiation of leading strand and Okazaki fragment synthesis (Figure 1.2). *S. cerevisiae* DNA pol ε is a complex with four subunits and DNA pol δ has three subunits. While the precise roles of DNA pol δ and ε in genome replication remain elusive (see below), it has been proposed that they carry out strand elongation at both leading and lagging strands once the short RNA-DNA primer has been synthesized by DNA pol α (Figure 1.2). In addition to genome replication, DNA pol δ and pol ε are also required for DNA recombination and repair synthesis (reviewed by Sugino, 1995; Burgers, 1998).

Besides the catalytic subunit (encoded by the *POL3* gene), the DNA pol δ in *S. cerevisiae* has two subunits encoded by the *POL31* and *POL32* genes, respectively. Whereas *POL3* and *POL31* are essential genes, *POL32* is dispensable for viability (Gerik *et al.*, 1998). A fourth subunit, p12, was identified in humans but not in budding yeast (Liu *et al.*, 2000). The DNA sequence upstream of the *POL3* gene contains a putative MCB (MluI cell cycle box) element, suggesting that transcription of *POL3* is cell-cycle regulated by the MCB-binding factor MBF at G1/S phase, in agreement with its critical role for chromosome replication (Boulet *et al.*, 1989). Similar regulation of expression is also found with DNA pol ε and α (Araki *et al.*, 1992). Reduction in the level of DNA pol δ leads to increased mutation rates and sensitivity to DNA damage, suggesting that genome stability requires that both the quantity and activity of DNA pol δ be properly regulated (Kokoska *et al.*, 2000).

Studies of a reconstituted *in vitro* SV40 replication system demonstrate that DNA pol δ can replicate the leading strand and complete Okazaki fragment synthesis of the lagging strand initiated by the DNA pol α-primase complex (Waga and Stillman, 1994),

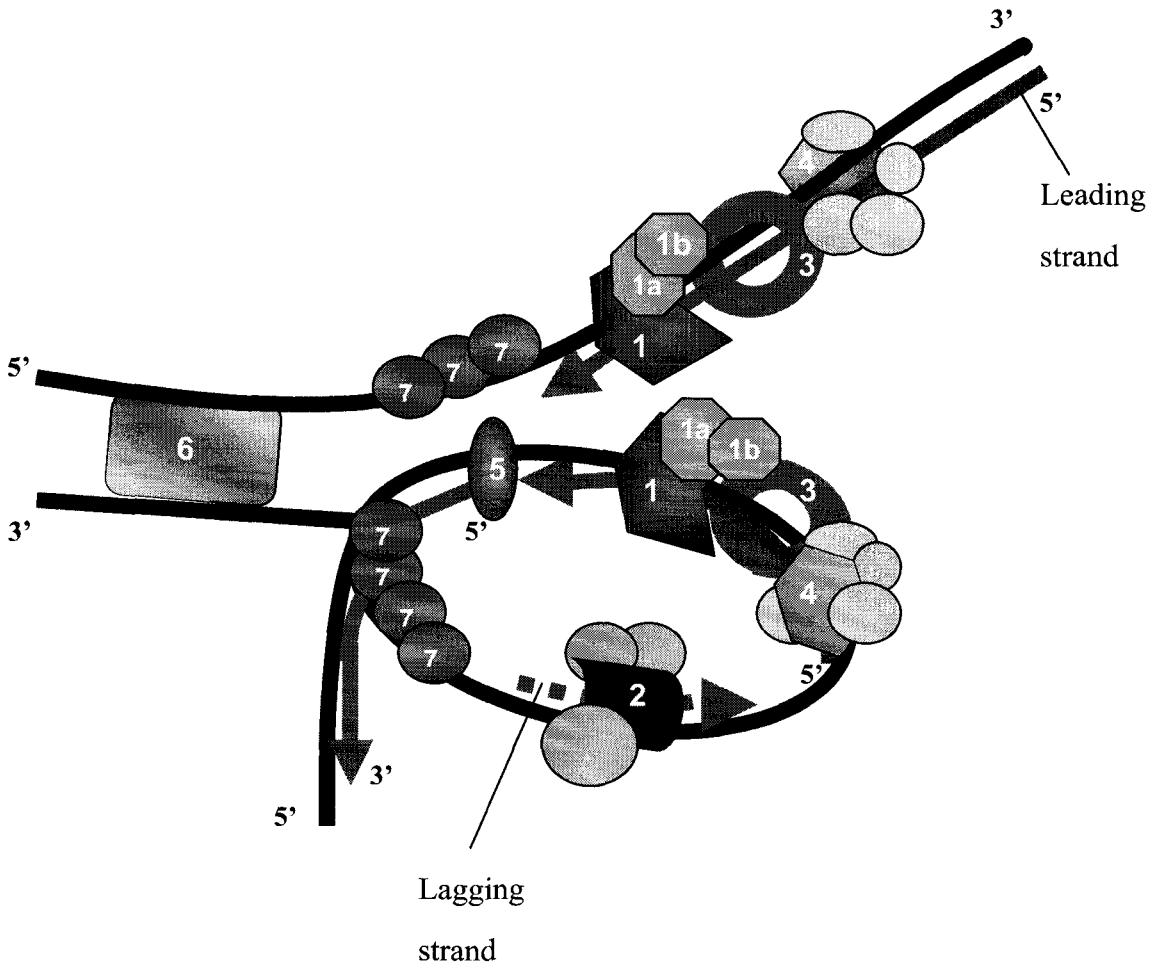

Figure 1.2 A simplified overview of DNA replication fork in eukaryotes. 1: DNA pol δ. 1a: DNA pol δ subunit Pol31p. 1b: DNA pol δ subunit Pol32p. 2: DNA pol α. 3: Replication clamp protein PCNA. 4: Clamp loader RF-C. 5: DNA ligase I. 6: DNA helicase. 7: Single-strand binding protein RP-A. Subunits of 2, 4 and 7 are not numbered. DNA pol δ is shown at both leading and lagging strands. However the exact roles of DNA pol δ and ε in genome replication still remain to be elucidated (see text). Drawing is based on Shevelev and Hubscher (2002).

suggesting that pol δ might synthesize both stands *in vivo* as well. Consistently, it has been demonstrated that DNA synthesis depends on pol δ both in *S. cerevisiae* and *S. pombe* (Budd and Campbell, 1993; Francesconi *et al.*, 1993). In line with its role in bulk DNA replication, inactivation of exonuclease activity of pol δ leads to significant compromise in genome integrity (Morrison *et al.*, 1993). Taken together, it is generally accepted that DNA pol δ is required for genome replication.

On the other hand, solid evidence has also been presented that chromosomal replication also involves DNA pol ε. First, in *S. cerevisiae*, pol ε is localized at or near replication forks during S-phase (Aparicio *et al.*, 1997). Temperature-sensitive (*ts*) pol ε mutants arrest DNA synthesis at restrictive temperatures (Araki *et al.*, 1992; Budd and Campbell, 1993). Using antibodies specific to pol ε to inhibit or deplete pol ε can also inhibit DNA synthesis both in human cell nuclei and in *Xenopus* egg extracts (Pospiech *et al.*, 1999; Waga *et al.*, 2001). Therefore, all of this evidence suggests that DNA synthesis depends on pol ε. In support of this notion, point mutations that inactivate polymerase activity of DNA pol ε cause lethality (Dua *et al.*, 1999), implying that normal pol ε functions in chromosome DNA replication and that inactive molecules are deleterious, presumably because they can block functional DNA pol δ from substituting for defective pol ε during genome replication. Consistently, although DNA pol ε mutants with a deletion of the catalytic domain are still viable, those mutants have serious defects in DNA replication (Kesti *et al.*, 1999; Dua *et al.*, 1999), suggesting that genome replication by DNA pol δ alone is rather inefficient. In addition to DNA replication, *S. cerevisiae* DNA pol ε also appears to be an active player in the S-phase checkpoint pathway (Navas *et al.*, 1995; Dua *et al.*, 1999). In this context, it might act in the classic *MEC1/RAD53* checkpoint pathway to sense DNA damage or replication blocks. The checkpoint activity of pol ε resides in the C-terminus of this enzyme (Kesti *et al.*, 1999). Taken together, these data indicate that both DNA pol δ and ε participate in the chromosomal DNA replication. Besides DNA synthesis, DNA pol ε may also be part of

the surveillance mechanism ensuring genome stability by sensing replication blocks and DNA damage.

Although it is well accepted that both DNA pol δ and ε are required for genome replication, their precise roles on leading and lagging strand synthesis remains elusive, because currently there is not an efficient method that allows us to study DNA pol δ or pol ε independently. Therefore, development of such a method will be important to further understand the precise function of DNA pol δ and pol ε.

1.3.2 Other DNA polymerases in S. cerevisiae

In addition to these three essential DNA polymerases, several other yeast DNA polymerases have also been identified, which are dispensable for viability but appear to have specific functions. For example, DNA pol γ is responsible for mitochondria DNA replication (reviewed in Burgers, 1998). DNA pol σ is required for the coordination between DNA replication and sister chromatid cohesion (Wang *et al.*, 2000). DNA pol IV is believed to be an ortholog of mammalian DNA pol β and appears to be involved in double strand break repair (Leem *et al.*, 1994). DNA pol ζ and η participate in trans-lesion synthesis (TLS) when template DNA contains altered or missing bases from damage to the DNA (Nelson *et al.*, 1996b; Johnson *et al.*, 1999). Because neither DNA pol ζ nor pol η DNA has exonuclease proofreading activity, they may be error-prone during DNA synthesis. Consistently, Pol ζ is required for UV-induced mutagenesis, implying that Pol ζ introduces replication errors during its synthesis across those UV-damaged sites (Morrison *et al.*, 1989). Another enzyme in TLS is the product of *REV1* gene, which encodes a deoxycytidyl transferase that is proposed to insert a C opposite an abasic site during DNA synthesis (Nelson *et al.*, 1996a).

1.3.3 Other proteins required for DNA replication

In addition to DNA polymerases, effective and accurate DNA replication depends on

a number of other proteins. ORC (origin recognition complex) proteins (Orc1p to Orc6p), MCM (minichromosomal maintainance) proteins (Mcm2p to Mcm6p), CDC proteins (Cdc6p and Cdc45p), the Cdc7p-Dbf4p kinase, the Cdc28p cyclin-dependent kinase and the B-type cyclins are all critical players for initiation of chromosome replication. Working together with checkpoint mechanisms, these proteins are also involved in prevention of replication origin re-firing, as well as temporal control of origin firing, i.e., regulation of early and late origin firing (reviewed by Kelly and Brown, 2000).

Replication origin firing is followed by the formation of single-stranded DNA (ssDNA), which is covered by ssDNA-binding protein RP-A (replication protein-A). DNA pol α - primase complexes initiate primer synthesis, which is elongated by DNA pol δ and ε. Clamp protein PCNA (proliferating cell nuclear antigen), which is loaded by the RF-C (replication factor-C) protein complex, ensures processive DNA replication. The replicative DNA helicase (presumably the MCM protein complex) is required to unwind upstream double-stranded DNA for chromosome replication. During lagging strand synthesis, DNA2 helicase/exonuclease, together with the flap-endonuclease (FEN1, encoded by the *RAD27* gene) removes single-stranded flaps of the RNA-DNA primers of Okazaki fragments abutting newly synthesized DNA, creating ligatable junctions that are subsequently sealed by DNA ligase I (Ayyagari *et al.*, 2003). DNA topoisomerases (top) release topological stresses during DNA replication and facilitate separation of the two DNA duplexes after semi-conservative DNA replication (reviewed in Kornberg and Baker, 1992). A simplified diagram of a eukaryotic replication fork is illustrated in Figure 1.2.

Among the proteins at the replication fork, particularly intriguing is the replication clamp, PCNA. DNA pol δ requires PCNA for processive DNA synthesis (Bauer and Burgers, 1988a; Bauer and Burgers, 1988b). Crystallography studies on PCNA indicate that it forms a homo-trimeric ring that encircles duplex DNA and tethers DNA polymerase to its template (Krishna *et al.*, 1994). However, no direct interaction has

been identified between DNA pol δ and PCNA, so their connection could be bridged by the pol δ subunit Pol32p in *S. cerevisiae* (Gerik *et al.*, 1998). However, the fact that the *POL32* gene is not essential suggests DNA pol δ may interact with PCNA through other proteins (Gerik *et al.*, 1998). In addition to increasing the processivity of DNA pol δ, PCNA is also involved in a variety of other activities, including interactions with DNA replication and repair proteins, such as DNA ligase I (Levin *et al.*, 1997) and FEN1 (Li *et al.*, 1995), clamp loader protein RF-C (Fotedar *et al.*, 1996) and the MMR proteins, Msh3p and Msh6p (Clark *et al.*, 2000; Flores-Rozas *et al.*, 2000). Consistently, point mutations in *POL30* (gene encodes PCNA in yeast) increase DNA polymerase misincorporations, slippage and also cause MMR defects (Chen *et al.*, 1999). Recent research has revealed that PCNA is a key player in the *RAD6*-dependent post-replication repair pathway (Torres-Ramos *et al.*, 1996) and the status of PCNA (mono-ubiquitination, SUMO modification and poly-ubiquitination at the lysine residue) determines cellular responses to DNA damaging agents (Hoege *et al.*, 2002).

1.4 Mismatch repair (MMR) in *S. cerevisiae*

While possible functional conservation can be predicted between the T4 DNA polymerase and *S. cerevisiae* DNA pol δ based on their sequence similarity, the interaction between DNA pol δ and the post-replication MMR cannot be anticipated from T4 studies because T4 phage does not have a MMR system itself and the host MMR system does not affect T4 DNA replication (Santos and Drake, 1994). Thus studies of the *pol3-612* yeast strain provide an opportunity to examine the interactions between DNA pol δ and MMR that are not possible with T4 phage.

In addition to high fidelity DNA polymerases, MMR is another important mechanism to ensure genome integrity. One of the major functions of MMR is to correct replication errors that escape proofreading of DNA polymerases. In *E. coli*, MMR increases replication fidelity by at least 100-fold. In *S. cerevisiae*, the magnitude of this

increase varies depending on the reporters used for fidelity measurement, but in general appreciable differences in genome stability are observed between genotypes where MMR is either present or absent (reviewed by Harfe and Jinks-Robertson, 2000). In addition, MMR also repairs mismatches generated by physical damage to DNA and suppresses recombination between non-identical sequences.

1.4.1 MMR in E. coli: a prototype

Most of our knowledge of bacterial MMR is based on the studies of the *E. coli* MMR system, which shares many similarities with eukaryotic MMR. *E. coli* requires functional Mut (mutator)S, MutL, MutH, MutU as well as a number of other proteins. Four basic steps are involved in the MMR process: (1) mismatch recognition, (2) assembly of a MMR complex, (3) excision of the mismatch and (4) re-synthesis and ligation. First, mismatches are recognized by the MutS proteins, which act as a homodimer to bind simple base mismatches as well as small insertion/deletion loops. Then MutL appears to work as a molecular switch with MutS to couple mismatch recognition to downstream MMR events (Acharya *et al.*, 2003). Subsequently MutH introduces a nick to the newly synthesized strand having a mismatch. *E. coli* MMR can distinguish mismatches in the newly synthesized strand by the presence of hemimethylated 5'-GATC-3' sequences in the template strand, because of the time lag before the newly synthesized strand is methylated. Once the nick is formed, MutU, a DNA helicase, unwinds DNA from the nick and works together with exonucleases to degrade the newly synthesized mismatch-containing strand. Different functionally redundant exonucleases have been identified that are involved in MMR. Following degradation, DNA re-synthesis occurs, catalyzed by the genome-replicating enzyme, DNA pol III holoenzyme, whose product is then ligated by DNA ligase (reviewed by Modrich and Lahue, 1996).

1.4.2 MMR in S. cerevisiae

Considerable similarities are found between *S. cerevisiae* and *E. coli* MMR systems. Six MSH (MutS homolog) proteins, Msh1p to Msh6p have been identified in *S. cerevisiae*. Like MutS in *E. coli*, *S. cerevisiae* MSH proteins also form dimers that recognize mismatches. Two heterodimers, MutSα (Msh2p-Msh6p) and MutSβ (Msh2p-Msh3p) exist in budding yeast that has overlapping functions in substrate recognition: MutSα preferentially binds to base-base mismatches and small insertion/deletion loops; MutSβ primarily recognizes both small and larger loops, but has low affinity for single base mismatches (Marsischky *et al.*, 1996; Sia *et al.*, 1997).

MLH (MutL homolog) proteins are also identified in *S. cerevisiae* and they form heterodimers as well: Mlh1p and Pms1p (post-meiotic segregation) form the MutLα complex (Prolla *et al.*, 1994) that interacts with MutSα and MutSβ when they are bound to mispaired bases (Habraken *et al.*, 1997; Habraken *et al.*, 1998). Consistently, inactivation of either *MSH2*, *MLH1* or *PMS1* genes generates a similar increase in mutation rates compared with the wildtype (Yang *et al.*, 1999). An interesting observation is that overexpression of yeast Mlh1p also leads to inactivation of MMR, suggesting that a quantitative balance between Mlh1p and Pms1p molecules might be important in keeping MutLα functional (Shcherbakova *et al.*, 2001). In addition to MutLα, MutLβ (Mlh1p-Mlh3p) has also been identified in *S. cerevisiae* (Flores-Rozas and Kolodner, 1998). MutLβ appears to mainly interact with MutSβ and functions to correct loops instead of the MutLα complex. However, as revealed by genetic analysis, it seems that only a very small portion of *MSH3*-dependent repair requires the MutLβ complex and in most cases the MutLα complex is involved (Flores-Rozas and Kolodner, 1998). A schematic diagram showing yeast MMR complex formation and substrate specificity is illustrated in Figure 1.3.

In addition to MSH and MLH proteins, other critical factors are also required in *S. cerevisiae* for MMR. PCNA is reported to be involved in MMR since mutant *POL30*

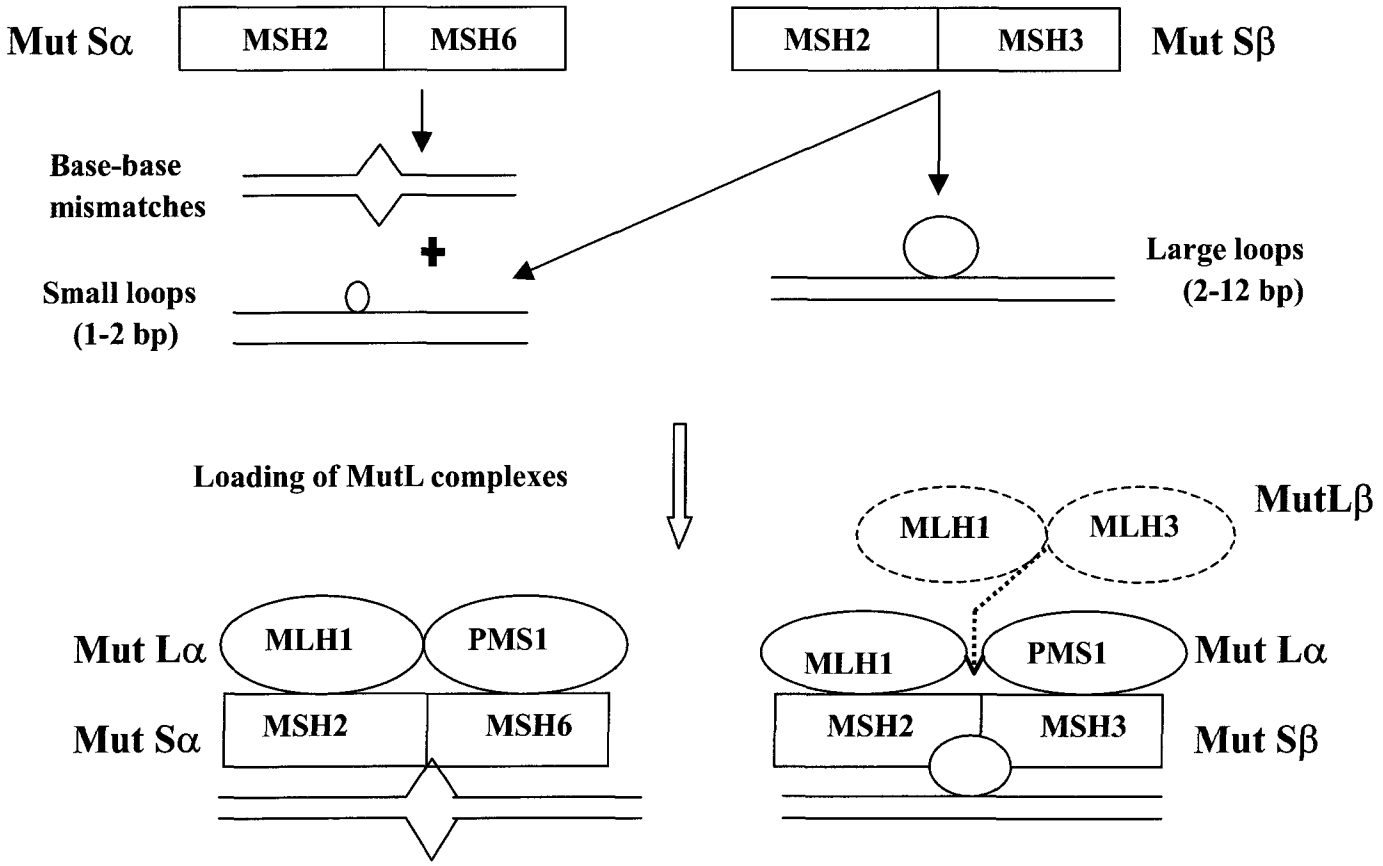

Figure 1.3 Components and substrates of mismatch repair complex in *S. cerevisiae*. Yeast homologs of *E. coli* MutS protein form two complexes in yeast: MutSα and MutSβ. Yeast homolgs of *E. coli* MutL protein also form two complexes: MutLα and MutLβ, with the latter functions less frequently in yeast cells (circled in dotted lines). Different MutS complexes recognize different substrates during MMR. See details in text (Section 1.4.2). Drawing is modified from Kolodner and Marsischky, 1999.

(encoding PCNA) alleles cause MMR defects (Chen *et al.*, 1999). While it is likely that PCNA plays a role during the DNA re-synthesis step because of its relationship with DNA polymerases (Gu *et al.*, 1998), a requirement for PCNA at a step prior to DNA re-synthesis has also been shown (Umar *et al.*, 1996). A possible explanation is that PCNA might help transfer the Msh2p-Msh6p complex to mismatches in DNA, thereby activating MMR (Lau and Kolodner, 2003). Other replication proteins, such as DNA pol δ and RPA, are also implicated in MMR, possibly during DNA re-synthesis (reviewed by Kolodner and Marsischky, 1999). As in *E. coli*, functional redundancy has also been found for *S. cerevisiae* exonucleases functioning in MMR. Exo1p (5'→3' exonuclease), exonuclease of DNA pol δ and ε (3'→5') all have been proposed to function in MMR (Tishkoff *et al.*, 1997; Tran *et al.*, 1999).

Despite their similarities, MMR in *S. cerevisiae* and *E. coli* are also different in some respects. One important difference is the mechanism that distinguishes between parental and newly synthesized strands. While hemi-methylation is used in *E. coli*, this is not so in budding yeast (Proffitt *et al.*, 1984). Furthermore no *S. cerevisiae* homolog of MutH endonuclease has been identified. A possible solution is that the presence of PCNA and the 5'-end of Okazaki fragments, which have higher density in the lagging strand, might serve as a signal for strand discrimination (Pavlov *et al.*, 2003).

MMR also functions in other processes besides DNA replication to maintain genome integrity. It is well documented that MMR corrects recombination intermediates and suppresses homeologous recombination, which occurs between similar but not identical sequences (reviewed by Harfe and Jinks-Robertson, 2000). Msh2p and Msh3p, but not other MMR proteins, also function in repairing double strand breaks (DSB) through single strand annealing (SSA) in *S. cerevisiae* (Sugawara *et al.*, 1997). Msh4p and Msh5p are related proteins required during meiosis (reviewed by Harfe and Jinks-Robertson, 2000).

Not surprisingly, MMR deficiencies increase genome instability in cells. In

mammalian cells, the importance of MMR has been demonstrated by a connection between MMR deficiency and hereditary nonpolyosis colorectal carcinoma (HNPCC) (Leach *et al.*, 1993; Bronner *et al.*, 1994). Germline mutations in the human MMR genes are found in the majority of HNPCC cases (reviewed in Prolla, 1998). The heterozygous status of MMR genes is sufficient for normal mismatch repair, however, upon loss of the wildtype allele, cells acquire a mutator phenotype and can lead to tumorigenesis.

1.5 Cellular responses to stalled DNA replication forks

DNA replication forks stall when DNA replication is disturbed by DNA damaging agents [for example, methyl methanesulphonate (MMS) or ultraviolet (UV) irradiation], or by replication inhibitors [for example, hydroxyurea (HU)]. Even in the absence of external stress, DNA replication forks in budding yeast (and probably in other organisms as well) may stall at replication slow zones, specific regions in chromosomes where replication fork progression is intrinsically slow (Cha and Kleckner, 2002).

Stalled replication forks are deleterious. For example, in *E. coli* they can be converted to double strand breaks (DSBs), which are highly unstable (Michel *et al.*, 1997). Cells have developed elegant mechanisms that respond to stalled replication forks. In *E. coli* stalled replication forks are mainly repaired through recombination (reviewed by Cox *et al.*, 2000) with TLS DNA polymerases as another option (Berdichevsky *et al.*, 2002).

Intensive studies have also been focused on the cellular response to stalled DNA replication fork in eukaryotes. Activation of the checkpoint pathway, a sophisticated surveillance mechanism that promotes cell cycle arrest and induces additional repair activities in response to DNA damage or other genotoxic stress (reviewed by Weinert, 1998; Gardner and Burke, 2000), is a common cellular response to stalled replication forks. It is generally accepted that S-phase checkpoint pathways regulating chromosome replication can be divided into two categories: (1) the DNA replication checkpoint, which

is activated when DNA synthesis is inhibited, for example by HU, (2) the DNA damage checkpoint, which is activated when DNA is modified by damaging agents, for example, UV and MMS (for review, see Kelly and Brown, 2000; Kolodner *et al.*, 2002). In *S. cerevisiae* these two checkpoint pathways overlap, for example, they both require Mec1p and Rad53p kinases for signal transduction and cell cycle arrest. On the other hand, differences also exist between these two pathways, for example, they may have different transducers (also called mediators) since *RAD9* inactivation only increases sensitivity to DNA damage but not to HU (Weinert and Hartwell, 1993).

Cell cycle checkpoint pathways have considerable impact on stalled replication forks. It has been demonstrated that checkpoint activation is essential to stabilize and prevent irreversible breakdown of stalled replication forks (Lopes *et al.*, 2001; Tercero and Diffley, 2001; Tercero *et al.*, 2003). In addition, checkpoint activation may facilitate DNA replication restart at the stalled replication forks. Furthermore, DNA replication proteins have been implicated in cell cycle checkpoint pathways. Mutations affecting the *POL2* gene (encoding DNA pol ε) show defects in the cell cycle checkpoint pathway and cause sensitivity to MMS and HU (Navas *et al.*, 1995). Similar roles in replication checkpoint are also proposed for DNA pol α in *S. pombe* (D'Urso *et al.*, 1995). In *S. cerevisiae*, phosphorylation of DNA pol α-primase complex is regulated by Rad53p-dependent checkpoint activation (Pellicioli *et al.*, 1999). More importantly, single-stranded DNA (ssDNA) coated by RP-A proteins has been implicated as a critical signal that triggers activation of the replication checkpoint both in budding yeast and human cells (Lee *et al.*, 1998; Zou and Elledge, 2003). In fact DNA replication forks have been proposed to play a central role in checkpoint activation and downstream responses (Tercero *et al.*, 2003).

CHAPTER 2 MATERIALS AND METHODS

2.1 Strains and microbiological media

2.1.1 Bacterial strains and media

The *Escherichia coli* strain DH5α was used in this study. Its genotype is F^- φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1. Bacterial cultures were grown at 30°C in Luria Broth (LB) or on solid LB medium as described (Ausubel, 1996). For plates supplemented with Ampicillin (Sigma), the antibiotic was added to a final concentration of 50 μg/ml.

2.1.2 S. cerevisiae yeast strains

Except for those specified otherwise, all *S. cerevisiae* strains used in this study were derived from MS71 (Strand *et al.*, 1995), which was kindly provided by Dr. T. Petes, University of North Carolina, Chapel Hill. EAS38 (*MATα msh6*), RJK256 (*MATα msh2*), EAS56 (*MAT***a** *msh2*), EAS74 (*MATα msh2)* and *pol3-t* were described previously (Sia *et al.*, 1997; Kokoska *et al.*, 1998; Hadjimarcou *et al.*, 2001). Strains used in this study are listed in Table 2.1.

2.1.2a Construction of the pol3-612 and msh2 pol3-612 strains

The LY103 (*MATα pol3-612*) strain was constructed as follows. The mutations encoding Leu612 to Met substitution were introduced into the wildtype *POL3* gene carried on the plasmid pBL304 by a PCR-based mutagenesis method (Cormack, 1996). The full-length *POL3* gene-containing plasmid pBL304 was obtained from Dr. P. Burgers, Washington University at St. Louis. The mutations encoding the L612M substitution were then confirmed by sequencing. Next, a 2.2 kb carboxy-terminal fragment containing the mutant *pol3* gene harboring the mutations was sub-cloned into a yeast integrating plasmid YIp 5, digested by *Hpa*I and transformed into MS71 cells. The

mutant *pol3-612* allele was then integrated into the yeast genome and replaced the wildtype *POL3* gene, which resulted in a full-length and a truncated copy of the *POL3* gene (Figure 3.1.1) (Scherer and Davis, 1979; Rothstein, 1983). Positive transformants were selected on the uracil drop-out media because integration of the YIp plasmid conferred the Ura$^-$ MS71 strain ability to grow in the absence of uracil. The chromosomal region neighboring the Leu612 position was amplified by PCR and then sequenced to confirm the presence of the mutations encoding the L612M substitution. The Ura$^+$ integrants were then plated on 5-FOA containing plates to select for chromosome restoration (Boeke *et al.*, 1984), after which a single copy at the *POL3* locus was obtained. The mutations encoding the L612M substitution were confirmed by sequencing.

Southern hybridization analysis was used to confirm the integration and restoration events. Southern hybridization analysis was performed using protocols described previously (Brown, 1996). Probes used for Southern hybridization experiments were ^{32}P-labelled PCR products. Primers LRK 236 and 241 were used for the PCR reaction, which generated 0.6 kb probes that can anneal to the C-terminal region of the *POL3* gene (Figure 3.1.1). In a 20 μl reaction, standard PCR conditions were used, except that unlabelled dCTP concentrations were lower than normal (10 nM), and 8 μl [α-^{32}P] CTP (NEN, 10 mCi/ml) was added to radiolabel the product. The radiolabeled PCR product was purified using a G-50 Sephadex column to remove unincorporated [α-^{32}P] CTP, following procedures described (Struhl, 1996). The activity of ^{32}P-labelled probes was quantified using a scintillation counter. Probes were denatured by incubating them at 100°C for 5 min followed by quenching in ice bath before use. Hybridization results were read from the Phosphorimager (Molecular Dynamics, Model 400E) and compared with photographs taken of the agarose gel to determine the size of labeled restriction fragments. The results of Southern hybridization analysis are shown in Figure 3.1.2.

LY104 (*MAT***a** *pol3-612*) and LY110 (*MAT***a** *msh2 pol3-612*) were derived by

sporulating the diploid generated by crossing haploid strains LY103 and EAS56. Standard procedures were used for mating, sporulation and dissection (Burke *et al.*, 2000). Primers used for PCR mutagenesis, sequencing and for labeling probes used for Southern analysis are listed in Table 2.2.

Due to the genome instability caused by mismatch repair deficiency alone, results reported in this study were all based on strains freshly inoculated from glycerol stocks. All strains derived from LY103 were sequenced to confirm the existence of the L612M-encoding mutations. PAA-sensitivity and a strong mutator phenotype [revealed by replica plating on synthetic complete plates containing 60 μg/ml canavanine (US Biological)] were used to monitor the genome instability of LY110 derivative strains.

2.1.2b Construction of deletion strains

Strains with knocked-out genes were constructed by a PCR-based one-step gene disruption method described previously (Wach *et al.*, 1994; Longtine *et al.*, 1998). Plasmids pFA6-*kanMX2* (provided by Dr. D. Stuart, University of Alberta) and pFA6-*kanMX6* (provided by Dr. B. Andrews, University of Toronto) were used as templates for amplifying a disruption cassette containing the G418-resistant gene.

The PCR primers for cassette amplification have 40 nucleotides at the 5'-end that correspond to the desired target gene sequence and 20 nucleotides (19 nucleotides for forward primers when using *kanMX2* as templates) at the 3'-end that anneal to amplification cassette. Reactions were carried out in a total volume of 50 μl: 160 nM of each dNTP, 2.5 mM $MgCl_2$, 100 pmol of each primer, 2.5 units Tag DNA polymerase (GIBCO), and 50 ng of template DNA. When using pFA6-*kanMX6* as templates, 1.5 unit of Pfu DNA polymerase (kindly provided by Dr. M. Pickard, Biological Sciences, University of Alberta) was also used in addition to Taq to facilitate primer extension during PCR amplification. For pFA6-*kanMX2*, the PCR amplification was performed under the following conditions: Stage 1: 94°C, 3 min; Stage 2: 92°C, 40 sec, 55 °C, 40

Strain	Genotype	Source
MS71	*MATα ade5-189 his7-2 trp1-2 ura3-52*	T. Petes
LY102	*MAT***a** MS71	This study
LY103	*MATα* MS71 *pol3-612*	This study
LY104	*MAT***a** MS71 *pol3-612*	This study
LY110	*MAT***a** MS71 *msh2Δ pol3-612*	This study
EAS56	*MAT***a** MS71 *msh2Δ*	T. Petes
EAS74	*MATα* MS71 *msh2Δ*	T. Petes
LY111	*MAT***a** MS71 *msh6::kanMX pol3-612*	This study
EAS38	*MATα* AMY125 *msh6::LEU2*	T. Petes
LY112	*MAT***a** MS71 *pms1::kanMX pol3-612*	This study
LY116	*MAT***a** MS71 *pms1::kanMX*	This study
KY507	*MAT***a** MS71 *mlh1::kanMX pol3-612*	K. Murphy
KY508	*MAT***a** MS71 *mlh1::kanMX*	K. Murphy
KY505	*MAT***a** MS71 *msh3::kanMX pol3-612*	K. Murphy
KY504	*MAT***a** MS71 *msh3::kanMX*	K. Murphy
LY113	*MAT***a** MS71 *exo1::kanMX pol3-612*	This study
LY117	*MAT***a** MS71 *exo1::kanMX*	This study
LY129	*MAT***a** MS71 *msh2Δ exo1::kanMX pol3-612*	This study
LY114	*MAT***a** MS71 *msh2Δ rev3::kanMX pol3-612*	This study
LY118	*MAT***a** MS71 *msh2Δ rev3::kanMX*	This study
LY115	*MAT***a** MS71 *msh2Δ rad30::kanMX pol3-612*	This study
LY119	*MAT***a** MS71 *msh2Δ rad30::kanMX*	This study
LY120	*MATα* MS71 *msh6::natMX*	This study

Table 2.1 a. Haploid *Saccharomyces cerevisiae* strains used in this study.

[Table 2.1 (a) continued]

Strain	Genotype	Source
LY121	*MAT***a** MS71 *msh6::natMX msh3::kanMX pol3-612*	This study
LY123	*MAT***a** MS71*msh2Δ rad52::kanMX pol3-612*	This study
LY124	*MAT***a** MS71 *msh2Δ rad51::kanMX pol3-612*	This study
LY125	*MAT***a** MS71 *rad52::kanMX pol3-612*	This study
LY126	*MAT***a** MS71*rad51::kanMX pol3-612*	This study
LY127	*MAT***a** MS71 *rad52::kanMX*	This study
LY139	*MAT***a** MS71 *msh2Δ rad52::kanMX*	This study
LY128	*MAT***a** MS71 *rad51::kanMX*	This study
LY122	*MAT***a** MS71 *msh6::LEU2 rad52::kanMX pol3-612*	This study
LY130	*MAT***a** MS71 *dun1::kanMX*	This study
LY131	*MAT***a** MS71 *dun1::kanMX pol3-612*	This study
LY134	*MAT***a** MS71 *msh2Δ dun1::kanMX*	This study
LY132	*MAT***a** MS71 *msh6::natMX dun1::kanMX pol3-612*	This study
LY133	*MAT***a** MS71 *msh2Δ dun1::kanMX pol3-612*	This study
LY138	*MAT***a** MS71 *rad27::kanMX*	This study
pol3-t	*MATα* MS71 *pol3-t*	T. Petes
LY135	*MATα* MS71 *pol3-t mlh1::kanMX*	This study
LY140	*MAT***a** MS71 *msh2Δ pol3-612,758*	This study
LY141	*MAT***a** MS71 *msh2Δ pol3-758URA3*	This study

Table 2.1 a. Haploid *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source/purpose
LY200	MS71 *POL3* MS71 *pol3-612*	This study / construction of LY102, 104, 130 and 131, dominance test of the *pol3-612* allele
LY201	MS71 *POL3 msh2Δ* MS71 *pol3-612 MSH2*	This study / construction of LY110, 114, 115, 118 and 119
LY202	MS71 *pol3-612 MSH6* MS71 *pol3-447 msh6::kanMX*	This study / construction of LY111
LY203	MS71 *POL3 pms1::kanMX* MS71 *pol3-612 PMS1*	This study / construction of LY112 and 116
LY204	MS71 *POL3 exo1::kanMX* MS71 *pol3-612 EXO1*	This study / construction of LY113 and 117
LY205	MS71 *POL3 rad52::kanMX* MS71 *pol3-612 RAD52*	This study / construction of LY125 and 127
LY206	MS71 *POL3 rad51::kanMX* MS71 *pol3-612 RAD51*	This study / construction of LY126 and 128
LY207	MS71 *msh2Δ DUN1* MS71 *MSH2 dun1::kanMX*	This study / construction of LY134
LY208	MS71 *pol3-612 exo1::kanMX MSH2* MS71 *POL3 EXO1 msh2Δ*	This study / construction of LY129
LY209	MS71 *pol3-612 msh3::kanMX MSH6* MS71*POL3 MSH3 msh6::natMX*	This study / construction of LY121
LY210	MS71 *pol3-612 dun1::kanMX MSH2* MS71 *POL3 DUN1 msh2Δ*	This study / construction of LY133
LY211	MS71 *pol3-612 rad51::kanMX MSH2* MS71 *POL3 RAD51 msh2Δ*	This study / construction of LY124
LY212	MS71 *pol3-612 RAD52 MSH2* MS71 *POL3 rad52::kanMX msh2Δ*	This study / construction of LY123
LY213	MS71 *pol3-612 rad52::kanMX MSH6* MS71 *POL3 RAD52 msh6::LEU2*	This study / construction of LY122
LY214	MS71 *pol3-612 dun1::kanMX MSH6* MS71 *POL3 DUN1 msh6::nat*	This study / construction of LY132
LY218	MS71 *POL3 MSH6* MS71 *pol3-447 msh6::kanMX*	This study / construction of LY120

Table 2.1 b. Diploid *Saccharomyces cerevisiae* strains used in this study.

[Table 2.1 (b) continued]

Strain	Genotype	Source/purpose
LY219	MS71 *msh2Δ rad52::kanMX* MS71 *MSH2RAD52*	This study / construction of LY139
LY220	MS71 *pol3-612 RAD27* MS71 *POL3 rad27::kanMX*	This study / construction of *rad27 pol3-612*
LY215	MS71 *pol3-612 msh6::kanMX* MS71 *pol3-612 msh6::kanMX*	This study / test for ploidy difference
LY216	MS71 MS71	This study / dominance test of *pol3-612* allele
LY217	MS71 *pol3-612* MS71 *pol3-612*	This study / dominance test of *pol3-612* allele

Table 2.1 b. Diploid *Saccharomyces cerevisiae* strains used in this study.

Primer	DNA sequence (5'→3')	Location and function
PCR mutagenesis		
LRK211	TCAATATTGACGGCCGATTAC	nt.1361-1381[a], forward primer
LRK213	TTCAATTCTATGTATCCAAGTATTATGATGG[b]	nt.1825-1855[a], forward primer
LRK212	ACTTGGATACATAGAATTGAAATCCAAAGTTG[b]	nt.1845-1814[a], reverse primer
LRK214	TCTTTTGAATGGATCCTTCTC	nt.2070-2050[a], reverse primer
Sequencing of POL3 alleles		
LRK234	AGCCAAAACGGCGTGCGGCTG	nt.2720-2701[a], reverse primer
LRK235	CAAGCCTCAACTTTTTTAATAAAG	nt.3026-3002[a], reverse primer
LRK236	ATAAATCCACCAACATGCAAAAAGT	nt.3365-3341[a], reverse primer
Gene knockout		
LRK271	ATACAAAACTACAAGTTGTGGCGAAATAAAATGTTTGGAA cagctgaagcttcgtacgc[c]	*REV3* knockout, forward primer
LRK272	ATAACTACTCATCATTTTGCGAGACATATCTGTGTCTAGAgcataggccactagtggatc[c]	*REV3* knockout, reverse primer
LRK277	CTGCTCATTTTTGAACGGCTTTGATAAAACAAGACAAAGCcagctgaagcttcgtacgc[c]	*RAD30* knockout, forward primer
LRK278	TTTAGTTGCTGAAGCCATATAATTGTCTATTTGGAATAGGgcataggccactagtggatc[c]	*RAD30* knockout, reverse primer
LRK284	ATGTCTCTCAGAATAAAAGCACTTGATGCATCAGTGGTTAcggatccccgggttaattaa[c]	*MLH1* knockout, forward primer
LRK285	TTAACACCTCTCAAAAACTTTGTATAGATCTGGAAGGTTGgaattcgagctcgtttaaac[c]	*MLH1* knockout, reverse primer
LRK288	GTAGTTATTTGTTAAAGGCCTACTAATTTGTTATCGTCAT cggatccccgggttaattaa[c]	*RAD51* knockout, forward primer
LRK289	GTAAACCTGTGTAAATAAATAGAGACAAGAGACCAAATAC gaattcgagctcgtttaaac[c]	*RAD51* knockout, reverse primer

Table 2.2 Primers used in this study

[Table 2.2 continued]

Primer	DNA sequence (5'→3')	Location and function
Gene knockout (Contd.)		
LRK305	TGCGATCACGTGAATTTTCAATGATAAAT AAGCTGGAACA cggatccccgggttaattaa[c]	*MSH3* knockout, forward primer
LRK306	ATGATAGTAATTTCGCGAGTTTATCCGTT GCTGTTATATT gaattcgagctcgtttaaac[c]	*MSH3* knockout, reverse primer
LRK307	GAAAAATATAGCGGCGGGCGGGTTACGC GACCGGTATCGAcagctgaagcttcgtacgc[c]	*RAD52* knockout, forward primer
LRK308	AATGATGCAAATTTTTTATTTGTTTCGGC CAGGAAGCGTT gcataggccactagtggatc[c]	*RAD52* knockout, reverse primer
LRK314	AGAAAAGACGCGTCTCTCTTAATAATCAT TATGCGATAAA cggatccccgggttaattaa[c]	*PMS1* knockout, forward primer
LRK315	GTATTTGTTAATTATATAATGAATGAATAT CAAAGCTAGA gaattcgagctcgtttaaac[c]	*PMS1* knockout, reverse primer
LRK316	AATAAAAGGAGCTCGAAAAAACTGAAA GGCGTAGAAAGGAcggatccccgggttaattaa[c]	*EXO1* knockout, forward primer
LRK317	TTTTCATTTGAAAAATATACCTCCGATATG AAACGTGCAG gaattcgagctcgtttaaac[c]	*EXO1* knockout, reverse primer
LRK320	CATTGGAAAGAAATAGGAAACGGACACC GGAAGAAAAAATcagctgaagcttcgtacgc[c]	*RAD27* knockout, forward primer
LRK321	AGGTGAAGGACCAAAAGAAGAAAGTGG AAAAAGAACCCCCgcataggccactagtggatc[c]	*RAD27* knockout, reverse primer
LRK343	ATGAGTTTGTCCACGAAAAGAGAGCACT CTGGTGATGTAAcagctgaagcttcgtacgc[c]	*DUN1* knockout, forward primer
LRK344	TTAGAGGCAAGATAATTCTGAGTATGTTT TGGGTATTTTA gcataggccactagtggatc[c]	*DUN1* knockout, reverse primer
PCR confirmation of knockout		
LRK276	AGTGATTTTGATGACGAGCG	*REV3, RAD30, RAD52, RAD27, DUN1* knockout, reverse primer
LRK275	GACGAGTGCAGTGCGTCTAG	*REV3* knockout, forward primer

Table 2.2 Primers used in this study

[Table 2.2 continued]

Primer	DNA sequence (5'→3')	Location and function
PCR confirmation of knockout (Contd.)		
LRK279	GGGCAATTCTTATAATTTCGG	*RAD30* knockout, forward primer
LRK309	CTACTCATCGCCAAAGAGT	*RAD52* knockout, forward primer
LRK345	GTCGAGAGTAACAAGTAAAG	*DUN1* knockout, forward primer
LRK300	CATACAATCGATAGATTGTCG	*MLH1, RAD51, MSH3, PMS1, EXO1* knockout, reverse primer
LRK298	TGCTAGGACAATTTAACTGCA	*MLH1* knockout, forward primer
LRK297	TAAAGGGGAATAGTGGGGA	*RAD51* knockout, forward primer
LRK310	TCCACGGATTCAAAATTGTC	*MSH3* knockout, forward primer
LRK326	CAACATGTACATAGCTAGAAC	*PMS1* knockout, forward primer
LRK327	TGCGTGATTGATAGAAGGC	*EXO1* knockout, forward primer

Table 2.2 Oligonucleotides used in this study

[a] Positions of primers are defined by their locations on the *POL3* gene starting with the ATG start codon.

[b] Underlined nucleotides indicate designed mismatch in order to introduce the L612M substitution.

c. For knockout primers, ORF-specific sequences are shown in capital letters and knockout cassette sequences are shown in small lower case.

sec, 72°C 90 sec, 30 cycles; Stage 3: 92°C, 40 sec, 55 °C, 40 sec, 72°C 5 min. For the pFA6-*kanMX6* plasmid, 5 additional cycles were inserted between Stages 2 and 3, so the complete process was: Stage 1: 94°C, 3 min; Stage 2: 95°C, 45 sec, 55 °C, 45 sec, 72°C 90 sec, 5 cycles; Stage 3: 92°C, 40 sec, 65 °C, 60 sec, 72°C 90 sec, 45 cycles; Stage 4: 72°C 5 min. The rationale for increasing annealing temperature at stage 3 is to minimize unspecific annealing during PCR amplification. Normally, 10 to 15 μl of each PCR product was used for transformation of gene inactivation. The transformants were plated on YPD plates and kept at 30°C for 2 days before being replica plated to YPD plates supplemented with G418 (geneticin). When the target gene was replaced by the amplified knockout cassette through homologous recombination, it conferred resistance of these transformants to G418. All putative positive transformants were streaked on G418-containg plates to obtain single colonies, which were then subject to further characterization as described below.

All gene disruptions were confirmed by PCR. Oligonucleotide primers hybridizing to sequences that are located upstream of the knocked-out gene were used for forward primers. Oligonucleotide primers hybridizing with sequences that are located within the *kanMX* cassette were used for reverse primers. The primers used for gene disruption and PCR confirmation are listed in Table 2.2.

Except where specified otherwise, gene disruption was carried out in diploids followed by sporulation and isolation of haploids to get the desired mutants. LY135 (*MATα* MS71 *pol3-t mlh1::kanMX*) and LY138 (*MAT***a** MS71 *rad27::kanMX*) were constructed by directly transforming *pol3-t* and LY102 (*MAT***a** MS71) haploid strains with knockout cassettes, respectively. A two-step strategy was used in the construction of LY121 (*MAT***a** *msh6 msh3 pol3-612*). First, the LY120 strain (*MATα msh6::natMX*) was constructed by using a switching plasmid p4339 (provided by Dr. B. Andrews, University of Toronto) to switch from G418 resistance (*kanMX*) to clonNAT resistance (*natMX*). To make this switch, 2.5 μg of plasmid p4339 DNA was completely digested

by *Eco*RI, then the 1.2 kb fragment was purified from an agarose gel and used to transform LY218 diploid cells. YPD plates supplemented with clonNAT were used for selection of colonies in which the *kanMX* cassettes were substituted by the *natMX* cassettes. Then, the confirmed diploid transformants were sporulated and dissected to obtain the LY120 strain. Next, the LY120 strain was crossed to KY505 (*MAT***a** *msh3 pol3-612*) followed by sporulating the diploid to obtain the LY121 strain. For triple mutants (LY115, LY121, LY122, LY123, LY124, LY132, LY133), a double mutant was constructed first and then crossed to the corresponding single mutant, followed by sporulation and tetrad dissection to isolate the desired mutants. The diploid strains used for haploid strain construction are listed in Table 2.1 (b).

2.1.3 S. cerevisiae yeast media

Standard methods were used for preparing yeast media (Wach *et al.*, 1994; Goldstein and McCusker, 1999; Burke *et al.*, 2000). YPD (2% glucose, 1% yeast extract, 2% peptone), synthetic dextrose complete (SD-complete) and dropout media, synthetic dextrose minimum medium and sporulation medium (1.5% KOAc, 0.25 x complete amino acid mix) were utilized during this study. Special supplements were added after autoclaving to the final concentrations indicated: 5-FOA (US Biological) at 1 mg/ml, canavanine (US Biological) at 60 μg/ml, G418 (geneticin, US Biological) at 200 μg/ml, clonNAT (nourseothricin-sulfate, HKI, Jena Germany) at 100 μg/ml.

SD-complete plates supplemented with PAA (Sigma) at the indicated concentrations (0.1-0.5 mg/ml) were made following the same protocol as regular SD-complete plates except that PAA was added after autoclaving, and 10 N NaOH was used to adjust the pH to 4.5.

The gradient PAA plates were made by using a method modified from one previously described (Woodworth and Kreuzer, 1996). 35 ml of synthetic complete medium with 2% Noble agar (Difco) and the indicated PAA concentration were poured

into square petri dishes, with one side of the plate elevated by resting on a pencil. After the agar solidified, the plates were placed flat on a table and 35 ml of synthetic complete medium without PAA was added, so that a gradient of PAA is formed across the plate. Plates were used on the same day as they were made.

2.2 Cell transformation and DNA isolation

Transformation of *S. cerevisiae* cells was performed according to the LiAc TRAFO method (Gietz and Woods, 2002). Cells were grown in YPD broth to mid-log phase (1 to 1.5 X 10^7 cells/ml). Cells from 5 ml cultures were centrifuged by using the clinical centrifuge at 1200 rpm for 5 min and washed with sterile water, then resuspended in 1 ml 100 mM LiAc. Cells were centrifuged by using the table microfuge at 6000 rpm for 15 sec and respuspended in 0.5 ml 100 mM LiAc. 50 µl of this cell suspension was briefly centrifuged and the supernatant was then removed using a micro-pipette. The pellet was then overlaid with 240 µl PEG 3350, 36 µl 1.0 M LiAc, 50 µl single-stranded DNA, 10 µl transforming DNA and 24 µl sterile water. This mixture was then vortexed vigorously until the cell pellet was evenly dispersed. The transformation mix was incubated at 30°C for 30 min, then heat-shocked at 42°C for 30 min. Cells were then centrifuged using the microfuge at 6000 rpm for 15 sec and resuspended in 1 ml sterile water. Twenty to 300 µl of the transformation mix were plated on proper media (dropout media or media supplemented with G418 or clonNAT) and subject to further characterization as described below. Standard procedures were used in transformation of *E. coli* cells and plasmid DNA isolation (Ausubel, 1996).

Isolation of yeast genomic DNA was performed by using a glass bead method desribed by Hoffman (1996), except that after adding 95% ethanol the sample was kept at -20°C for one hour in order to increase the yield. The genomic DNA from 10 ml of overnight yeast culture was suspended in 80 µl TE (pH 8.0). Two µl DNA (approximately 500 ng, estimated from the agarose gel containing 0.5 µg/ml ethidium

bromide) was used for each PCR reaction.

2.3 Replication fidelity measurements for DNA polymerases

2.3.1 Measurement of spontaneous mutation rates

Spontaneous mutation rates were measured by a procedure modified from previously described (Hadjimarcou *et al.*, 2001). For strong mutator mutants such as the *msh2-, pms1-, mlh1 pol3-612* strains, mutation rates of *trp1-289* and *his7-2* loci were measured by inoculating a single fresh colony (incubated at 30°C for 3 days) into 2 ml YPD and incubating at 30 °C for 6 hours. Cells were then centrifuged using the clinical centrifuge at 1200 rpm for 5 min and re-suspended in 1 ml of 2mM EDTA before plating aliquots onto selective media to identify revertants, and on SD-complete medium to titer the concentrations of cultures that were plated. To measure mutation rates at the *CAN1* locus, a single fresh colony (incubated at 30°C for 3 days, diameter of approximately 1 mm) was picked and suspended in 0.2 ml 2mM EDTA. Then, appropriate amount of cells were plated on plates supplemented with canavanine to select for mutants and on SD-complete plates to titer concentrations of cultures that were plated. For the *msh2, pms1* and *mlh1* strains, single colonies (3 days incubation at 30°C) were directly suspended in 0.2 ml 2mM EDTA and then plated onto plates supplemented with canavanine to measure mutation rates at the *CAN1* locus. For mutation rates measurement of other strains reported at the *trp1-289, his7-2, CAN1* and *ade5-1* loci (except for *CAN1* locus of the *msh2, pms1* and *mlh1* strains, which is described above), cultures grown at 30°C for 24 hours and then were diluted and plated. All plates were incubated at 30°C for 3 days before counting colony formation. Both *MAT***a** and *MAT*α strains were used to determine the mutation rates of MS71, *msh2* and *pol3-612* strains and no differences were found between two mating types.

The method of the median was used to calculate the mutation rate (Lea and Coulson, 1949). In this method, the numbers of mutants (r_0) and viable cells per culture (x) were

determined by counting colony numbers on selective and permissive media, respectively, followed by multiplying dilution factors. Then, using the r_0 value and Table 3 of Lea and Coulson (1949), the number of mutations per culture (m) were calculated. By dividing m by x, the mutation rate of that culture was determined. At least ten independent cultures were measured for each strain and the median number was chosen as mutation rate of that strain unless otherwise specified.

The "bootstrap" method (Efron and Tibshirani, 1993) was used to calculate the 95% confidence interval for each of the mutation rates. A computer program for calculating these bootstrap values was provided by Dr. P. Hurd, Department of Psychology, University of Alberta. In this program, mutation rates of independent cultures of a specific strain were randomly sampled with replacement to generate a large number (in present study, this number is 1000) of independent bootstrap samples. For each of the 1000 samples, the median was determined and ranked by its value. Then the 95% confidence interval was determined from the top and bottom 2.5 percentile breaks in this set of values.

2.3.2 Measurement of replication fidelity in the presence of PAA

To measure the replication fidelity of DNA pol δ in the presence of 1 mg/ml PAA, 2 fresh (incubated at 30 °C for 3 days) *msh2* colonies were inoculated into 10 ml YPD broth and grown at 30°C overnight. Then, 1 ml of the overnight culture was taken and cells were collected by centrifuging at 5,000 rpm for 5 min using the micro-centrifuge. Next, cells were washed in 1 ml sterile water and resuspended in 1 ml SD-complete broth. These cells were inoculated into 10 ml (final volume) SD-complete broth with 1 mg/ml (final concentration) PAA and grown at 30°C for 8 hours. The cells were then collected and appropriate numbers of cells were plated on selective and permissive media. Plates were incubated at 30°C for 3 days before counting colony formation. As experimental controls, overnight cultures of *msh2* cells were also treated with the same

procedures except that no PAA was added. To minimize experimental variables, all cultures were processed in the same way and at the same time for these assays.

The mutation frequency was calculated by dividing the number of colonies grown on selective media by the number of viable cells plated, which was determined from the permissive media. Then mutation frequencies at the reporter loci of *msh2* cells with PAA treatment were used to compare with that of no PAA-treatment in order to determine the effect of PAA on replication fidelity.

2.4 Test of cellular sensitivity to PAA, HU and UVC

In this study, PAA-sensitivity was tested in both liquid and solid media. For PAA-sensitivity tests in liquid media, cells were grown to logarithmic phase in SD-complete broth and were then diluted to approximate 10^6 cells/ml using pre-warmed SD-complete broth. Before inoculation, 10 μl of 0.5 mg/ml PAA stock solution was added to a final concentration of 0.5 mg/ml and 30 μl 2N NaOH was used for adjusting the pH. Cells were grown at 30°C and sampled at the indicated time points. The number of cells were counted by using a heamocytometer (see Section 2.5) and then tittered for the number of viable cells by plating onto SD-complete plates. Plates were then incubated at 30°C for 3 days before counting colonies.

For PAA-sensitivity tests on PAA-gradient plates, approximate 100 viable cells (by adjusting number of cells plated based on the viability of that strain) in a volume of ~10 μl at logarithmic phase of each strain were spotted across the gradient plates and plates without PAA. PAA-sensitivity was also tested on 0.1 mg/ml to 0.5 mg/ml PAA (non-gradient) plates. Approximate 100 viable cells in logarithmic phase for each strain were plated on the non-gradient PAA plates and SD-complete plates without PAA. All plates were incubated for 3 days at 30°C before taking photographs to document the results of these assays.

To examine cellular sensitivity to HU, cells were grown in YPD broth to mid-log

phase and a 10-fold serial dilution of samples was made (10^3 to 10^7 cells/ml dilution range were used). Ten μl of each different dilution was spotted on SD-complete and on 100 mM HU plates (approximately 10 to 10^5 cells per spot). For UVC-sensitivity assays, the same procedure was used except that cells were only spotted on SD-complete plates. After the liquid was absorbed, cells on plates were irradiated with a UV lamp (Ultra-Violet Products, model UVGL-58, 254 nm wave-length) at the distance of 20 cm for 60 seconds (equals 36 J/m^2) and 120 seconds (equals 72 J/m^2). Then plates were wrapped with aluminum foil and kept at 30°C for 3 days.

2.5 Cell viability test

Cells in logarithmic phase were used for these viability tests. Cell samples were counted under compound microscope with a heamocytometer to determine their density and approximately 100 to 200 cells were plated onto SD-complete plates in duplicate for each sample. These plates were incubated for 3 days at 30°C before counting the colonies. Viability was determined by dividing colony formation by cell numbers deduced from heamocytometer counting. At least 4 different isolates for each strain [except for MS71, EAS56 (*msh2*) and LY104 (*pol3-612*)] were examined for viability and the average viability was reported. For the MS71, EAS56 and LY104 strains, the viability was measured in repeated experiments and the average was taken. The standard error was calculated by using the Excel software of Microsoft®.

To test the viability of MS71, EAS56, LY104 and LY110 strains in SD-complete broth containing 0.5 mg/ml PAA, cells were grown at 30°C and sampled at the time points indicated. Samples were diluted in SD-complete broth without PAA and counted using a heamocytometer. One to four hundred cells were plated onto SD-complete medium in duplicate. These plates were incubated for 3 days at 30°C before counting colony formation. Because the PAA-containing cultures were diluted at least 1000-fold during these viability measurements before plating on SD-complete medium that has no

PAA, the viability test under those situations can also be considered as an indication of reversibility from PAA-inhibition.

For viability tests of temperature sensitive mutants, cells were sampled at the indicated time points. Samples were first counted under heamocytometer, then diluted and plated on YPD plates in duplicate. Plates were kept at 23°C for 5 days before counting colony formation. For the same reasons stated above, viability measurements determined by this assay can also be considered as an indication of reversibility from inhibition at restrictive temperature.

2.6 Temperature sensitivity test

The temperature sensitive *pol3* mutant strain, *pol3-t*, and its mismatch repair deficient derivatives LY135 (*mlh1 pol3-t*) were tested for temperature sensitivities. For *pol3-t* strain, 30°C is reported to be a permissive temperature (Kokoska *et al.*, 1998), so during growth curve experiments using this strain, cells were pre-incubated at 30°C (permissive temperature), then half of each culture was shifted to 37°C (restrictive temperature) while the remaining half was incubated at 30°C. Cells were sampled at the time points indicated and countered under light microscope followed by plating on YPD plates. The plates were incubated at room temperature for 5 days before counting colony formation.

2.7 DNA sequencing

Both manual and automated DNA sequencing were used in this study. For manual sequencing, AMV reverse transcriptase (AMV-RT, Roche) was used. Single-stranded sequencing DNA templates were made by either asymmetric PCR (using a single primer) (Dorit *et al.*, 1996), or alkaline denaturation of double-stranded plasmid DNA. The same methods described in Hadjimarcou (1999) were used in manual sequencing. Sequencing results were read manually from the Phosphorimager (Molecular Dynamics,

Model 400E).

Automated sequencing was carried out using reagents from a DYEnamic ET sequencing kit (Amersham). The protocol used for the sequencing reactions followed the manufacture's instructions. Both plasmid DNA and PCR products were used as sequencing templates. When using PCR products, template DNA was first purified from an agarose gel using a "glassmilk" method. Briefly, the DNA band was cut out of the gel and transferred to a microfuge tube, then 2-3 volumes of freshly made 6 M NaI solution and 5 μl glassmilk (kindly provided by the Molecular Biology Service Unit of Biological Sciences, University of Alberta) were added. The tube was kept at 55°C for 10 min and then centrifuged at 13,000 rpm for 3 min. The supernatant was removed and the pellet was washed with New Wash buffer [9.5 mM Tris.Cl (pH 7.2), 95 mM NaCl, 0.95 mM EDTA and 50% ethanol] three times. Then, the pellet was dried and the DNA was resuspended in 30 μl sterile water, which was then kept at 55°C for 10 min before collecting the supernatant for use as a sequencing template.

CHAPTER 3 RESULTS

3.1 The *pol3-612* strain is PAA-sensitive

3.1.1 Construction of the pol3-612 strain:

The *S. cerevisiae* counterpart of T4 L412M mutant, *pol3-612*, was constructed through PCR-based mutagenesis (see Chapter 2). Southern hybridization analysis was used to monitor chromosome configuration at the *POL3* locus throughout the strain construction process. As predicted, two bands with sizes of 3.9 kb and 6.8 kb, respectively, were observed after plasmid integration, while only one 3.9 kb band was seen after the 5-FOA selection (Figure 3.1.1 and 3.1.2), confirming the restoration of single copy status at the *POL3* locus in the *pol3-612* strain. Genome sequencing was used to verify the presence of mutations encoding the L612M substitution in the *pol3-612* strain.

3.1.2 The pol3-612 strain is moderately PAA sensitive:

The *pol3-612* strain was examined for PAA sensitivity by spotting cells on gradient PAA plates. While no growth difference was found between the MS71 and *pol3-612* strains on SD-complete plates, *pol3-612* cells were inhibited on PAA gradient plates at concentrations that did not restrict MS71 cells (Figure 3.1.3).

PAA-sensitivity of the *pol3-612* strain was also confirmed by growth in liquid medium. In the absence of PAA, the MS71 and *pol3-612* strains showed similar doubling time and growth rates (Figure 3.1.5 a). In the presence of 0.5 mg/ml PAA the MS71 strain showed almost no inhibition, while the *pol3-612* strain had a markedly longer doubling time during the first six hours (Figure 3.1.5 a). After six hours, *pol3-612* cells appeared to adapt and resumed growth, although more slowly than the MS71 strain. In addition to slower growth, a significant portion of the *pol3-612* cells (up to 30% in an asynchronized culture) showed a "dumbbell-like" morphology in the presence of 0.5 mg

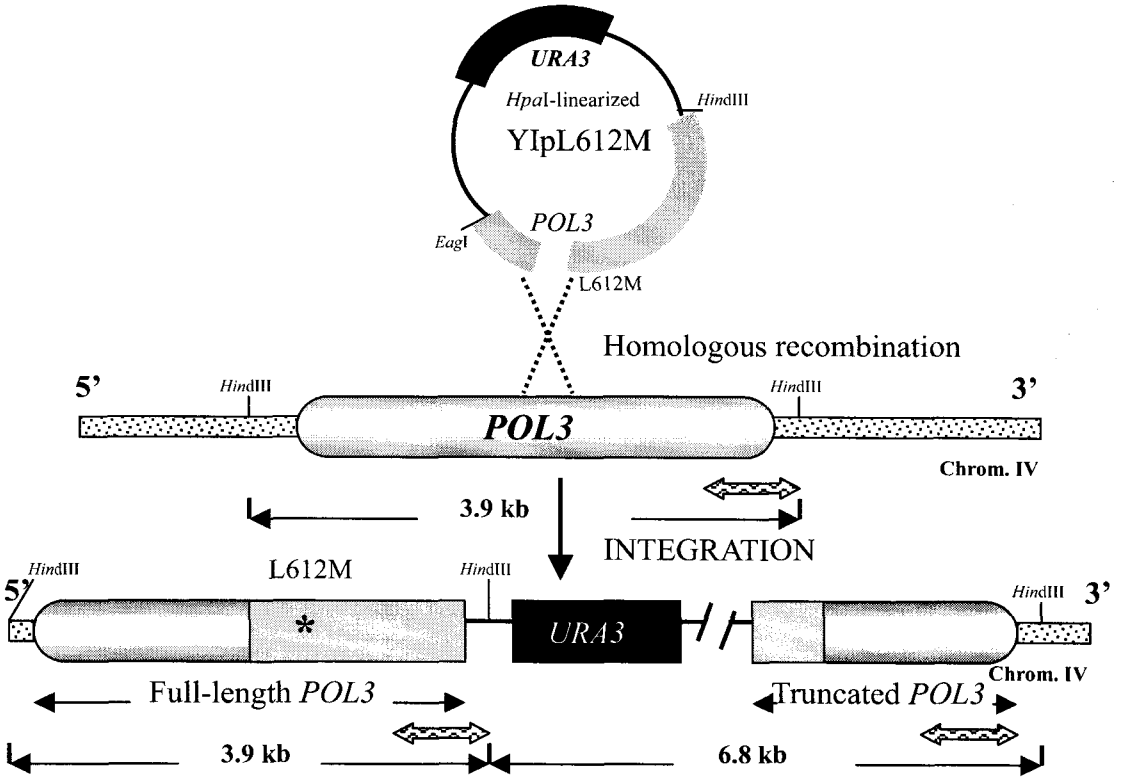

Figure 3.1.1 Integration at the chromosomal *POL3* locus. The YIpL612M plasmid containing the C-terminus of the *POL3* gene (*Eag*I – *Hin*dIII fragment, the mutations encoding the L612M substitution are represented as * in the figure) was linearized with *Hpa*I and transformed to the MS71 cells. After integration, two copies of *POL3* exist in the genome: one full-length copy harboring the mutations and one truncated copy containing the *Eag*I – *Hin*dIII fragment of the wildtype *POL3* gene. As a result, when hybridized with a probe (shown in double-sided arrows) specific for the C-terminus of the *POL3* gene, the presence of an extra 6.8 kb *Hin*dIII fragment is predicted after the YIpL612M plasmid integration, while only a 3.9 kb fragment is expected before plasmid integration and after restoration to the single copy status following selection on 5-FOA. Predictions were confirmed by Southern hybridization analysis (Figure 3.1.2).

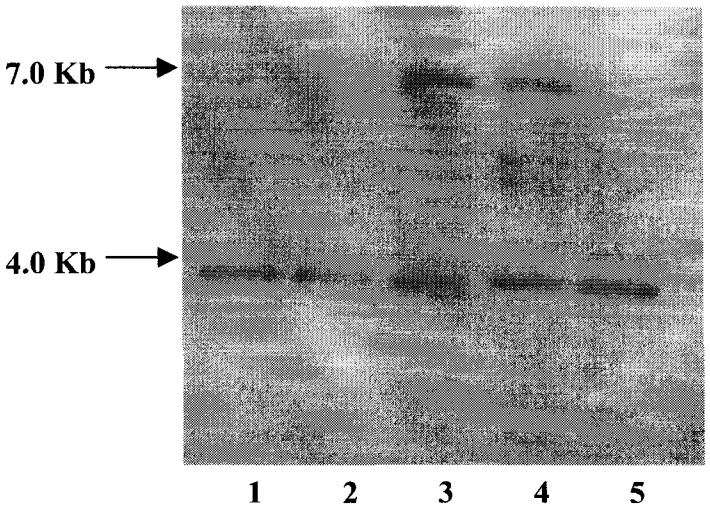

Figure 3.1.2 Southern hybridization analysis of the *pol3-612* strain. The yeast genomic DNA was digested by *Hin*dIII and hybridized with a probe specific for the C-terminus of *POL3* gene (Figure 3.1.1). The sample in lane 5 was from a wildtype MS71 strain. Samples in lane 3 and 4 were from two Ura^+ transformants, in which the YIpL612M plasmid integrated into the yeast genome. Lane 1 and 2 were from two $5\text{-}FOA^r$ popouts, in which the single copy status was recovered. The isolate in lane 2 was confirmed by genomic sequencing to have the mutations encoding the L612M substitution and was chosen as the *pol3-612* strain.

/ml PAA, while only approximately 5% of the MS71 cells have this "dumbbell-like" shape in the presence of PAA (Figure 3.1.5 b). No difference was found between MS71 and *pol3-612* cells with respect to the proportion of cells with "dumbbell-like" shaped cells in the absence of PAA. The "dumbbell" morphology is defined as a swollen mother cell with a large bud and undivided nucleus, and has been proposed to be an indication of cell cycle arrest at the S/G2 phase (Weinert and Hartwell, 1993). Observation of the "dumbbell-like" *pol3-612* cells suggested that PAA triggered cell cycle arrest, presumably by inhibition of the L612M-DNA pol δ. Consistent with the adaptation shown by the growth curve (Figure 3.1.5 a), the percentage of "dumbbell-like" *pol3-612* cells also dropped as being incubated in the presence of PAA for longer time (Figure 3.1.5 b). Thus, as predicted, similar to the T4 L412M DNA polymerase mutant, this particular single amino acid substitution in *S. cerevisiae* DNA pol δ confers sensitivity to PAA. It should be noted that, although *pol3-612* cells demonstrated pronounced PAA-sensitivity, this inhibition was fully reversible because there was no loss in viability of the *pol3-612* cells even after 24 hours incubation with PAA (Figure 3.1.5 c).

To confirm that the observed PAA-sensitivity was solely caused by a single amino acid substitution in Motif A of DNA pol δ, mutations encoding the L612M substitution were replaced by the wildtype codon and subsequently PAA-sensitivity was examined. A strategy similar to the one used to generate the *pol3-612* strain was used to eliminate the mutations: a linearized YIp plasmid containing the C-terminus of a wildtype *POL3* gene was transformed into *pol3-612* cells. After integration of the YIp plasmid into Chromosome IV, mutations encoding the L612M substitution might be replaced by a wildtype codon, which was confirmed by sequencing. As shown in Figure 3.1.4, PAA-sensitivity disappeared when the mutations encoding the L612M substitution were eliminated by transformation ($^{\#}$1 transformant), while PAA-sensitivity remained if mutations were still present after transformation ($^{\#}$2 transformant). Thus this experiment provided convincing evidence that it was the L612M substitution that conferred sensi-

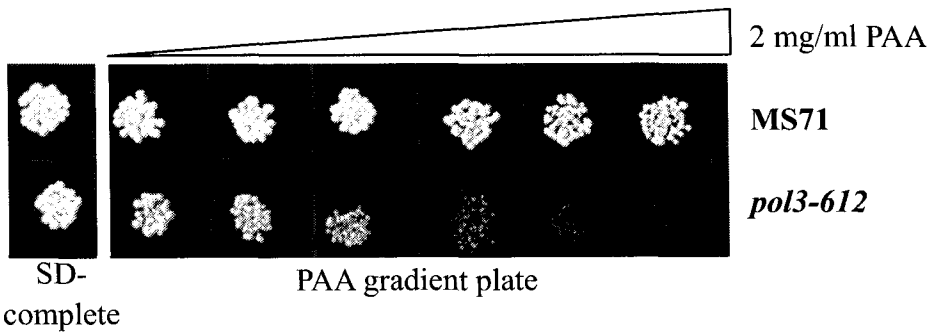

Figure 3.1.3 The *po3-612* strain is PAA-sensitive. Cells in logarithmic phase were diluted and approximately 100 wildtype MS71 and *pol3-612* cells were plated on SD-complete and PAA gradient plates. Plates were incubated at 30°C for 3 days.

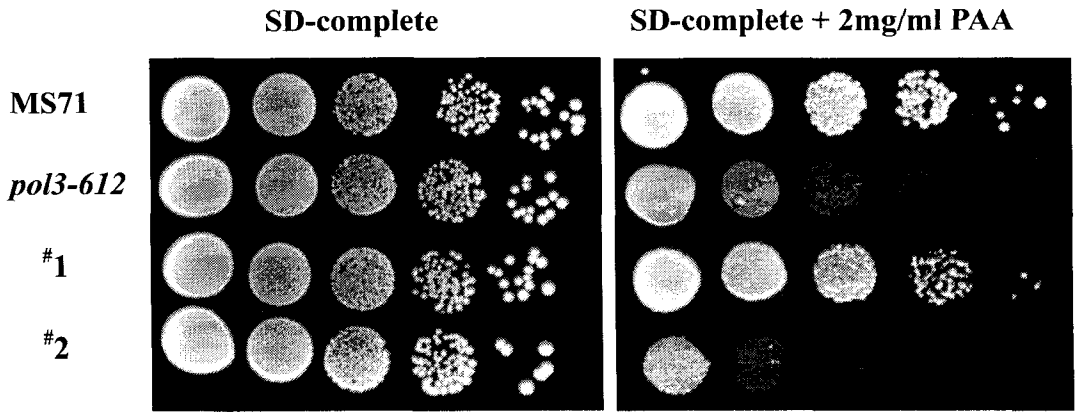

Figure 3.1.4 The L612M substitution confers PAA-sensitivity. Cells in logarithmic phase were diluted and spotted on SD-complete medium, with or without PAA. #1 and #2 are different transformants made by transforming *pol3-612* cells with a YIp plasmid that contained the C-terminus of wildtype *POL3* gene. #1 restored the wildtype *POL3* sequence while #2 remained L612M substitution after transformation. Plates were incubated at 30°C for 3 days.

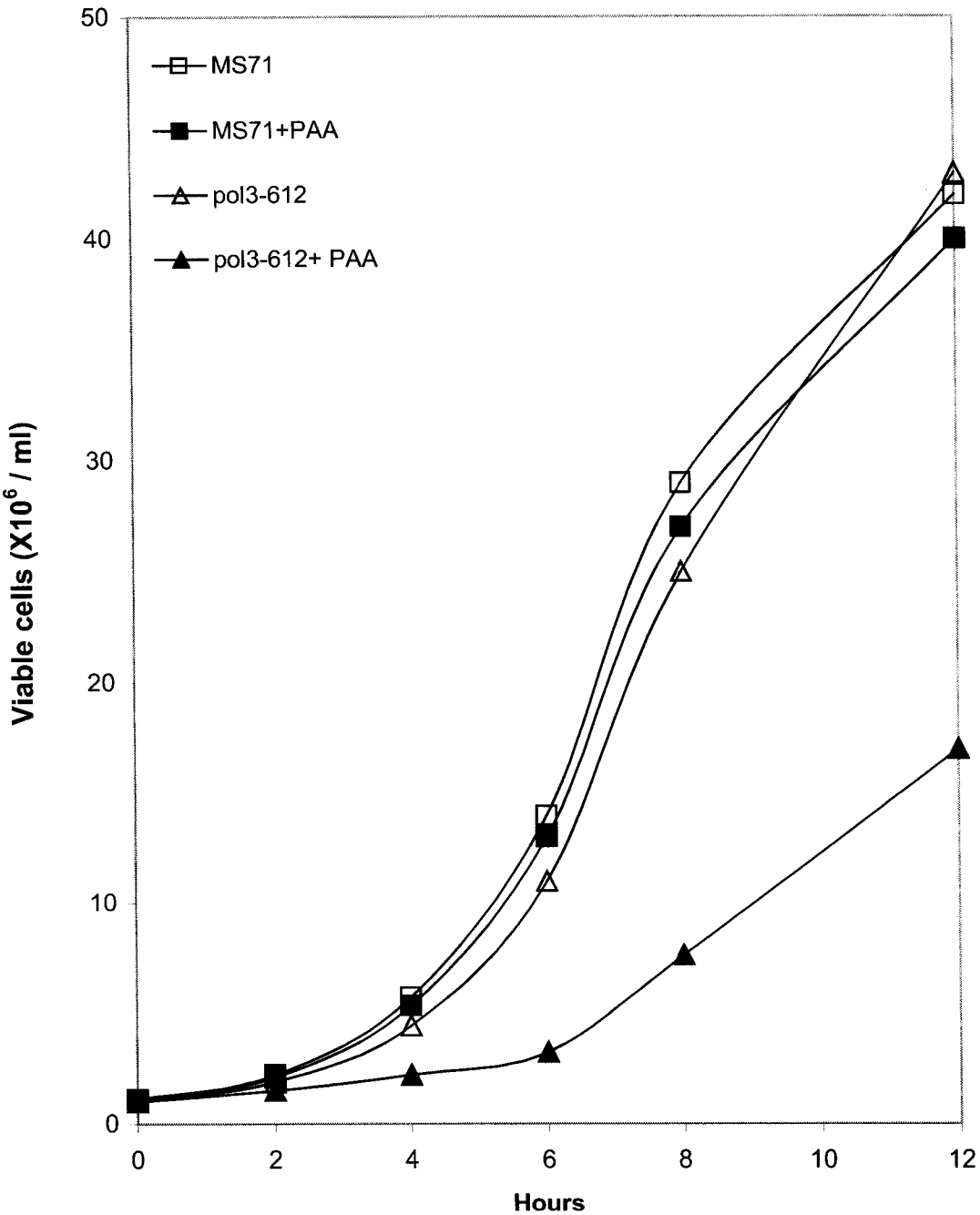

Figure 3.1.5 a

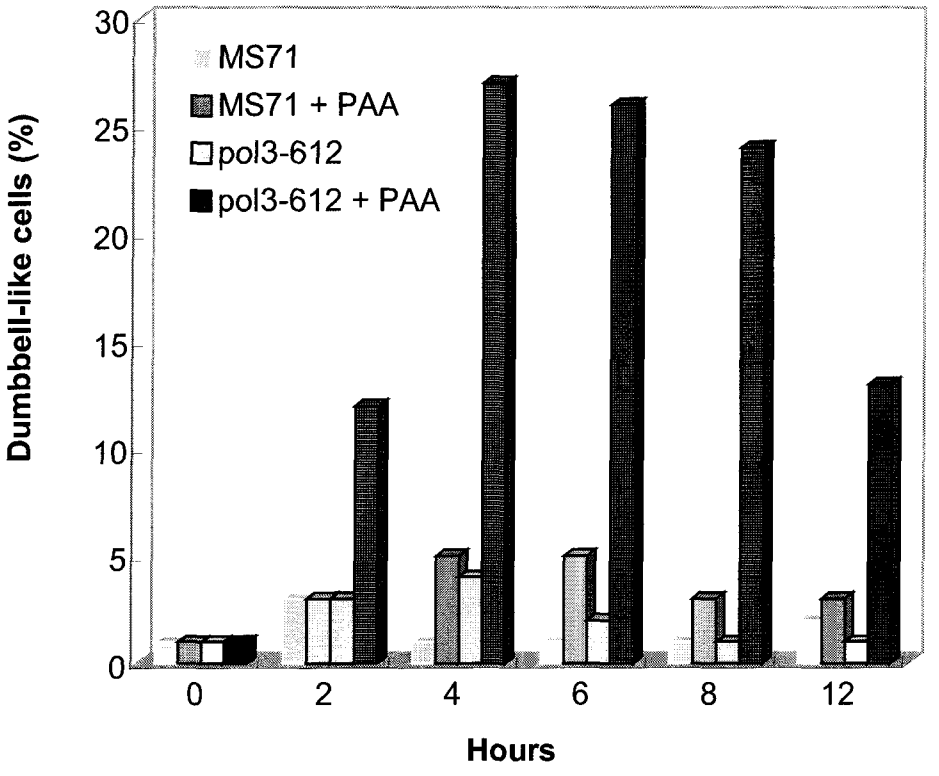

Figure 3.1.5 b

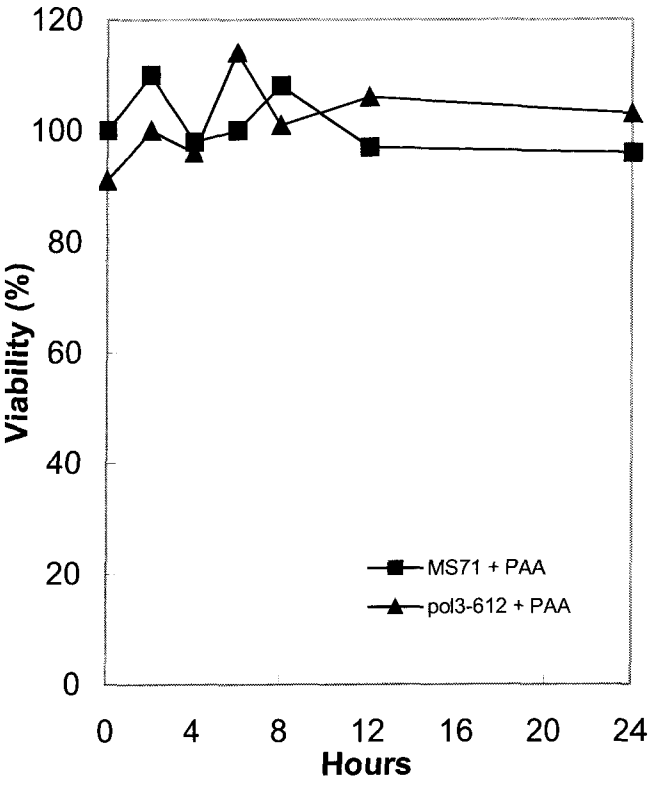

Figure 3.1.5 c

Figure 3.1.5 Cell growth of wildtype MS71 and *pol3-612* in the presence and absence of PAA. (a) Cells in logarithmic phase were inoculated into SD-complete broth with or without 0.5 mg/ml PAA. Numbers of viable cells were determined at the time points indicated. The percentage of "dumbbell-like" cell and viability of the MS71 and *pol3-612* strains was also determined at each time point indicated and is shown in (b) and (c), respectively.

tivity to PAA to the yeast cells.

Taken together, the above data demonstrated that the L612M substitution in the *S. cerevisiae* DNA pol δ confers yeast cells sensitivity to PAA. Because the wildtype DNA pol α, δ and ε do not appear to be inhibited by PAA at the concentration that restricts the L612M-DNA pol δ, the PAA-sensitivity of the *pol3-612* mutant provides a tool to study DNA pol δ separately from DNA pol α and ε.

3.1.3 The pol3-612 allele is recessive:

To further characterize the *pol3-612* allele, a heterozygous diploid *pol3-612/POL3* strain was constructed by crossing the haploid *pol3-612* strain with a haploid wildtype MS71 strain. Diploid strains that were homozygous for the *POL3* and *pol3-612* alleles were also constructed for comparison. Unlike the haploid *pol3-612* and homozygous diploid *pol3-612/pol3-612* strains, heterozygous diploid *pol3-612/POL3* cells did not show appreciable PAA-sensitivity (Figure 3.1.6), indicating that *pol3-612* is recessive to the wildtype *POL3* allele.

3.2. Characterization of the mutator activity of the L612M-DNA pol δ

3.2.1 The pol3-612 strain has a weak mutator phenotype:

In addition to PAA-sensitivity, the L412M T4 DNA polymerase shows decreased replication fidelity compared with the wildtype enzyme, i.e., a mutator phenotype (Reha-Krantz and Nonay, 1994). To examine whether this was also true for the L612M-DNA pol δ, we carried out a replication fidelity study in yeast. Three reporters were used to measure replication fidelity. Two of them measure reversion mutations: *trp1-289* has an *amber* mutation in the coding region, which can be reverted by base substitutions; *his7-2* has a –1 deletion in a tract of eight As, which can be reverted through +1 frameshift mutations (Figure 3.2.1). The third reporter, *CAN1*, measures forward mutations. Canavanine is a lethal chemical that acts as an arginine analog. Both

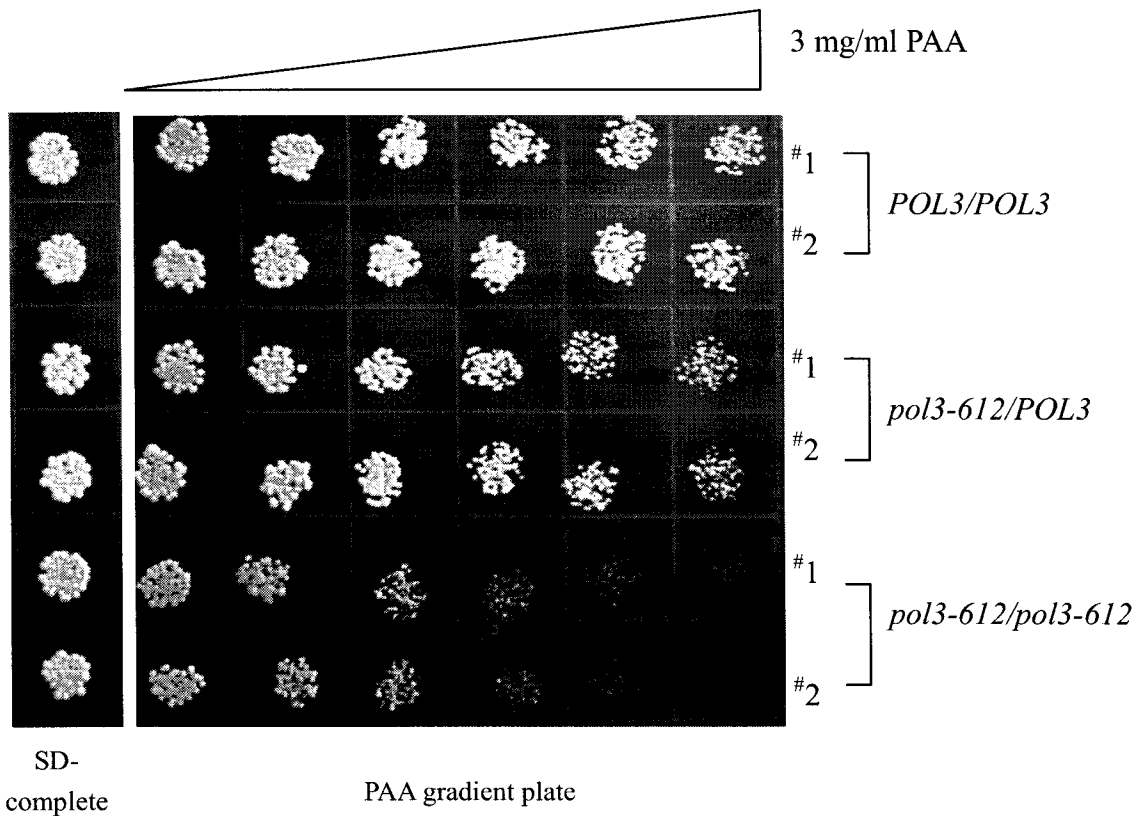

Figure 3.1.6 The *pol3-612* is a recessive allele. Two independent isolates (#1 and #2) of each diploid strain were tested. Approximate 100 cells of each isolate were spotted onto SD-complete plates in the absence or presence of PAA. Plates were incubated at 30°C for 3 days.

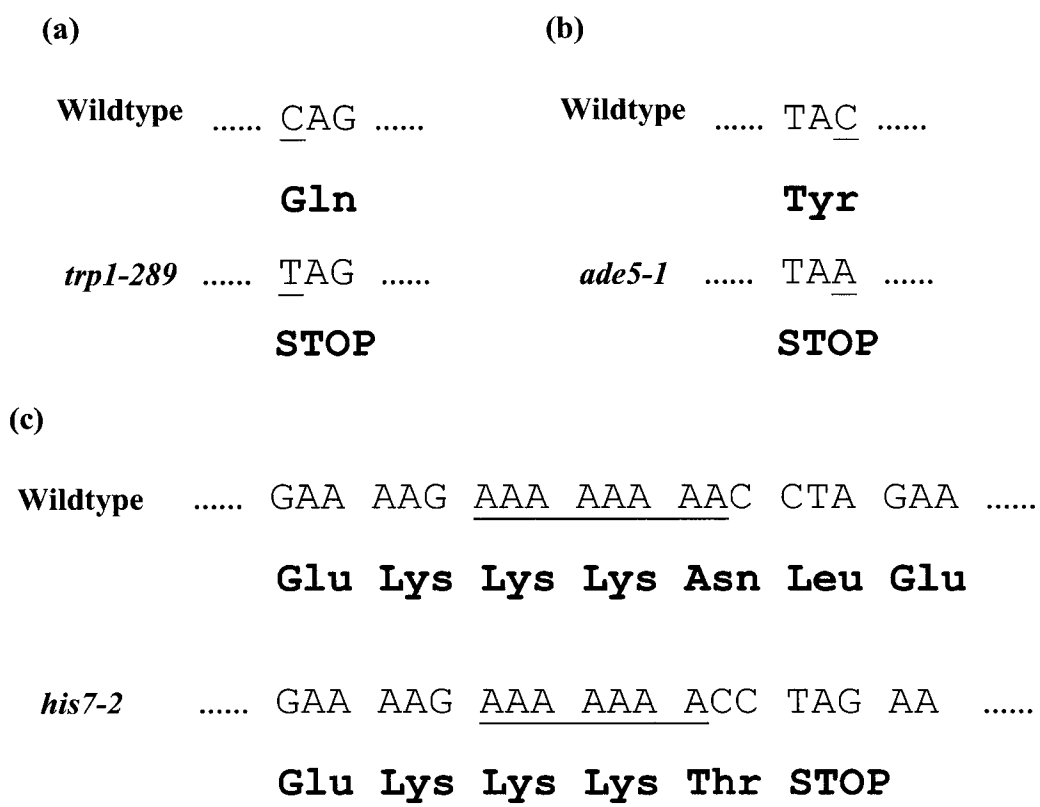

Figure 3.2.1 Sequence of mutant alleles used for replication fidelity test. (a) The *trp1-289* allele has a C to T transition at nucleotide 403, which causes a premature *amber* stop codon (Pavlov, personal communications). (b) The *ade5-1* allele has a C to A transversion at nucleotide 1159, resulting in a premature *ochre* stop codon (Hadjimarcou, 1999). Both mutations in (a) and (b) can be reversed by base substitutions that change the stop codon to codons encoding amino acids. (c) The *his7-2* allele has a deletion of A at nucleotide 472 from the tract of eight As, which results in a premature *amber* stop codon downstream (Hadjimarcou, 1999). This mutation could be reversed by +1 frameshift mutations that restore reading frame. The nucleotide sequence affected by mutations is underlined in (a), (b) and (c).

arginine and canavanine are transported into yeast cells through a permease encoded by the *CAN1* gene. Whereas yeast cells with a wildtype *CAN1* allele are sensitive to canavanine, mutations inactivating the *CAN1* gene confer resistance to the drug (Grenson *et al.*, 1966; Ono *et al.*, 1983). Thus, the *CAN1* locus can readily be used to detect base substitution, frameshift mutations, gross insertions/deletions as well as other complex mutational events (Marsischky *et al.*, 1996; Chen *et al.*, 1999).

Spontaneous mutation rates using these reporters were determined as described in the Materials and Methods section. Compared with the wildtype MS71 strain, the *pol3-612* strain had a modest decrease in replication fidelity. An approximate six-fold elevation in mutation rate was observed at the *his7-2* locus and a four-fold increase for the *CAN1* locus (Table 3.2.1). A smaller increase (1.6-fold) in mutation rate was seen at the *trp1-289* locus (Table 3.2.1). Although small, the increase in mutation rate at the *trp1-289* locus was statistically significant, because the 95% confidence intervals for the mutation rates of MS71 and *pol3-612* strains were non-overlapping (Table 3.2.1). Hence, similar to the observations made of the T4-L412M DNA polymerase, the single amino acid change in the Motif A of yeast DNA pol δ also reduced replication fidelity. Taken together, the data described above suggested a similarity in Motif A function between yeast DNA pol δ and T4 DNA polymerase.

3.2.2 Construction of the msh2 pol3-612 strain:

The post-replication mismatch repair (MMR) system corrects misincorporations that escape proofreading by DNA polymerases (reviewed by Modrich and Lahue, 1996), so in order to uncover the full mutator activity of L612M-DNA pol δ, the MMR system needs to be inactivated.

The *MSH2* gene encodes an essential component of the MMR system (Figure 1.2) and MMR can be inactivated by disrupting the *MSH2* gene (Marsischky *et al.*, 1996). The *msh2* strain used in this study has a deletion of an *Eco*RI fragment in the coding

Strain	Mutation rate (X 10^{-8})					
	trp1-289 (base substitution)		*his7-2* (frameshift)		*CAN1* (base substitution + frameshift)	
MS71	2.9	(2.7-3.6)	1.1	(0.9-1.6)	31	(27-39)
pol3-612	4.5	(4.1-6.2)	6.3	(4.6-7.4)	108	(104-122)
Relative rate	1.6		5.8		3.5	

Table 3.2.1 The *pol3-612* strain has a mutator phenotype. The method of the median was used to calculate mutation rates. Mutation rates shown here were based on data from 20 to 25 independent cultures. 95% confidence intervals are shown in parentheses. Relative rate was calculated by dividing mutation rate of the *pol3-612* strain by that of the MS71 strain.

region of the *MSH2* gene, thus inactivating the gene (Sia *et al.*, 1997). To study the mutator activity of L612M-DNA pol δ without the "masking" effect of MMR, a double mutant *msh2 pol3-612* strain was constructed by crossing the *pol3-612* strain with the *msh2* strain, followed by tetrad dissection. Three types of tetrads were generated in tetrad dissection: the parental ditype (PD), the tetratype (TT) and the non-parental ditype (NPD). The desired *msh2 pol3-612* strain was identified from both TT and NPD tetrads. The strain construction scheme is shown in Figure 3.2.2. For simplicity, only the TT tetrad is drawn.

3.2.3 The pol3-612 MMR-deficient strains display strong mutator phenotypes

Four *msh2 pol3-612* segregants from four tetrads were randomly chosen for characterization. Spot assays on synthetic complete medium supplemented with 60 μg/ml canavanine were used to approximate the mutator phenotype. As shown in Figure 3.2.3, all four putative *msh2 pol3-612* segregants had significantly elevated mutator activity compared with the *msh2* strain, evidenced by an increase in canavanine resistant mutant cells in the spotted culture. Interestingly, all four *msh2 pol3-612* segregants also exhibited significantly enhanced PAA-sensitivity (see Section 3.3 for details).

To quantitatively characterize the mutator activity of the L612M-DNA pol δ, spontaneous mutation rates for the *msh2 pol3-612* strain were measured at the *trp1-289*, *his7-2* and *CAN1* loci. The *msh2* strain carrying wildtype DNA polymerases was used for comparison. Mutation rates are shown in Table 3.2.2.

The *amber* codon of *trp1-289* allele appeared to be replicated with high fidelity in the wildtype MS71 strain, since removing MMR only increased the mutation rate 3-fold (from 2.9×10^{-8} to 8.4×10^{-8}, Table 3.2.2). In contrast, elimination of MMR caused a 45-fold and a 17-fold increase in mutation rates at the *his7-2* locus and *CAN1* locus, respectively (from 1.1×10^{-8} to 50×10^{-8} and 31×10^{-8} to 527×10^{-8}, Table 3.2.2), revealing levels of inaccurate replication by wildtype DNA polymerases at these sites.

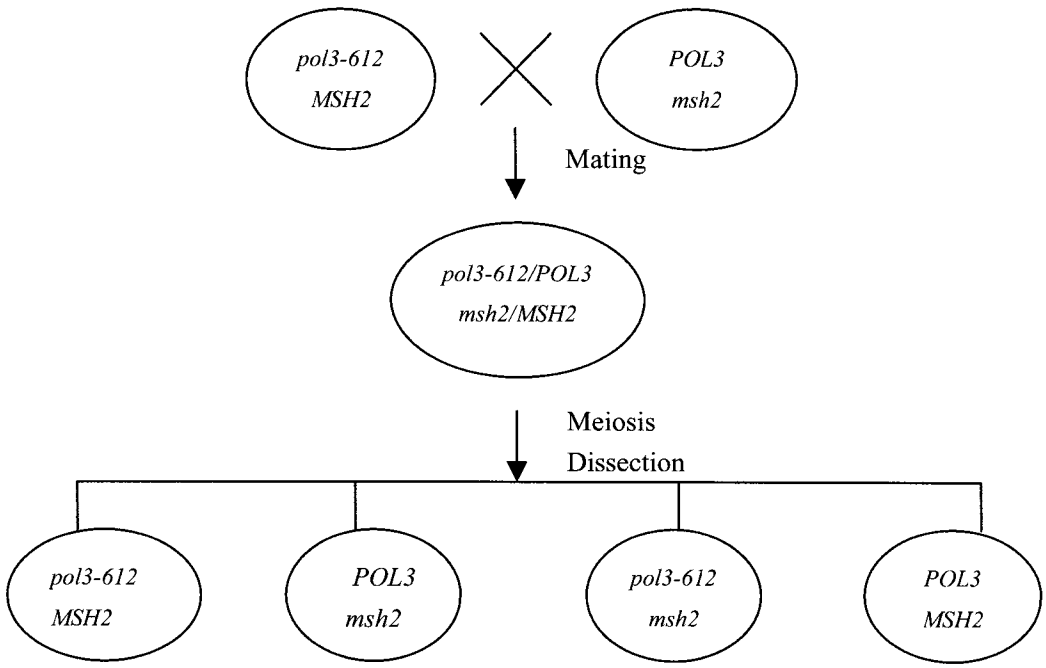

Figure 3.2.2 Diagram outlining the construction of the *msh2 pol3-612* strain. See text for details.

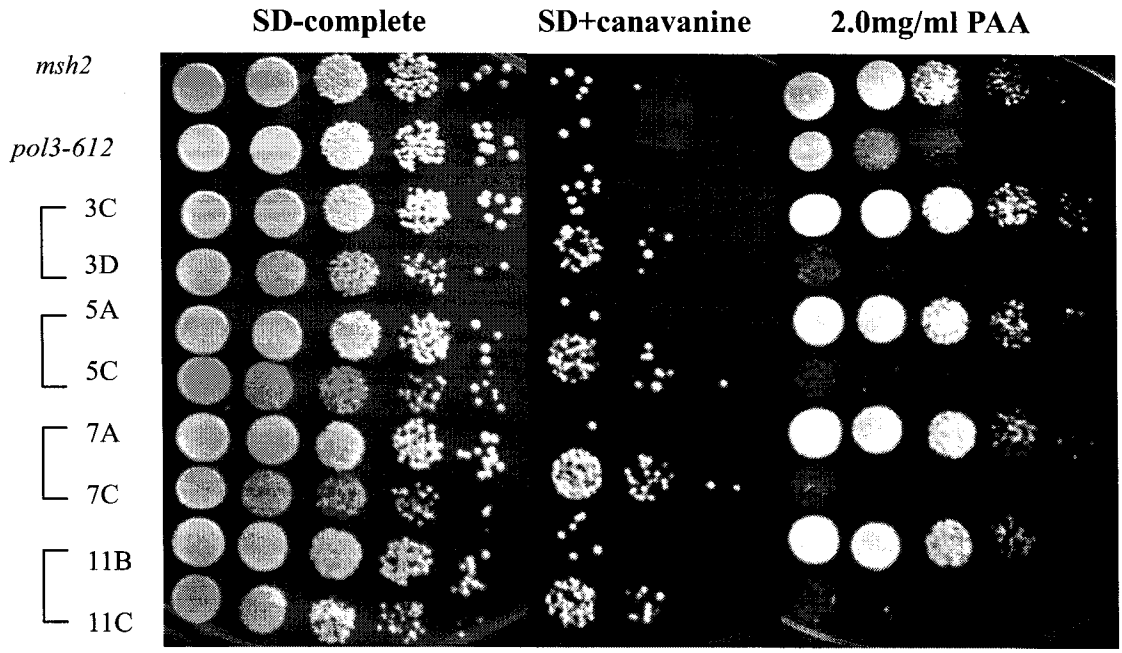

Figure 3.2.3 Characterization of different *msh2 pol3-612* and *msh2* segregants. The *msh2 pol3-612* (3D, 5C, 7C and 11C) and *msh2* segregants (3C, 5A, 7A and 11B) from four tetrads were randomly chosen for characterization (segregants from the same tetrad are grouped by brackets). Tenfold serial dilutions of each strain were spotted onto SD-complete plates in the presence of PAA and canavanine. Plates were incubated at 30°C for 3 days.

Strain	Relative Genotype	Mutation rate (X 10^{-8})		
		trp1-289 (base substitution)	*his7-2* (frameshift)	*CAN1* (base substitution + frameshift)
MS71	Wildtype	2.9 (2.7-3.6)	1.1 (0.9-1.6)	31 (27-39)
LY104	*pol3-612*	4.5 (4.1-6.2)	6.3 (4.6-7.4)	108 (104-122)
EAS56	*msh2*	8.4 (6.9-10.6)	50 (43-53)	527 (432-664)
LY111	*msh2 pol3-612*	215 (177-271)[26]	1204 (734-1736) [24]	8803 (7961-12405) [17]
KY508	*mlh1*	7.3 (5.6-12.4)	35 (29-38)	342 (270-425)
KY507	*mlh1 pol3-612*	242 (160-440) [33]	1263 (940-1500) [36]	8889 (5759-12617) [26]
LY116	*pms1*	5.2 (3.8-6.6)	31 (25-37)	435 (308-494)
LY112	*pms1 pol3-612*	172 (145-262) [33]	1124 (927-1506) [36]	9781 (6249-16278) [22]
EAS38	*msh6*	5.0 (3.9-6.6)	3.1 (2.9-4.1)	180 (128-250)
LY111	*msh6 pol3-612*	223 (147-368) [45]	34 (31-54)[11]	4150 (3563-5275) [23]
KY504	*msh3*	2.2 (1.9-2.6)	7.6 (6.6-9.6)	33 (28-36)
KY505	*msh3 pol3-612*	2.5 (1.5-2.9) [1]	12 (10-14)[1.6]	151 (110 –210) [5]
LY117	*exo1*	8.7 (7.8-12.7)	2.4 (2.3-5.3)	89 (82-99)
LY113	*exo1 pol3-612*	35 (25-56) [4]	167 (145-300) [70]	603 (563-665) [7]
LY121	*msh3 msh6 pol3-612*	214 (157-313)	1057 (950-1348)	10075 (9192-11565)

Table 3.2.2 Mutation rates for the wildtype and the L612M-DNA pol δ in MMR-deficient background. The "method of the median" was used to calculate mutation rates. Mutation rates shown were based on data from 10 to 20 independent cultures. 95% confidence intervals are shown in parentheses. Numbers given in [] indicate fold increases in mutation rate, which is calculated by dividing the mutation rates of the *pol3-612* MMR-defective double mutants with that of the MMR-defective single mutants immediately above. The results of KY strains are based on the experiments done by K. Murphy.

Not surprisingly, without the MMR present to correct replication errors, the L612M-DNA pol δ had significantly reduced DNA replication fidelity. In the absence of MMR, while wildtype DNA pol δ had a mutation rate of 527×10^{-8} at the *CAN1* locus, the L612M-DNA pol δ increased mutation rate to 8803×10^{-8}; in other words, the L612M-DNA pol δ lowered the replication fidelity by 17-fold (Table 3.2.2). Similarly, a 26-fold and 24-fold elevation in mutation rates was seen for base substitutions and +1 frameshift mutations respectively in the *msh2 pol3-612* double mutant compared to the *msh2* strain, as shown at the *trp1-289* and *his7-2* loci (Table 3.2.2).

Besides disrupting the *MSH2* gene, MMR can also be inactivated by knocking out other critical MMR components such as the *PMS1* and *MLH1* genes (Figure 1.2). To confirm the strong mutator activity of L612M-DNA pol δ in the absence of MMR, the *PMS1* and *MLH1* genes were knocked out through one-step gene disruption (see Materials and Methods). Similar to what was seen in the *msh2* background, replacement of wildtype DNA pol δ with L612M-DNA pol δ led to dramatic increases (20 to 30-fold) in mutation rates at all three loci tested in both the *pms1* and the *mlh1* mutant backgrounds (Table 3.2.2). Note that although *msh2 pol3-612*, *pms1 pol3-612* and *mlh1 pol3-612* had fluctuating mutation rates at the *trp1-289*, *his7-2* and *CAN1* loci, the 95% confidence intervals of these three strains had significant overlaps for all of the loci (Table 3.2.2), suggesting that the differences in mutation rates for each marker between the three strains are insignificant.

The proteins encoded by the *MSH6* and *MSH3* genes form complexes with Msh2p. The Msh6p-Msh2p complex mainly recognizes base-base mismatches and small loops, whereas the Msh6p-Msh3p complex binds preferentially to small and large loops (Figure 1.2), suggesting there is functional redundancy between Msh6p-Msh2p and Msh3p-Msh2p complexes in MMR (Marsischky *et al.*, 1996). The combination of the *pol3-612* and *msh6* knockout strain led to a dramatic increase in mutation rate at the *trp1-289* locus, comparable to that of the *msh2-*, *pms1-*, *mlh1 pol3-612* strains (Table

3.2.2). A significant elevation (23-fold) was also observed at the *CAN1* locus in the *msh6 pol3-612* strain, while a moderate increase (11-fold) was observed at the *his7-2* locus. In contrast to the large effects described above, inactivation of the *MSH3* gene in a *pol3-612* background only resulted in a slight increase in spontaneous mutation rates for the *his7-2* and *CAN1* loci (Table 3.2.2), and no change was observed for the *trp1-289* locus, which was consistent with previous reports on the subtle effects of an *MSH3* deletion (Marsischky *et al.*, 1996; Tran *et al.*, 1999b). Inactivation of both the *MSH6* and *MSH3* genes in a *pol3-612* background (the *msh3 msh6 pol3-612* strain) led to high mutation rates that were comparable to the *msh2-*, *pms1-*, *mlh1 pol3-612* strains (Table 3.2.2), consistent with previous studies suggesting functional redundancy between *MSH6* and *MSH3* (Marsischky *et al.*, 1996; Tran *et al.*, 1999b).

The product of the *EXO1* gene has been proposed to function in the *MSH2*-dependent MMR pathway (Tishkoff *et al.*, 1997a). Interactions of Exo1p with both Msh2p and Mlh1p were reported based on two-hybrid studies (Tran *et al.*, 2001) and co-immunoprecipitation assays (Tishkoff *et al.*, 1997a). Exo1p possesses an intrinsic 5'→ 3' exonuclease activity, as well as a flap-endonuclease activity (Tran *et al.*, 2002), which may account for its role during MMR and other DNA metabolic processes (Tran *et al.*, 1999b). In the presence of wildtype DNA pol δ, inactivation of the *EXO1* gene only produced a 2 to 3-fold increases in mutation rates using all three reporters (Table 3.2.2, compare the *exo1* strain to the MS71 strain), consistent with previous reports (Tishkoff *et al.*, 1997a). The relatively subtle increase compared with other MMR gene deficiencies suggested functional redundancy with other exonucleases involved in MMR, as observed in prokaryotic MMR (Modrich and Lahue, 1996). A similar scenario was also seen for L612M-DNA pol δ at the *trp1-289* and *CAN1* loci (Table 3.2.2). Only 4 and 7-fold increases were observed when the wildtype DNA pol δ was replaced by the L612M-DNA pol δ, suggesting that replication errors made by L612M-DNA pol δ at those loci might be processed by exonucleases other than Exo1p in MMR. In contrast, a

70-fold increased *his7-2* mutation rate was observed for the L612M-DNA pol δ in the absence of Exo1p (Table 3.2.2), implying that L612M-DNA pol δ might generate aberrant DNA structures that, if not corrected by Exo1p, lead to +1 frameshift mutations. A requirement of Exo1p for preventing +1 frameshifts in homonucleotide repeats was also reported previously in a DNA pol ε mutant (Kirchner *et al.*, 2000). The underlying mechanism could reflect the role played by Exo1p in removing 5'-flaps generated during processing of Okazaki fragments. Synthetic lethality is observed when *exo1* is combined with *rad27*, an important gene required for Okazaki fragment processing (Tishkoff *et al.*, 1997a). Overexpression of Exo1p partially suppresses some of the phenotypes caused by *RAD27* inactivation (Tishkoff *et al.*, 1997a; Qiu *et al.*, 1999). Inappropriate processing of Okazaki fragments has been proposed to be responsible for minisatellite and microsatellite instability in yeast (Kokoska *et al.*, 1998).

Taken together, these data indicated that replacement of wildtype DNA pol δ by L612M-DNA pol δ results in a significant elevation in spontaneous mutation rates. In order to investigate whether the strong mutator phenotype is caused by the L612M -DNA pol δ itself, or is an indirect consequence of other factors, the TLS DNA polymerases and the *DUN1*-dependent checkpoint pathway were examined.

3.2.4 The effects of inactivation of TLS DNA polymerases on mutation rates

TLS DNA polymerases (see Section 1.3.2) are specialized DNA polymerases normally involved in replicating the template strand that contains DNA damage (reviewed by Friedberg *et al.*, 2002; Kunkel *et al.*, 2003). They may transiently replace the genome-replicating DNA polymerases when the latter encounter DNA damage in the template strand. After a short synthesis across the damage, the TLS DNA polymerases may be replaced by major DNA polymerases, which will continue the strand elongation. Because most of these specialized DNA polymerases do not possess a 3'→ 5' proofreading activity, they display low replication fidelity as shown by analysis of both *S.*

cerevisiae (Washington *et al.*, 1999; Harfe and Jinks-Robertson, 2000) and human TLS DNA polymerases (Matsuda *et al.*, 2000).

One possible scenario accounting for the strong mutator phenotype observed in the *pol3-612* MMR-defective strains is that the L612M-DNA pol δ may frequently cause replication fork stalling, then the TLS DNA polymerases may transiently replace DNA pol δ and carry out genome replication. If that is true, then the observed strong mutator phenotype is indeed caused by the TLS DNA polymerases.

In order to investigate the possible contribution of the TLS polymerases to the strong mutator phenotype observed in *pol3-612* MMR-deficient strains, two major TLS DNA polymerases, DNA pol ζ and pol η, were inactivated in both *msh2* and *msh2 pol3-612* strain backgrounds. DNA pol ζ was inactivated by knocking out the *REV3* gene, which encodes the catalytic subunit of the enzyme (Nelson *et al.*, 1996). DNA pol η was disabled by knocking out the *RAD30* gene (Johnson *et al.*, 1999). If the DNA pol ζ and/or η are responsible for the observed strong mutator phenotypes, then removal of those DNA polymerases should eliminate or alleviate the mutator phenotypes.

Compared with the *msh2* strain, the *msh2 rev3* strain had a slightly decreased mutation rate at all three loci tested (Table 3.2.3), consistent with previous reports (Datta *et al.*, 2000) and the proposed error-prone nature of DNA pol ζ. In the absence of DNA pol ζ, substitution of wildtype DNA pol δ with L612M-DNA pol δ still appeared to generate a strong mutator phenotype: compared with the *msh2 rev3* strain, the *msh2 rev3 pol3-612* strain increased mutation rates by 52-fold at the *trp1-289* locus, 25-fold at the *his7-2* locus and 17-fold at the *CAN1* locus (Table 3.2.3), similar to the increase observed in the presence of pol ζ (i.e., compared to the *msh2* strain, 26-fold, 24-fold and 17-fold increases were seen for the *msh2 pol3-612* strain, respectively). Likewise, a *RAD30* gene knockout did not exhibit suppression: the significant increase in mutation rates still existed even in the absence of DNA pol η (Table 3.2.3). Thus the strong mutator phenotypes of *pol3-612* MMR-deficient strains appeared to be independent of

Strain	Relative Genotype	Mutation rates (X 10^{-8})		
		trp1-289 (base substitution)	*his7-2* (frameshift)	*CAN1* (base substitution, frameshift)
Effects of error-prone DNA pols				
EAS56	*msh2*	8.4 (6.9-10.6)	50 (43-53)	527 (432-664)
LY110	*msh2 pol3-612*	215 (177-271) [26]	1204(734-1736) [24]	8803 (7961-12405)[17]
LY119	*msh2 rad30*	5.9 (5.1-6.8)	29 (24-35)	265 (218-320)
LY115	*msh2 rad30 pol3-612*	285 (211-371) [48]	834 (680-1580) [29]	6967 (5095-9741) [26]
LY118	*msh2 rev3*	4.3 (3.2-5.2)	33 (27-38)	365 (313-480)
LY114	*msh2 rev3 pol3-612*	227 (193-249) [52]	815(639-919) [25]	6266 (4695-9113) [17]
Effects of *DUN1*-dependent checkpoint				
LY130	*dun1*	nd	nd	24 (16-28)
LY131	*dun1 pol3-612*	nd	nd	51 (31-54) [2.1]
LY134	*msh2 dun1*	2.7 (1.5-3.9)	21 (16-23)	288 (217-367)
LY133	*msh2 dun1 pol3-612*	138 (103-197) [51]	354 (316-575) [17]	4724 (3775-5891) [16]

Table 3.2.3 Mutation rates determined in the absence of DNA pol η, DNA pol ζ or Dun1p. The "method of the median" was used to calculate mutation rates. Mutation rates shown here were based on data from 10 to 20 independent cultures. 95% confidence intervals are shown in parentheses. Numbers given in [] indicate fold increases in mutation rate relative to the strain immediately above. For certain strains, mutation rates at the *trp1-289* and *his7-2* loci were not determined (nd).

DNA pol ζ and η.

3.2.5 The effects of DUN1 deletion on mutation rates

In addition to testing TLS DNA polymerases, the possible effects of the *DUN1*-dependent checkpoint pathway on mutation rates were also examined. Dun1p functions in the *MEC1, RAD53*-dependent checkpoint pathway and is required for up-regulating dNTP pool concentrations in response to DNA damage or replication inhibition (Zhao and Rothstein, 2002; Chabes *et al.*, 2003). As a result, a *dun1* mutant is sensitive to DNA damaging agents and HU (Zhou and Elledge, 1993). The reason why this pathway was examined was because of previous studies on the *pol3-01* mutator strain, in which the 3' → 5' exonuclease activity of DNA pol δ is abolished by replacement of the key residues in the exonuclease active center with alanine residues (Morrison *et al.*, 1993). Datta *et al.* (2000) reported that the strong mutator phenotype of the *pol3-01* strain was at least partially dependent on *DUN1*-dependent checkpoint activation. Deletion of the *DUN1* gene led to a 8-fold decrease in mutation rate at the *CAN1* locus in a *pol3-01* background. Decreased mutation rates (2-fold) were also seen with other reporters (Datta *et al.*, 2000).

To examine whether or not Dun1p also plays a role in *po3-612* MMR-deficient strains, a series of *DUN1* knock out strains were constructed (Table 3.2.3). In the presence of a functional MMR system, deletion of the *DUN1* gene had little effect on the mutator phenotype of *pol3-612* (2.1-fold increase in mutation rate for the *CAN1* marker, compared to a 3.5-fold increase in the presence of *DUN1*, Table 3.2.3 and 3.2.1). In the absence of a functional MMR and the *DUN1*-dependent pathway, replacing wildtype DNA pol δ with L612M-DNA pol δ still led to 16 to 51-fold increase in mutation rates (mutation rates of *dun1 msh2 pol3-612* strain divided by that of *dun1 msh2* strain, Table 3.2.3), similar to the increase seen when the *DUN1* pathway was present (compare the *msh2 pol3-612* strain to the *msh2* strain, Table 3.2.2). Therefore, the *DUN1*-dependent

checkpoint pathway seemed to be dispensable for the strong mutator phenotypes observed for the L612M-DNA pol δ.

Taken together, these data suggested that the strong mutator phenotypes observed in the *pol3-612* MMR-deficient strains is mainly due to the L612M-DNA pol δ itself. In other words, the mutator phenotype appears due to a significant compromise in replication fidelity of DNA pol δ caused by the single amino acid substitution in the polymerase active center. The strong mutator activity observed in *pol3-612* MMR-deficient strains could still be an underestimation of inaccuracy of L612M-DNA pol δ in genome replication, because it is reported that DNA pol ε can proofread DNA that is replicated by DNA pol δ and mask replication errors generated by pol δ (Morrison and Sugino, 1994).

3.3. PAA-sensitivity of *pol3-612* MMR-deficiency strains

3.3.1 MMR-deficiency causes dramatic increases on PAA-sensitivity in the pol3-612 background

In addition to assessing mutator activity, PAA-sensitivity of *msh2 pol3-612* segregants was also examined during strain construction. Interestingly, the strong mutator phenotype was linked to significantly enhanced PAA-sensitivity for all *msh2 pol3-612* segregants that were randomly chosen (Figure 3.2.3). None of the *msh2* segregants appeared to be sensitive to PAA (Figure 3.2.3), suggesting that MMR deficiency causes a dramatic increase of PAA-sensitivity in a *pol3-612* background. This was an unexpected observation that was not foreshadowed by studies of the T4 L412M mutant, because T4 phage does not have a MMR system itself and the host MMR system does not affect T4 DNA replication (Santos and Drake, 1994).

To further investigate the relationship between MMR and *pol3-612* with respect to PAA-sensitivity, a series of MMR-deficient strains were constructed by inactivating different components of the yeast MMR system. Deletion of either the *PMS1* gene

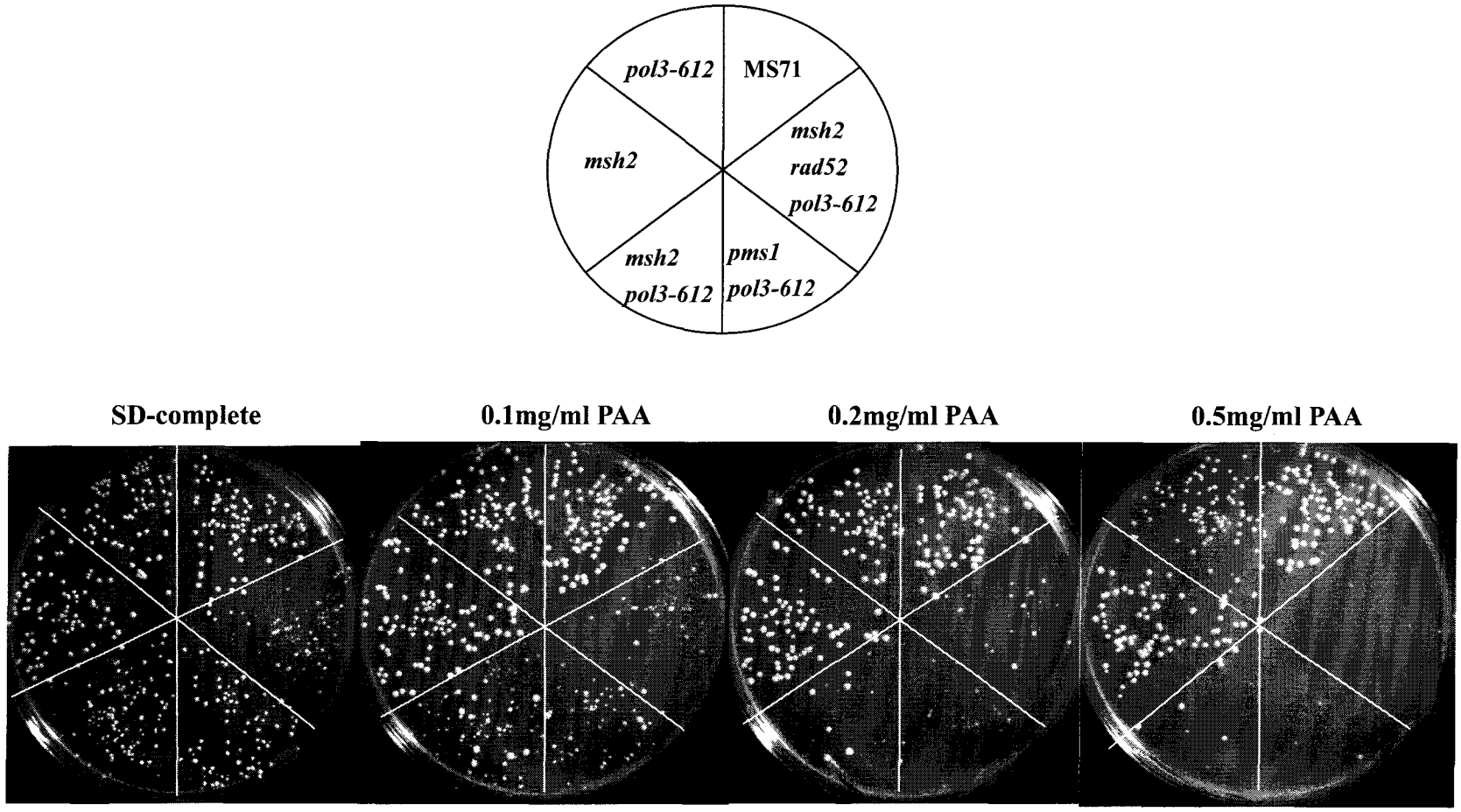

Figure 3.3.1 PAA-sensitivity of *msh2 pol3-612*, *pms1 pol3-612* and *msh2 rad52 pol3-612* strains. Cells in logarithmic phase were diluted and approximately 100 viable cells of each tested strain were spotted on SD-complete plates with or without PAA. Plates were incubated at 30°C for 3 days.

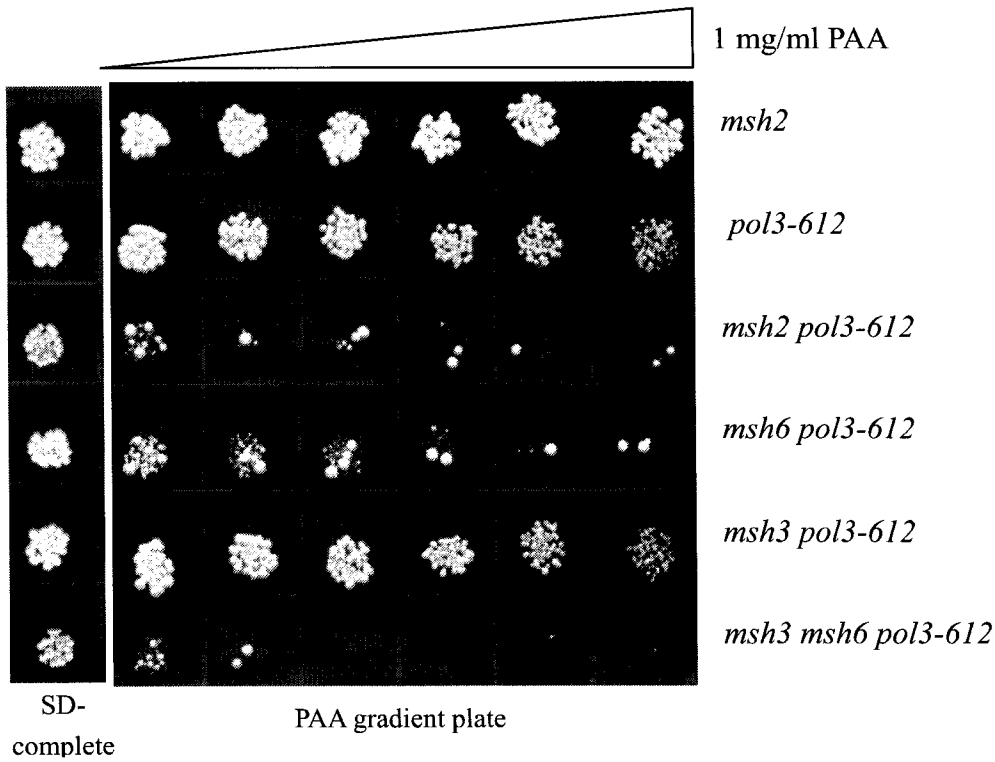

Figure 3.3.2 MMR-deficiency increases PAA-sensitivity in the *pol3-612* strain background. Cells were grown at 30°C to logarithmic phase and then diluted. Approximately 100 viable cells of each strain were spotted on SD-complete plates with or without PAA gradient. Plates were incubated at 30°C for 3 days.

(Figure 3.3.1) or the *MLH1* gene (data not shown) in a *pol3-612* background caused a dramatic increase in PAA-sensitivity, comparable to that seen for the *msh2 pol3-612* strain. The combination of a *MSH6* deletion and *pol3-612* also led to significantly elevated sensitivity to PAA (Figure 3.3.2). However, compared to *msh2-*, *mlh1-*, *pms1 pol3-612* strains, the *msh6 pol3-612* strain seemed to be less PAA-sensitive, as shown by growth of small colonies of *msh6 pol3-612* at PAA concentrations that completely inhibited *msh2 pol3-612* cells on the gradient PAA plate (Figure 3.3.2). The appearance of a few PAA-resistant colonies in the *pol3-612* MMR-deficient strains can be noted (Figure 3.3.1 and 3.3.2), likely a consequence of the strong mutator activity in those strains. The PAA-resistance could be caused by two reasons: either spontaneous revertants or second-site mutations that suppress PAA-sensitivity conferred by the L612M substitution (see Section 3.6 for details). From Figure 3.3.1, it appeared that the second-site mutations were more likely to be the case, since only partial resistance to PAA was observed. If spontaneous reversions occurred, then PAA-resistance comparable to *msh2* cells was expected. Unlike other components of MMR, inactivation of the *MSH3* gene did not seem to appreciably affect the PAA-sensitivity of the *pol3-612* mutant (Figure 3.3.2), reminiscent of the subtle effects seen on mutation rates after knocking out the *MSH3* gene in a *pol3-612* strain (Table 3.2.2). When both the *MSH6* and *MSH3* genes were inactivated, a dramatic increase in PAA-sensitivity was observed as seen in the *msh2-*, *mlh1-*, *pms1 pol3-612* strains (Figure 3.3.2). Thus MMR appears to play a critical role in cellular responses to PAA in *pol3-612* cells.

3.3.2 MMR deficiency impairs cell cycle arrest and reversibility from PAA inhibition

PAA-sensitivity of the *msh2 pol3-612* strain was also examined in liquid medium. Even in the absence of PAA, *msh2 pol3-612* cells demonstrated a much lower titer compared to the *pol3-612* cells (number of viable cells at 12 and 24 hours, Figure 3.3.3 a). Consistently, *msh2 pol3-612* strain only had approximately 50% viability (Figure

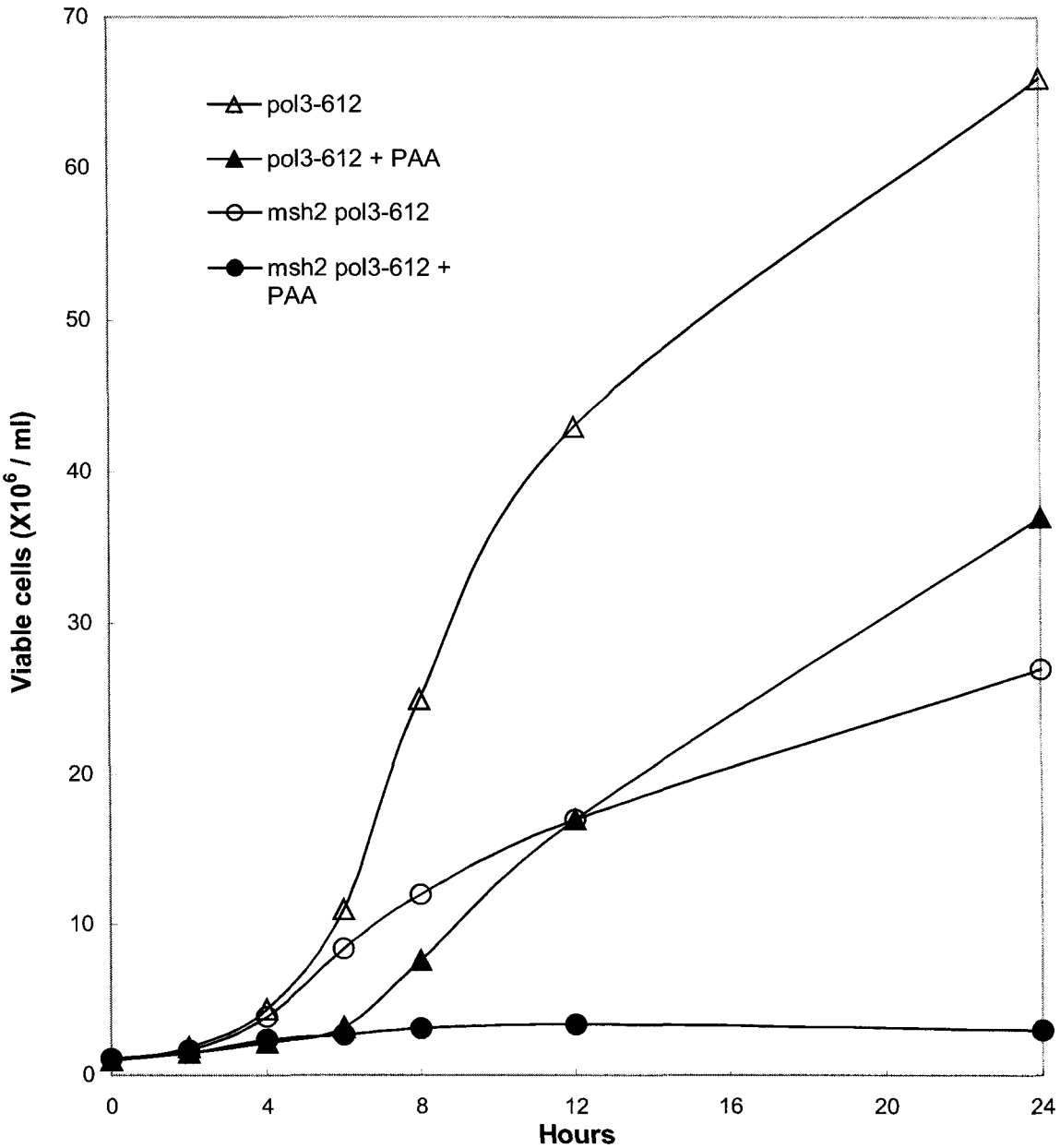

Figure 3.3.3 a

Figure 3.3.3 Growth of *pol3-612* and *msh2 pol3-612* cells in the presence and absence of PAA. Cells in logarithmic phase were inoculated to SD-complete broth with or without 0.5 mg/ml PAA. (a) Numbers of viable cells were determined at time points indicated. (b) The total number of cells was determined by counting cells with a hemocytometer. The number of viable cells determined in (a) is also plotted for comparison. (c) Viability of *msh2 pol3-612*strain in the presence and absence of PAA. (d) The percentage of "dumbbell-like" cells of *pol3-612* and *msh2 pol3-612* in the presence of PAA.

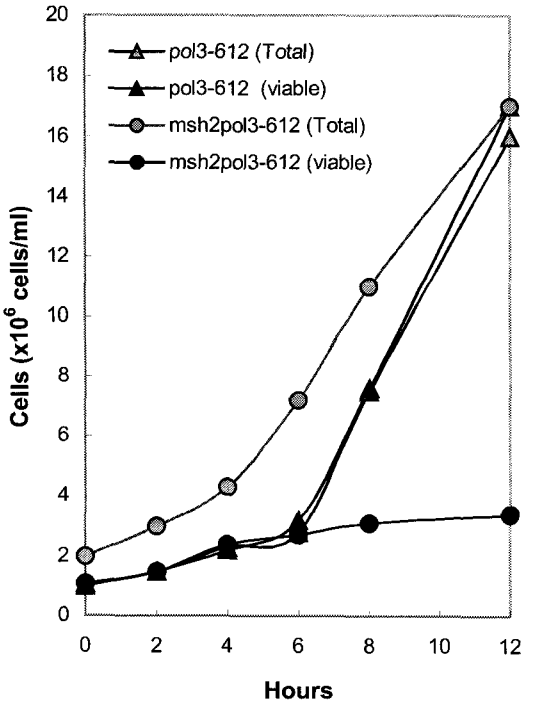

Figure 3.3.3 b

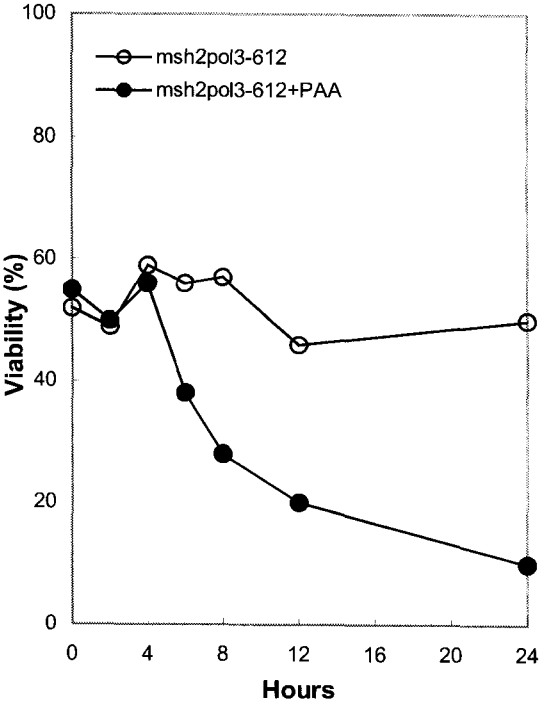

Figure 3.3.3 c

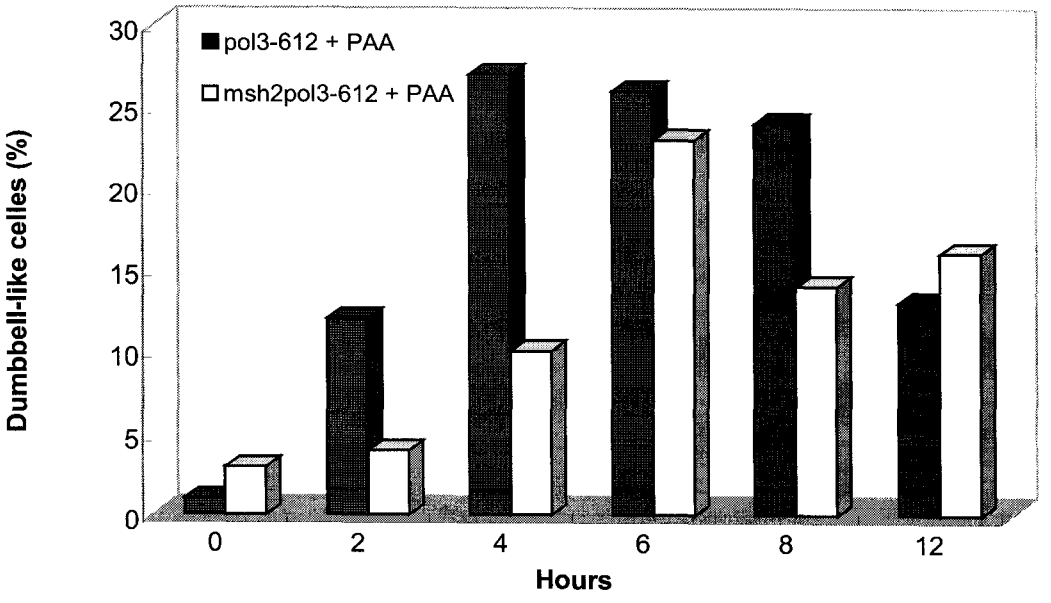

Figure 3.3.3 d

3.3.3c), while viability of the *pol3-612* strain was about 100% (Figure 3.1.5 b).

More appreciable differences were observed between the *msh2 pol3-612* and *pol3-612* strains in the presence of 0.5 mg/ml PAA. First, for the *pol3-612* strain, cell proliferation was largely arrested for the first 6 hours as shown by slow increase of total number of cells in asynchronized cultures (Figure 3.3.3 b). In contrast, this arrest was much weaker for *msh2 pol-612* cells since cells still kept dividing as revealed by the increase of total cell numbers (Figure 3.3.3 b), especially considering that *msh2pol-612* cells grew more slowly than *pol3-612* cells even in the absence of PAA (Figure 3.3.3 a). Consistently, compared with the PAA-treated *pol3-612* cells, fewer *msh2 pol3-612* cells displayed the "dumbbell-like" morphology in the presence of PAA, an indication of cell cycle arrest (Figure 3.3.3 d). Thus, it appeared that inactivation of MMR impaired the PAA-induced cell cycle arrest in *pol3-612* cells. Second, as shown by the number of viable cells, *pol3-612* cells largely adapted and resumed growing after this arrest, while no adaptation to PAA inhibition was observed for *msh2 pol3-612* cells (Figure 3.3.3 a and b). Third, there was a difference in viability of the two strains in the presence of PAA: while there was no viability change for the *pol3-612* strain (about 100%, Figure 3.1.5 b), loss of viability was observed for the *msh2 pol3-612* strain after 6 hours, and viability dropped to 10% after 24 hours (Figure 3.3.3 c), suggesting that PAA inhibition was fully reversible in *pol3-612* cells even after 24 hours, whereas *msh2 pol3-612* cells started losing reversibility after 6 hours.

3.3.3 The role of Exo1p in PAA-sensitivity

Due to the role of Exo1p in MMR and in light of the findings outlined above, it was expected that inactivation of the *EXO1* gene should also enhance PAA-sensitivity in the *pol3-612* strain background. In order to test this hypothesis, *exo1* and *exo1 pol3-612* strains were constructed and examined for PAA-sensitivity.

Like the other MMR-deficient strains, *exo1* itself was not sensitive to PAA at the

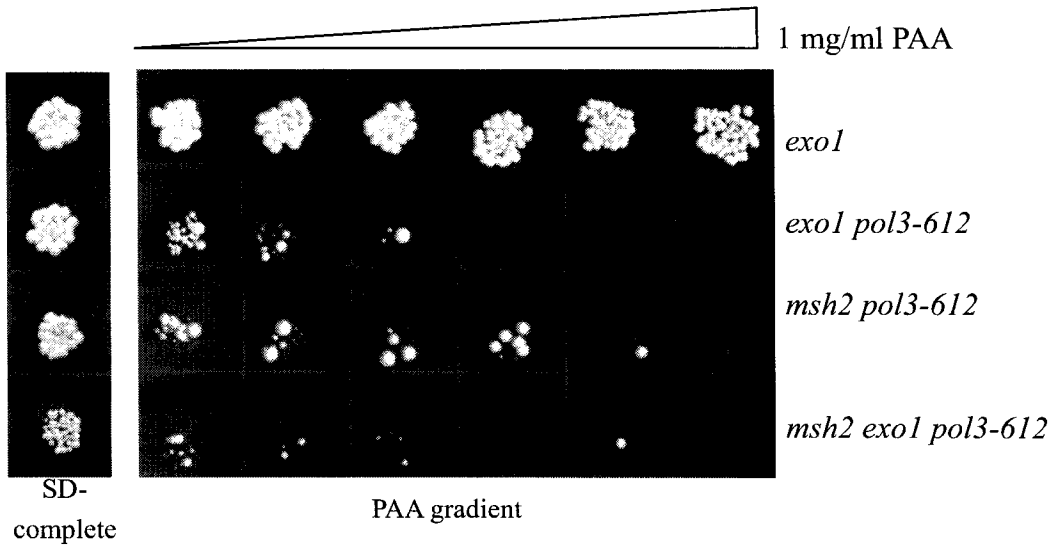

Figure 3.3.4 The role of Exo1p in PAA-sensitivity. Approximate 100 viable cells from each strain were spotted on SD-complete plates and those with PAA gradient. Plates were incubated at 30°C for 3 days.

concentration tested (Figure 3.3.4). But the combination of *exo1* and *pol3-612* resulted in dramatic enhancement of PAA-sensitivity; the PAA-sensitivity was similar to that seen in the MMR knockout (Figure 3.3.4): levels were slightly more than that seen in the *MSH6* knockout (compare Figure 3.3.4 to Figure 3.3.2), and slightly lower than that observed in the *MSH2* knockout (Figure 3.3.4). So, unlike its minor role in mutation prevention, which was shown in Table 3.2.1, Exo1p appeared to play an important role in response to the PAA-induced inhibition on L612M-DNA pol δ. The involvement of Exo1p in the PAA-response might be due to its catalytic exonuclease or flap-endonuclease activity (Tran *et al.*, 2002), or the role of Exo1p in maintaining structural integrity of a MMR complex (Amin *et al.*, 2001) considering the dramatic increase in PAA-sensitivity after *EXO1* deletion (see Section 4.2.2 for more discussion).

In addition to its role in MMR, Exo1p is also well known for its function in DNA recombination (Fiorentini *et al.*, 1997) and repair of DNA double strand breaks (Tsubouchi and Ogawa, 2000). Therefore, in order to further understand the role of Exo1p in PAA response, the triple mutant strain *msh2 exo1 pol3-612* strain was constructed by crossing the *exo1 pol3-612* strain with the *msh2* strain, followed by sporulation and tetrad dissection. If the roles that Exo1p plays in processes other than MMR contributed towards PAA-sensitivity, then inactivation of the MMR system in the *exo1 pol3-612* strain should further increase sensitivity to PAA. Alternatively, if PAA-sensitivity of the *exo1 pol3-612* strain was due to the role of Exo1p in MMR, then no further increase in sensitivity should occur.

The triple mutant was examined for PAA-sensitivity. Compared with the *msh2 pol3-612* strain, no additional elevation in PAA-sensitivity was shown in the *msh2 exo1 pol3-612* strain (Figure 3.3.4). Therefore, the role of Exo1p in PAA response appears to reside in MMR.

3.3.4 Homozygous diploid strain is less PAA-sensitive than its haploid

Previous studies indicated that isogenic haploid and diploid yeast strains had different sensitivities to spontaneous DNA damage (Morey *et al.*, 2003) as well as DNA damage induced by ultraviolet irradiation (Siede and Friedberg, 1990). In addition, ploidy difference was also reported for mutants with a combination of MMR deficiency and a DNA polymerase with inactive proofreading function (Morrison *et al.*, 1993; Morrison and Sugino, 1994; Tran *et al.*, 1999b). Thus, to examine whether or not there are any ploidy differences in the role of MMR in the PAA-response, a diploid *msh6/msh6, pol3-612/pol3-612* strain was constructed by crossing two *msh6 pol3-612* isolates with opposite mating types and subsequently examined for PAA-sensitivity. For this study, *msh6 pol3-612* was chosen over *msh2 pol3-612*, as the former had considerable PAA-sensitivity but better viability than the latter, which lowered the likelihood of getting suppressors that might have obscured observations.

Two isolates of *pol3-612/pol3-612, msh6/msh6* strains were tested for PAA-sensitivity on PAA gradient plates. Both of them demonstrated considerably reduced PAA-sensitivity compared to the haploid parent strains (Figure 3.3.5). Hence similar to previous reports with other DNA polymerase mutants, there appeared to be a ploidy difference in PAA response in *pol3-612* MMR-deficient strains as well. A second set of chromosomes appeared to protect cells from the PAA-induced stress.

3.3.5 PAA is not a strong mutagen

Since MMR plays such a critical role in the PAA response, and the major role of MMR is to maintain genome integrity, it is logical to examine whether or not PAA is a strong mutagen that induces genome instability. If indeed the case, then the role of MMR in PAA-response could be ascribed to maintenance of genome stability.

Because the MMR system may "cover" replication errors that are induced by PAA, and because of the PAA-sensitive nature of the L612M-DNA pol δ, the effects of PAA on replication fidelity of wildtype DNA pol δ were examined in the *msh2* strain.

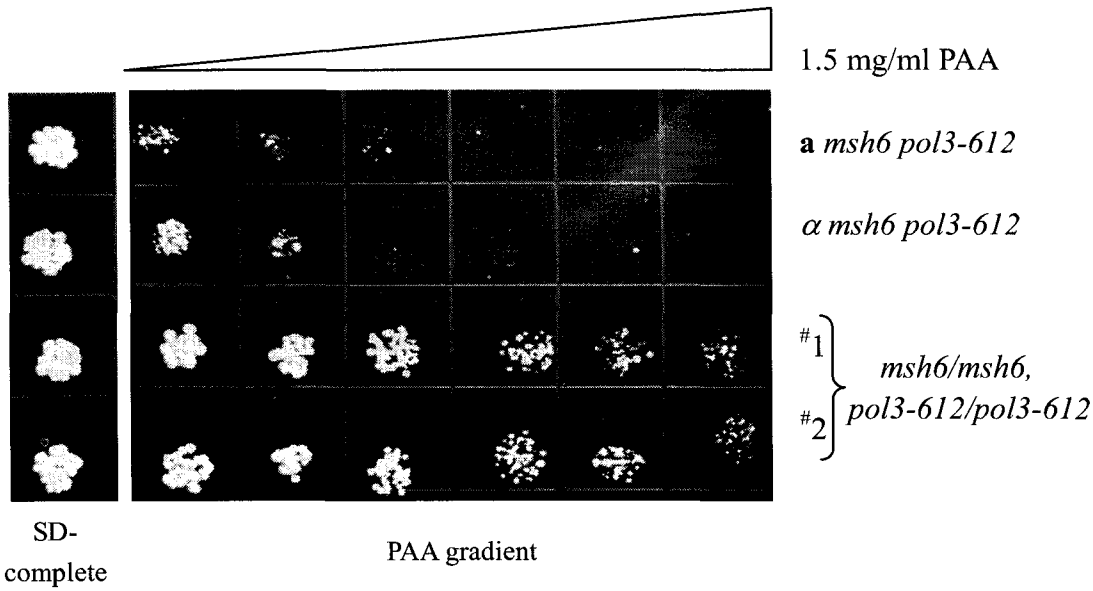

Figure 3.3.5 The haploid *msh6 pol3-612* strain is more PAA-sensitive than the diploid. Two *msh6 pol3-612* isolates with opposite mating types (**a** and α) were used to generate diploids. Two independent isolates ($^{\#}1$ and $^{\#}2$) of diploid were tested for PAA-sensitivity. Approximately 100 viable cells of each strain were spotted on SD-complete plates alone or with a PAA gradient. Plates were incubated at 30°C for 3 days

Compared with the spontaneous mutation frequencies determined at three reporter loci, *trp1-289, his7-2* and *CAN1*, the presence of 1 mg/ml PAA only caused a 3-fold increase in replication errors (Table 3.3.1). Thus, the dramatic increase in PAA-sensitivity accompanied by inactivation of MMR does not appear to be due to a compromise in replication fidelity in the presence of PAA.

3.3.6 Isolation of a second-site suppressor of PAA-sensitivity

Previous studies on the T4 L412M-DNA polymerase mutant showed that second-site mutations in T4 DNA polymerase gene (the first mutation encoding L412M) could suppress the PAA-sensitivity conferred by L412M substitution (Reha-Krantz and Nonay, 1994; Reha-Krantz and Wong, 1996). Therefore, if there is a functional similarity in the polymerase active centers between T4 DNA pol and *S. cerevisiae* DNA pol δ, then second-site suppressors of PAA-sensitivity could also be isolated in yeast and they should have properties similar to their T4 counterparts. Second-site suppressors were selected by plating *msh2 pol3-612* cells on 2 mg/ml PAA plates. Cells with the *msh2 pol3-612* genotype were chosen because of their strong PAA-sensitivity and mutator phenotype, which increased the likelihood of spontaneous mutation occurring in the *POL3* gene.

A second-site suppressor was isolated. Compared to the *msh2 pol3-612* strain, this suppressor had appreciably reduced PAA-sensitivity (Figure 3.3.6, compare *msh2 pol3-612* to *msh2 pol3-612,758*). Sequencing of the mutant revealed that this suppressor isolate had a G to A transition at nucleotide position 2272 and caused a Val to Met substitution at the 758th amino acid (Figure 3.3.7). Interestingly, like the L612M substitution, this second-site mutation also caused an amino acid substitution in a highly conserved motif (Motif C) in Family B DNA polymerases (Figure 3.3.7).

Due to the strong mutator phenotype of *msh2 pol3-612*, it was possible that this second-site suppressor mutant might harbor other mutations in addition to the one

Condition	Mutation frequency		
	trp1-289 (base substitution)	*his7-2* (frameshift)	*CAN1* (base substitution + frameshift)
msh2 (- PAA)	4.1×10^{-7}	5.3×10^{-6}	3.4×10^{-5}
msh2 (+ PAA)	13.4×10^{-7}	16.9×10^{-6}	10.3×10^{-5}
Relative fidelity	3.3	3.2	3.0

Table 3.3.1 The effects of PAA on replication fidelity in the absence of MMR. Mutation frequencies were measured as describe in Materials and Methods. Data shown here were the average of 2 independent cultures grown in the absence or presence of 1mg/ml PAA each. To minimize experimental variables, all cultures were processed at the same time. Relative fidelity was calculated by dividing the mutation frequencies in the presence of PAA by that obtained in the absence of PAA.

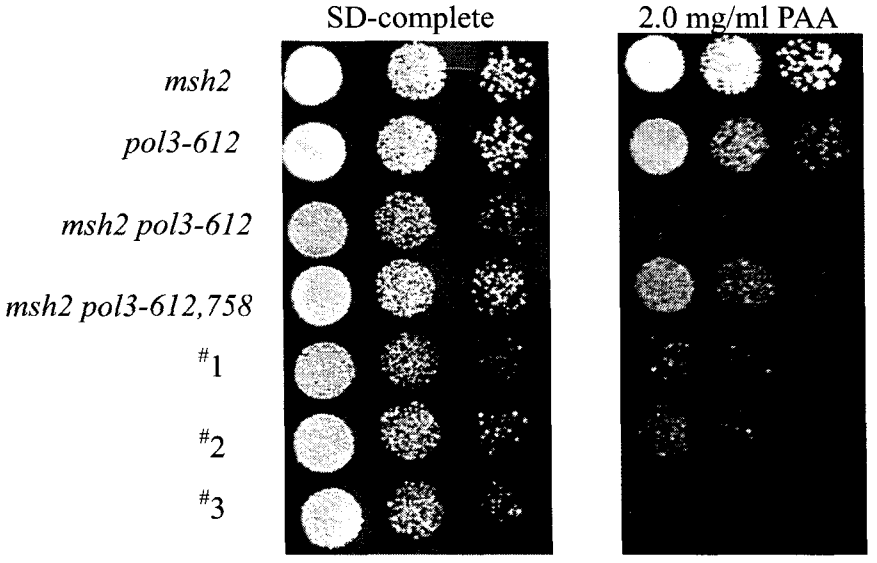

Figure 3.3.6 Suppression of PAA-sensitivity by a second-site mutation. Tenfold serial dilutions of each strain were spotted onto SD-complete plates with or without PAA. The *msh2 pol3-612,758* strain had a spontaneous second-site mutation encoding the V758M substitution in the L612M-DNA pol δ, which suppressed PAA-sensitivity. #1, #2 and #3 were independent isolates obtained after transforming the *msh2 pol3-612,758* strain with linearized plasmid to remove the second-site mutation. Plates were incubated at 30°C for 3 days.

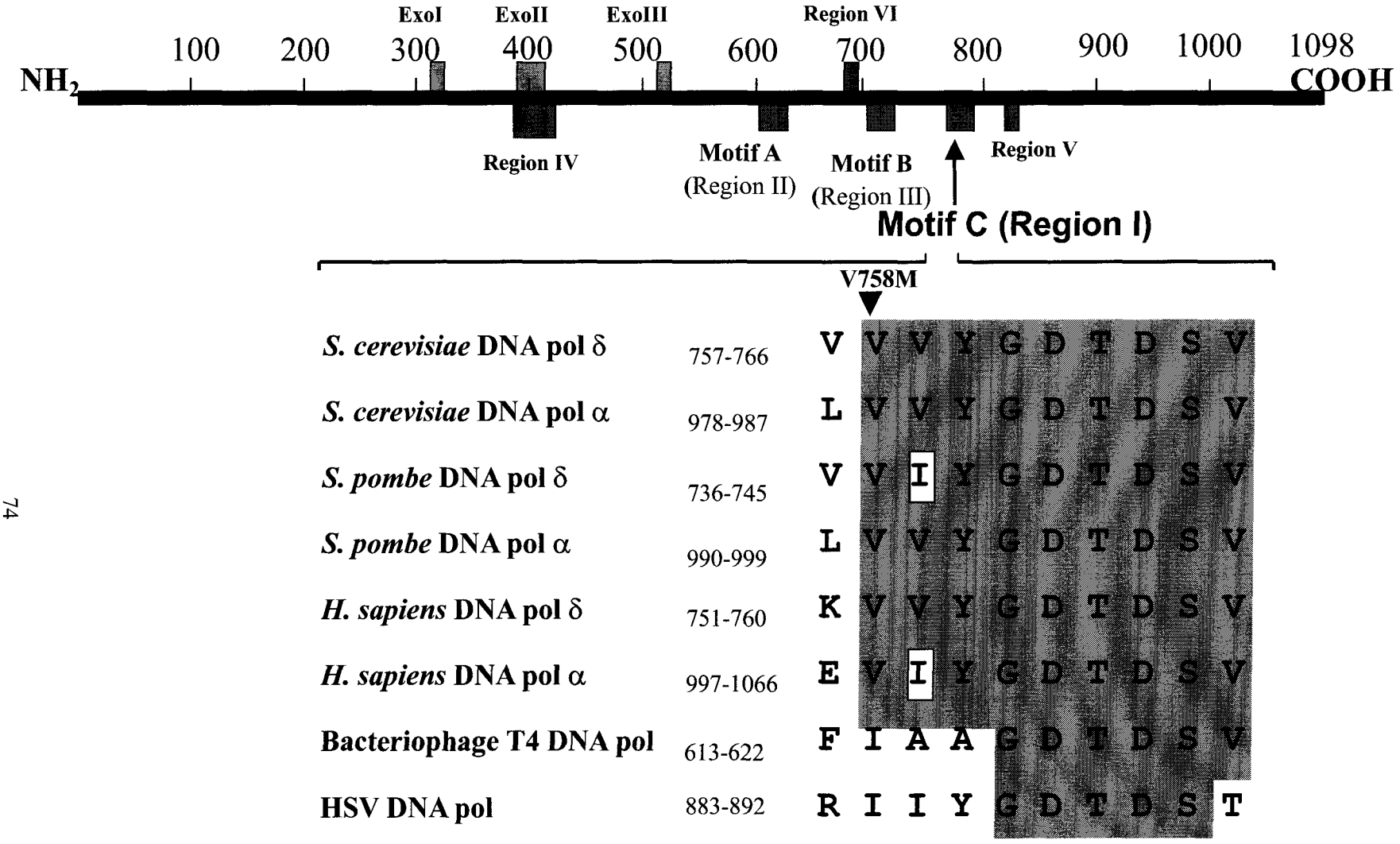

Figure 3.3.7 Identification of a second-site mutation in Motif C that suppresses PAA-sensitivity caused by the L612M substitution. The second-site mutation encoding a V758M substitution is located in Motif C (also called Region I) and indicated by an arrow. Sequence alignment is taken from Braithwaite and Ito (1993). The numbering of amino acids shown above the bar is based on the sequence of *S. cerevisiae* DNA pol δ.

encoding the V758M substitution. In order to confirm that suppression was only due to the V758M substitution, the second-site mutation was replaced by the wildtype codon by transforming *msh2 pol3-612,758* cells with the *Hpa*I linearized C-term portion of the L612M-*POL3* allele. Three independent transformants were chosen and tested for PAA-sensitivity. All three transformants displayed PAA-sensitivity comparable to the *msh2 pol3-612* strain (Figure 3.3.6). Sequencing results indicated that the second-site mutation encoding the V758M substitution was replaced by the wildtype codon, demonstrating that suppression was only due to this specific DNA mutation.

3.3.7 The V758M-DNA pol δ demonstrates an antimutator phenotype

Previous studies in T4 by our laboratory indicated that when isolated independently, second-site T4 DNA polymerase mutant alleles demonstrated antimutator phenotypes (i.e., increased replication fidelity compared to the wildtype enzyme) for certain genetic markers (Reha-Krantz and Nonay, 1994; Reha-Krantz and Wong, 1996). To characterize the V758M-DNA pol δ, the second substitution V758M should be separated from the L612M substitution in the *msh2 pol3-612,758* strain. A strategy similar to that used in the *pol3-612* strain construction was used to isolate V758M from L612M, after which the mutations encoding the L612M substitution were replaced by the wildtype codon (Figure 3.3.8).

The *msh2 pol3-758 URA3* intermediate was used in preliminary characterization of V758M-DNA pol δ for replication fidelity. Three reporters, *ade5-1*, *trp1-289* and *his7-2*, were used (Table 3.3.2). The *ade5-1* mutant allele has a premature *ochre* stop codon in its coding region (Figure 3.2.1), which can be reverted by base substitutions that change the *ochre* into sense codons. An antimutator phenotype (about 5-fold increase in replication fidelity) was observed for V758M-DNA pol δ at the *ade5-1* locus (Table 3.3.2). A slight increase in mutation rate was seen at *trp1-289* and no difference was found for +1 frameshift mutations using the *his7-2* reporter gene (Table 3.3.2). The

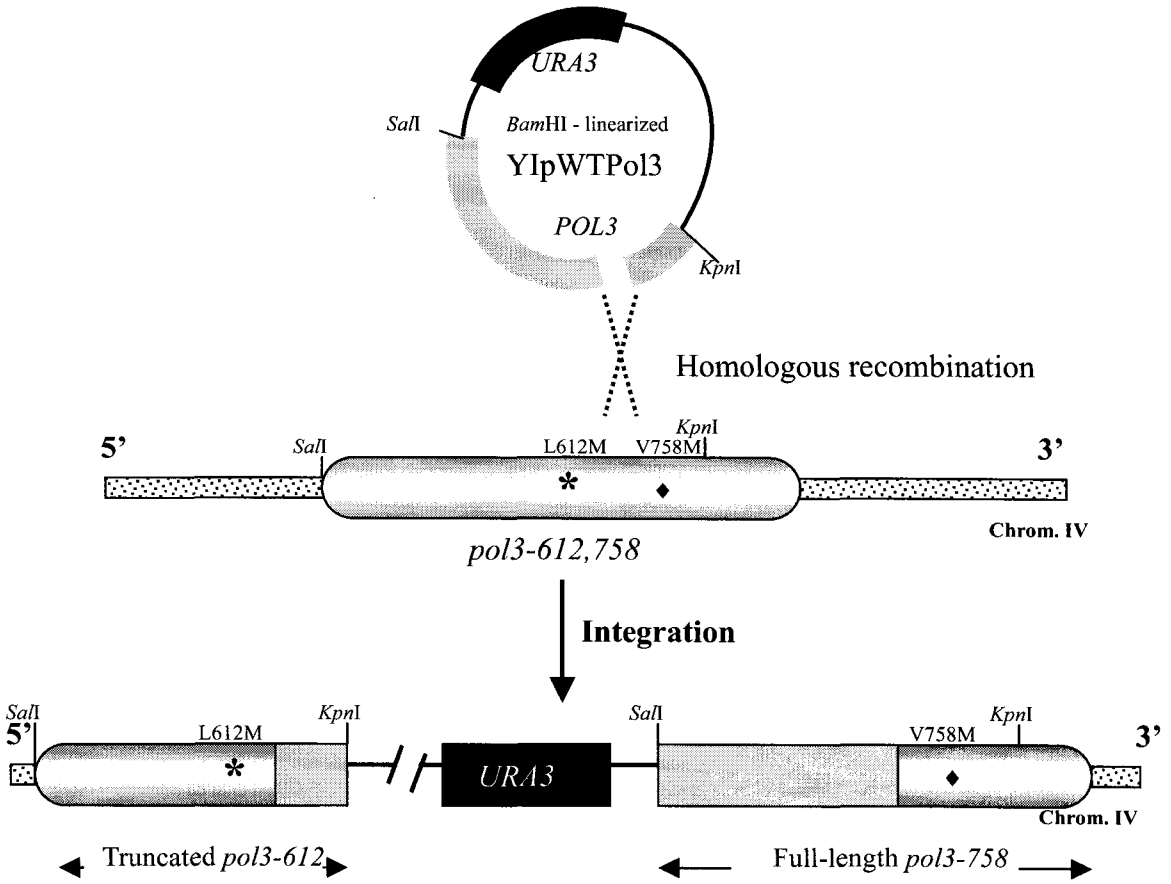

Figure 3.3.8 Strategy used for isolation of the *pol3-758* mutant allele from the double mutant *pol3-612,758* allele. The double mutant *pol3-612,758* allele was first isolated in a selection for spontaneous second-site suppressors for PAA-sensitivity of *msh2 pol3-612* (L612M is represented as * and V758M is represented as ♦ in the figure). To isolate the *pol3-758* allele, a strategy similar to that used in *pol3-612* construction (Figure 3.1.1) was employed. A plasmid YIpWTpol3 was constructed containing the N-terminus (*Sal*I – *Kpn*I fragment) of the wildtype *POL3* gene. This plasmid was digested by *Bam*HI and then transformed to *msh2 pol3-612,758* cells. Through homologous recombination, YIpWTpol3 integrated into the *pol3-612,758* locus, resulting in a full-length *pol3-758* allele (confirmed by sequencing) and a truncated *pol3-612* allele. This *msh2 pol3-758 URA3* intermediate was used to characterize the *pol3-758* allele in preliminary experiments.

	Mutation rate (X 10^{-8})		
	ade5-1	*trp1-289*	*his7-2*
Strain	(TAA→sense codon)	(TAG→sense codon)	(frameshift)
msh2	5.1 (3.7-6.7)	8.4 (6.9-10)	50 (43-53)
msh2 pol3-758	1.0 (0.9-3.2)	28 (18-43)	50 (28-59)
Relative rate	0.2	3.3	1.0

Table 3.3.2 Replication fidelity of the V758M-DNA pol δ. The method of the median was used to calculate mutation rates. Mutation rates shown here were based on data from 5 to 10 independent cultures. 95% confidence intervals were shown in parentheses. Relative rate was calculated by dividing mutation rate of the *msh2 pol3-758* strain by that of the *msh2* strain.

observation that the V758M-DNA pol δ demonstrated an antimutator effect only with certain but not all reporters was consistent with previous reports that T4 antimutator polymerase mutants reduce mutations only at certain sites (e.g., AT→GC transitions), but increase mutations at other sites (Drake *et al*, 1969; Ripley, 1975).

Taken together, isolation of a second-site suppressor of PAA-sensitivity in the *POL3* gene and preliminary characterization of V758M-DNA pol δ provided further evidence that there are functional similarities between yeast DNA pol δ and T4 DNA polymerases.

3.4. The L612M-DNA pol δ appears to be "problematic"

3.4.1 Viability loss in pol3-612 MMR-deficient strains

In addition to dramatic increased PAA-sensitivity observed in the *msh2 pol3-612* strain, another unexpected observation was the reduced (~ 50%) viability (Figure 3.3.3c). This viability loss was surprising because neither *po3-612* nor *msh2* strain showed any viability problem independently (Figure 3.4.1). This suggested that L612M-DNA pol δ depended on MMR system for viability. To investigate this further, different *pol3-612* MMR-deficient strains were examined for viability. As shown in Figure 3.4.1, inactivation of the *PMS1* or *MLH1* genes both led to severe viability loss that was similar to that seen in the *msh2 pol3-612* strain. The consequence of deleting the *MSH6* or *MSH3* genes was less severe in the *pol3-612* background: both *msh6 pol3-612* and *msh3 pol3-612* cells were approximately 80% viable, which was consistent with their functional redundancy revealed by genetic studies. When both *MSH6* and *MSH3* genes were inactivated, viability dropped to about 50% again (Figure 3.4.1). Hence, MMR appeared to be required to maintain normal viability when genomic DNA was replicated by L612M-DNA pol δ.

3.4.2 The rad27 pol3-612 strain is synthetic lethal

Previous studies on the *pol3-01* mutant (the proofreading deficient pol δ mutant)

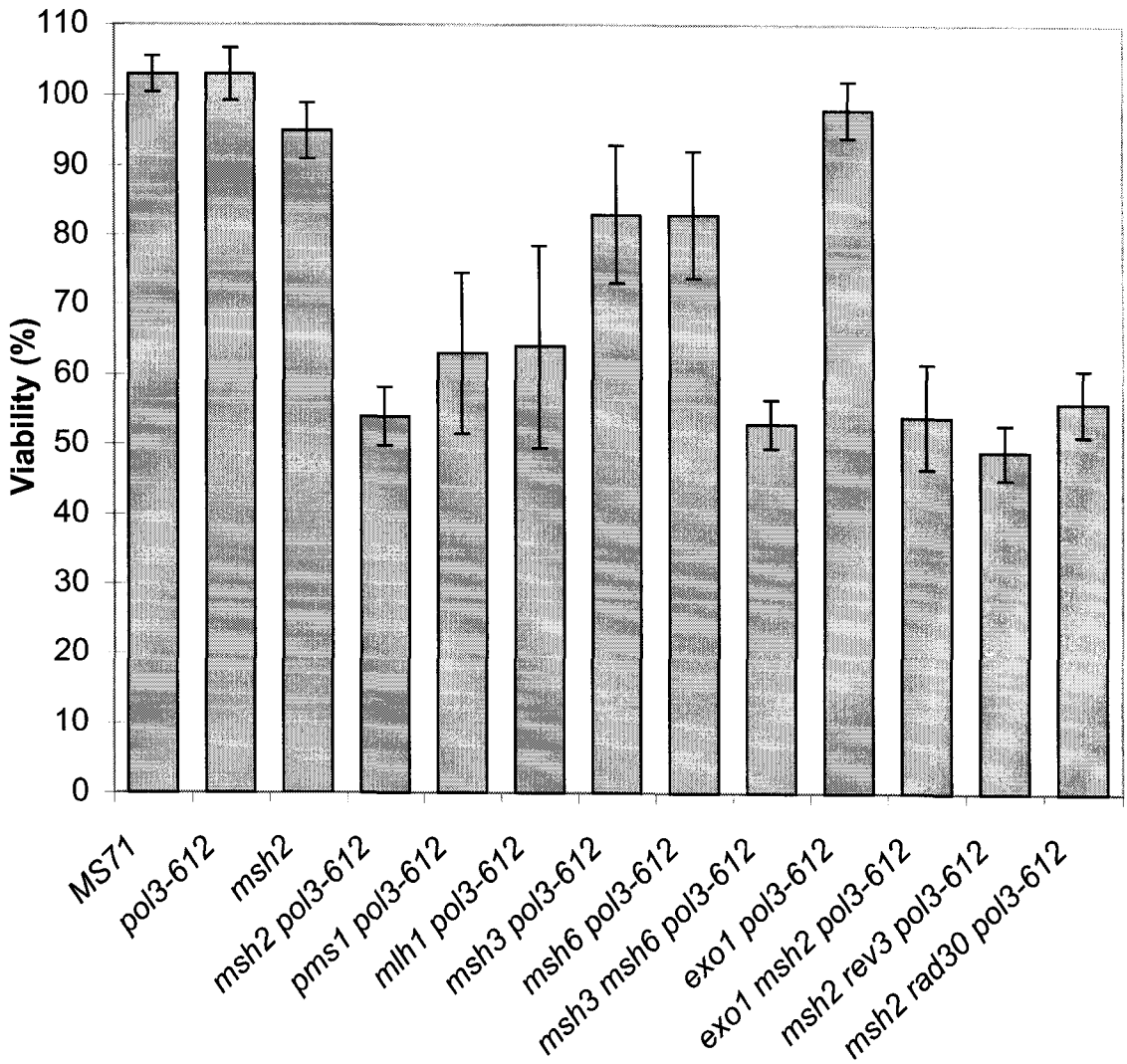

Figure 3.4.1 Viability of the *pol3-612* strain in the presence and absence of MMR. Viability presented here was the average of 6 to 10 independent isolates except for MS71, *msh2* and *pol3-612* strains. For MS71, *msh2* and *pol3-612* strains, the viability was measured in repeated experiments and the average was taken. Error bars indicate the standard error of the mean. The viabilities of the *mlh1 pol3-612* and *msh3 pol3-612* strains are based on experiments done by K. Murphy.

also demonstrated loss in viability when this mutant allele was combined with MMR deficiency. Deletion of *MSH2, MLH1, PMS1, MSH6* genes all led to synthetic lethality in the *pol3-01* background in haploids (Morrison *et al.*, 1993; Tran *et al.*, 1999a; Sokolsky and Alani, 2000; Argueso *et al.*, 2002). *In vitro* experiments using the *pol3-01* mutant demonstrate that the mutant DNA polymerase is more efficient in initiation of strand displacement synthesis compared with the wildtype enzyme (Jin *et al.*, 2003), which is consistent with *in vivo* studies (Jin *et al.*, 2001). Interestingly the L412M-T4 DNA polymerase also increases strand displacement synthesis *in vitro* compared with the wildtype DNA polymerase (Reha-Krantz, unpublished observation). Thus *pol3-612* and *pol3-01* share certain similarities.

In addition to MMR deficiency, the *pol3-01* mutant is also synthetic lethal with the *RAD27* gene deletion (Gary *et al.*, 1999). The *RAD27* gene product has been proposed to play important roles during DNA replication and repair (Reagan *et al.*, 1995; Gary *et al.*, 1999; Ayyagari *et al.*, 2003). Biochemical analysis indicates that Rad27p and its mammalian ortholog FEN1 have multiple activities, including 5'-flap endonuclease, 5'→3' exonuclease and other functions (Harrington and Lieber, 1994; Sommers *et al.*, 1995). Deletion of the *RAD27* gene causes instability on simple repetitive sequences and other complex mutations (Johnson *et al.*, 1995; Gary *et al.*, 1997), however the mechanism is believed to be distinct from mismatch repair (Tishkoff *et al.*, 1997b).

Because of the similarities shared by *pol3-01* and *pol3-612,* deletion of the *RAD27* gene was attempted in the *pol3-612* background to further investigate DNA replication abnormalities caused by the L612M-DNA pol δ. The construction of the *rad27 pol3-612* strain was carried out by crossing a *rad27* (null mutant by knockout) strain with the *pol3-612* strain, followed by sporulation and tetrad dissection. The *rad27* knockout and the *pol3-612* allele were traced by their associated genetic markers (G418-resistence for *rad27* knockout and PAA-sensitivity for the *pol3-612* allele). As revealed by the genetic markers, among the 47 tetrads dissected, 34 tetrads had the tetratype (TT), 8 tetrads had

the non-parental ditype (NPD) and 5 tetrads had the parental ditype (PD). No putative *rad27 pol3-612* mutant was identified among the TT and NPD tetrads. Both 30°C and room temperature were tested since *rad27* has been reported to be temperature sensitive (Reagan *et al.*, 1995), however no *rad27 pol3-612* spore was found at either temperature. Thus, like *rad27 pol3-01*, *pol3-612* and *rad27* null alleles are also synthetic lethal. Because Rad27p is believed to play critical roles in Okazaki fragment maturation during lagging strand synthesis (Kao *et al.*, 2002; Ayyagari *et al.*, 2003), the observed synthetic lethality of *rad27 pol3-612* implied that the L612M-DNA pol δ might cause abnormalities during lagging strand synthesis. One likely scenario could be an excessive number of 5'-flaps formation caused by displacement synthesis, which occurs when the 3' primer terminus of newly synthesized DNA meets the 5'-end of downstream Okazaki fragment. This hypothesis was consistent with the observation on the +1 frameshift mutations of the *exo1 pol3-612* strain.

3.4.3 The L612M-DNA pol δ generates replication abnormalities that are subject to MMR correction

The combination of L612M-DNA pol δ with MMR deficiency resulted in a strong mutator phenotype, a dramatic increase in PAA-sensitivity and severe viability loss. The above abnormalities were not observed in the *msh2, mlh1,* or *pms1* strains, in which the wildtype DNA pol δ was present. In order to confirm that the single amino acid substitution, L612M, was responsible for those phenotypes, this mutation was replaced by the wildtype codon by transforming *msh2 pol3-612* cells with the YIp plasmid that contained the C-terminus of the wildtype *POL3* gene. Transformants were randomly chosen, examined and sequenced for codons encoding the 612th amino acid residue.

As shown in Figure 3.4.2, restoration of the leucine residue at the 612th position was accompanied by resistance to PAA, considerably weakened mutator activity and normal viability ($^{\#}$1and $^{\#}$2 transformants). Meanwhile, the transformant that retained the L612M

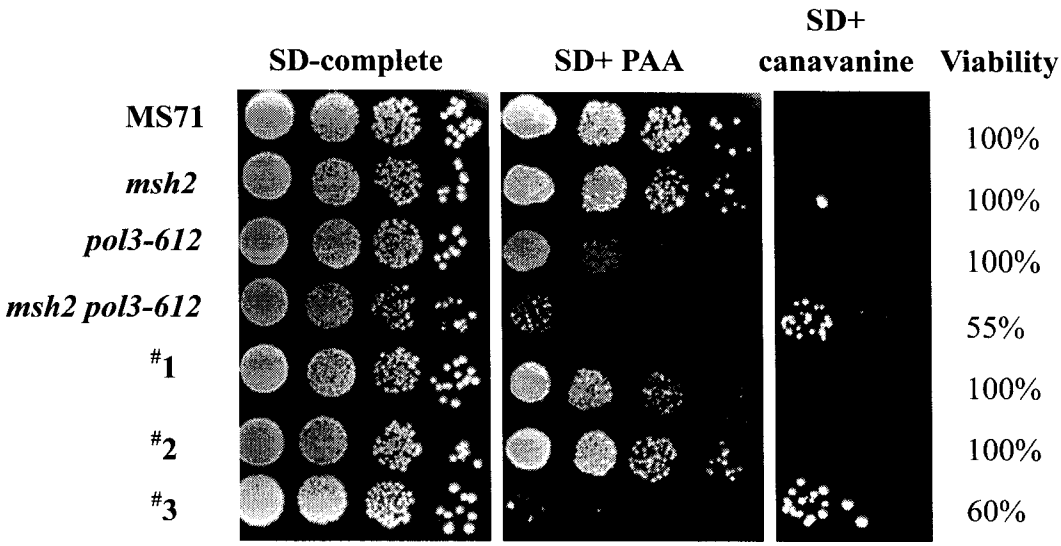

Figure 3.4.2 The L612M substitution confers elevated PAA-sensitivity and viability loss in the absence of MMR. Cells in logarithmic phase were serially diluted and spotted on SD-complete plates as well as plates supplemented with 2 mg/ml PAA and 60 μg/ml canavanine. Approximately, 10 to 10^4 cells of each strain were spotted. #1, #2 and #3 were independent transformants from transforming *msh2 pol3-612* cells with the YIp plasmid that contained the C-terminus of wildtype *POL3* gene. #1 and #2 transformants restored the wildtype *POL3* sequence while 3# transformant remained the L612M substitution after transformation. Plates were incubated at 30°C for three days.

In order to determine the viabilities of #1, #2 and #3 transformants, about 200 cells of each transformants were plated on SD-complete plates. The viability was determined by dividing the number of colonies by number of cells plated. Experiments were done in duplicates and the average was taken. The viability of the MS71, *msh2*, *pol3-612* and *msh2 pol3-612* strains are taken from previous experiments (Figure 3.4.1) for comparison.

substitution after transformation ($^{\#}$3 transformant) did not have any visible changes compared to cells without transformation. Therefore, the L612M-DNA pol δ appeared to cause detrimental effects during DNA replication and PAA might greatly exacerbate the situation (for example, cause replication fork stalling). In both cases, a functional MMR system appears to be required to solve the *pol3-612*-meditated genotoxic stress.

3.5. DNA Recombination is also involved in the PAA-induced cellular responses

3.5.1 Recombination deficiency also increases PAA-sensitivity

In bacteria, the recombination pathway is the major means of repair when DNA replication forks are arrested by DNA damage, replication inhibitors or other factors (reviewed by Michel, 2000; Kuzminov, 2001). Similarly, in eukaryotes, the recombination process is the main pathway to repair double strand breaks (DSBs) caused by DNA damage and aberrant DNA replication intermediates (reviewed by Haber, 2000). It has been estimated that more than 90% of the DSB in budding yeast and up to 50% of the DSB in mammalian cells are repaired through recombination (Johnson and Jasin, 2000; Valencia *et al.*, 2001).

In order to investigate if recombination plays a role in response to the PAA-inhibited L612M-DNA pol δ, two critical genes functioning in recombination, *RAD51* and *RAD52*, were knocked out and the consequences were examined. Rad51p is critical for homologous recombination by promoting DNA strand exchange between homologous sequences (Sung, 1994; Sung and Robberson, 1995). Rad52p is a central player for recombination and is required for nearly all types of DSB repair in yeast (reviewed by Haber, 1999). Biochemical studies reveal that Rad52p targets Rad51p to a complex consisting of replication protein A (RP-A) and single-stranded DNA in an early step of recombination (New *et al.*, 1998).

In the presence of wildtype DNA pol δ, the recombination pathway seemed to be dispensable because *rad52* cells were not inhibited by PAA at the tested concentration

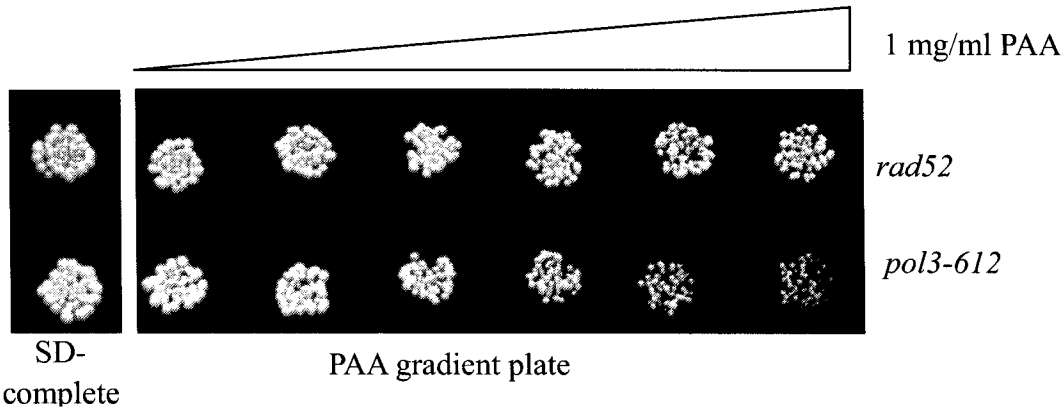

Figure 3.5.1 (a)

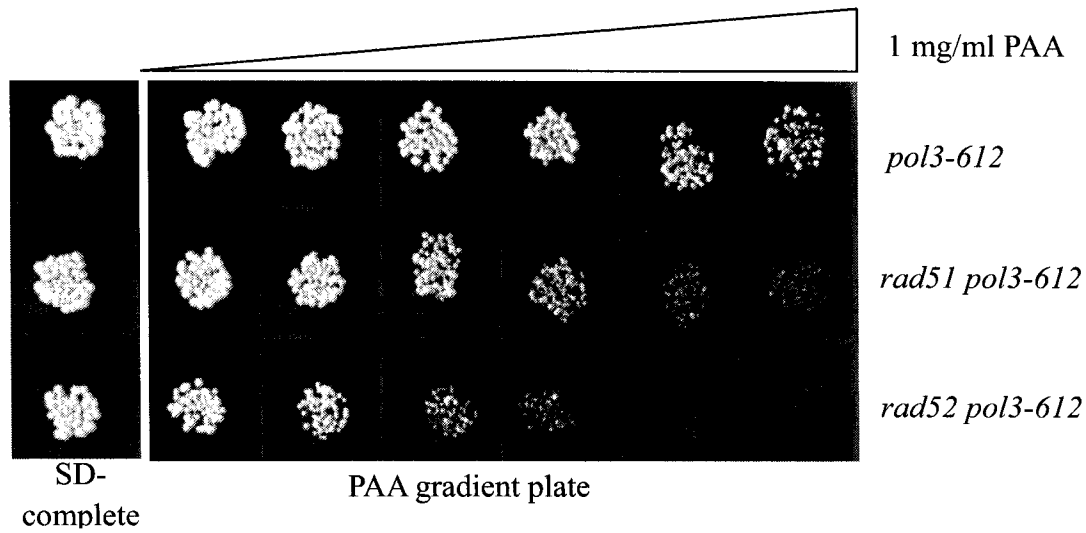

Figure 3.5.1 (b)

Figure 3.5.1 The recombination pathway is involved in the cellular responses in the presence of PAA. (a) *rad52* itself was not PAA-sensitive. (b) Inactivation of the recombination pathway increased PAA-sensitivity in the *pol3-612* background. Approximately 100 viable cells of each strain were spotted on SD-complete plates with or without a PAA gradient. Plates were incubated at 30°C for 3 days.

(Figure 3.5.1a), which slightly restricted growth of the *pol3-612* cells that had intact recombinational repair (see smaller colonies at the highest PAA concentration on the gradient plate, Figure 3.5.1a). The same effect was seen in *rad51* cells (data not shown). In contrast, DNA recombination did appear to be involved in cellular responses when genomic DNA was replicated by L612M-DNA pol δ in the presence PAA. As shown in Figure 3.5.1b, *rad51 pol3-612* cells were restricted at a lower PAA concentration compared with *pol3-612* cells. More pronounced inhibition was seen when the *RAD52* gene was deleted in a *pol3-612* background. The higher PAA-sensitivity seen in the *rad52 pol3-612* strain compared with the *rad51 pol3-612* strain was consistent with previous studies that Rad52p plays a more critical role than Rad51p in the recombination process (Rattray and Symington, 1995; Sugawara *et al.*, 1995; Malkova *et al.*, 1996). However compared to MMR, recombination only seemed to play a secondary role, because *msh2 pol3-612* cells were much more PAA-sensitive than *rad52 pol3-612* (compare Figure 3.3.2 to Figure 3.5.1b).

Since both MMR and recombination repair systems were involved in the cellular responses to PAA, it is necessary to know whether or not two systems work together in the same pathway. This possibility was examined by constructing a triple mutant strain *msh2 rad52 pol3-612* by crossing the *msh2 rad52* strain with the *pol3-612* strain. As shown in Figure 3.3.1, the triple mutant did not show further increase in PAA-sensitivity compared with the *msh2 pol3-612* or *pms1 pol3-612* strains, suggesting that MMR and recombination may work in the same pathway when the L612M-DNA pol δ was inhibited by PAA.

3.5.2 The role of recombination in maintaining cell viability

Deletion of the *RAD52* gene alone led to viability loss (viability was about 80%, Figure 3.5.2), indicating the importance of recombination in the normal DNA replication process. Consistently, in bacteria, the recombination deficient *recA* strain of *E. coli* has

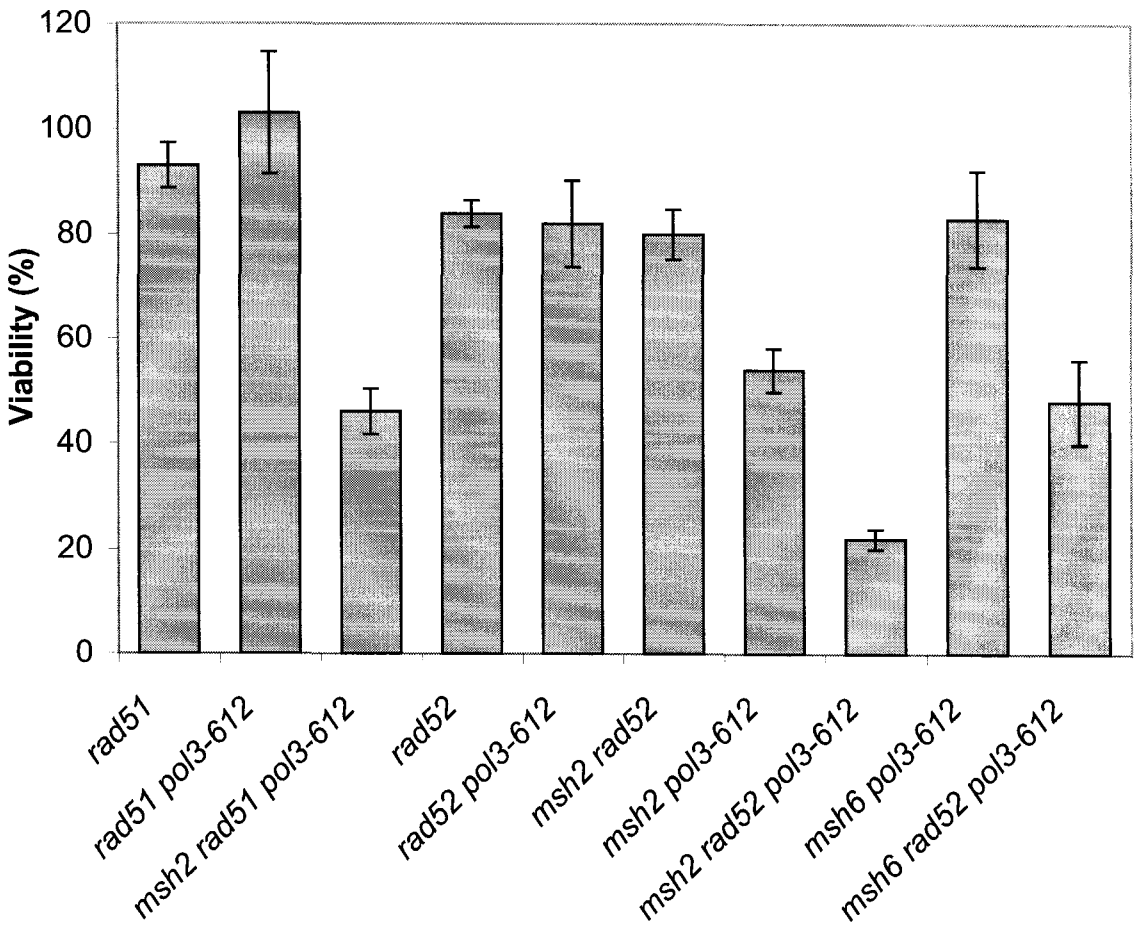

Figure 3.5.2 Viability of *pol3-612* cells in the presence and absence of DNA recombination and MMR. Viability shown here is the average of 2 to 8 independent isolates. Error bars indicate the standard error of the mean.

reduced (~50%) viability (Capaldo *et al.*, 1974). Unlike what was observed in the MMR-deficient strains, no further viability loss was seen when *rad52* was combined with *pol3-612* (viability was 80% for the *rad52 pol3-612* strain, Figure 3.5.2). But in the absence of MMR, recombination became important to survival, because strain viability dropped from approximately 50% (*msh2 pol3-612*) to only 20% (*msh2 rad52 pol3-612*). Considering the strong mutator activity of *msh2 pol3-612*, the small percentage of viable *msh2 pol3-612 rad52* cells could be due to second-site suppressors. A significant decrease in viability was also seen in the *msh6 rad52 pol3-612* strain when compared to *msh6 pol3-612* (survival decreased from 80% to 50%, Figure 3.5.2). Thus it appeared that L612M-DNA pol δ might produce detrimental replication intermediates that were primarily prevented or repaired through an MMR–mediated pathway. Recombination might also participate in solving the replication stress caused by the L612M DNA pol δ, but like in the PAA-response, recombination only appeared to play a secondary role.

Although DNA recombination is usually used to sustain cell viability, aberrant recombination is detrimental. It has been proposed that the *RAD51*-dependent aberrant recombination may account for the viability loss observed in some DNA topoisomerase and helicases mutants. For example, Top3p is a topoisomerase that displays a relaxing activity on negatively supercoiled DNA and is involved in genome replication (Kim and Wang, 1992). Deletion of the *TOP3* gene leads to severe viability loss and a slow growth phenotype (Chakraverty *et al.*, 2001; Shor *et al.*, 2002), which is rescued by inactivation of the *RAD51*-dependent recombination pathway. Similarly, synthetic lethality is observed for deletion of both *SGS1* and *SRS2* genes encoding two DNA helicases, Sgs1p and Srs2p, which is required for normal DNA replication (Lee at al., 1999). This synthetic lethality can also be rescued by deletion of the *RAD51* gene (Gangloff *et al.*, 2000). In order to examine whether or not aberrant recombination could account for the viability loss seen in the *pol3-612* MMR-deficient strains, the *RAD51* gene was deleted in the *msh2 pol3-612* strain. The triple mutant *msh2 pol3-612 rad51* had about 50%

viability compared to the *pol3-612* strain and similar to that observed in the *msh2 pol3-612* strain (Figure 3.5.2). Therefore, unlike what was observed in the *top3* and *sgs1 srs2* mutant strains, the *RAD51*-dependent aberrant recombination did not appear to contribute to the viability loss observed in the *pol3-612* MMR-deficient strains.

3.6. The role of *DUN1* in the PAA response

3.6.1 Inactivation of the DUN1 gene increases PAA-sensitivity

Inactivation of the *DUN1* gene results in sensitivity to DNA damaging agents and the replication inhibitor HU (Zhou and Elledge, 1993), presumably due to the inability to increase dNTP levels in response to the genotoxic insults (Chabes *et al.*, 2003). Consistent with this, *dun1* mutants also showed considerable sensitivity to PAA, which was even more pronounced than *pol3-612* (Figure 3.6.1), implying that a high dNTP pool concentration is important in alleviating the effects of PAA on DNA replication.

Combination of *dun1* and *pol3-612* resulted in a further increase in PAA-sensitivity. The *dun1 pol3-612* cells appeared to be as PAA-sensitive as the *msh2 pol3-612* cells (Figure 3.6.1). Thus, unlike the slight effects seen on mutation rates after *DUN1* deletion (Table 3.2.2), the *DUN1*-dependent pathway appeared to be important in protecting cells when treated with PAA. Compared with *dun1 pol3-612*, no further increase in PAA-sensitivity was observed in the triple mutant *msh2 dun1 pol3-612* (Figure 3.5.2), suggesting that Dun1p and Msh2p might act in the same pathway in response to PAA, which is in accordance with the notion that the *msh2 pol3-612* mutant might be defective in triggering cells cycle arrest in the presence of PAA (see Section 3.3.2), since Dun1p plays an important role in the checkpoint pathway (Zhou and Elledge, 1993; Zhao and Rothstein, 2002).

3.6.2 The role of DUN1 in maintaining cell viability

Deletion of the *DUN1* gene resulted in severe viability loss (less than 40% of the

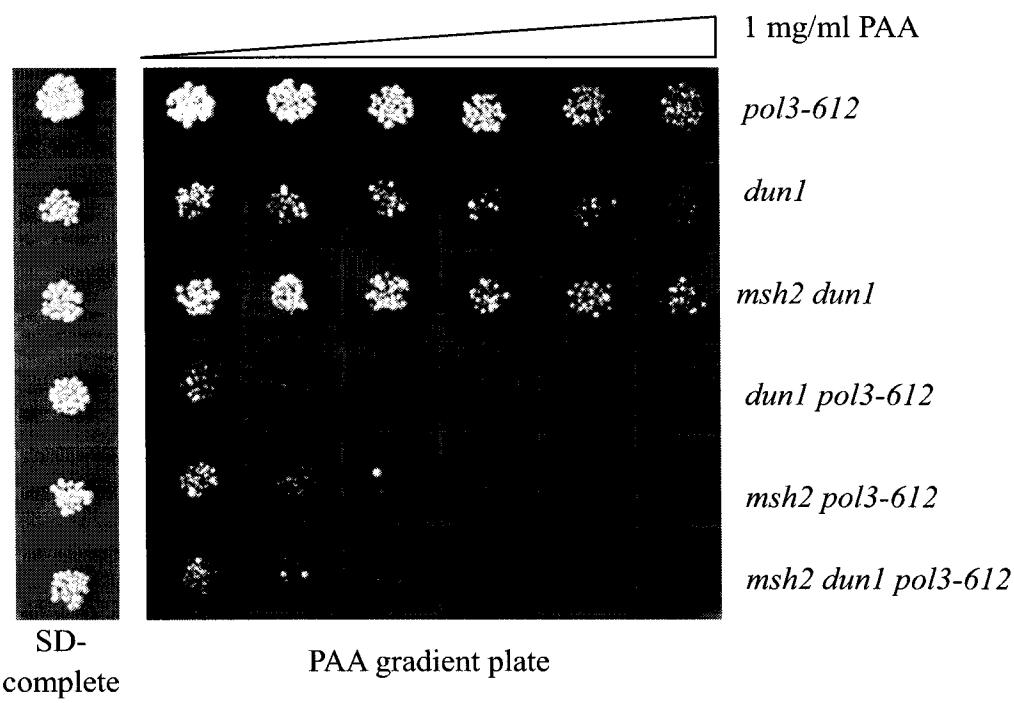

Figure 3.6.1 Inactivation of the *DUN1* gene increased PAA-sensitivity of the *pol3-612* mutant. Approximately 100 viable cells of each strain were spotted on SD-complete plates and those with a PAA gradient. Plates were incubated at 30°C for 3 days.

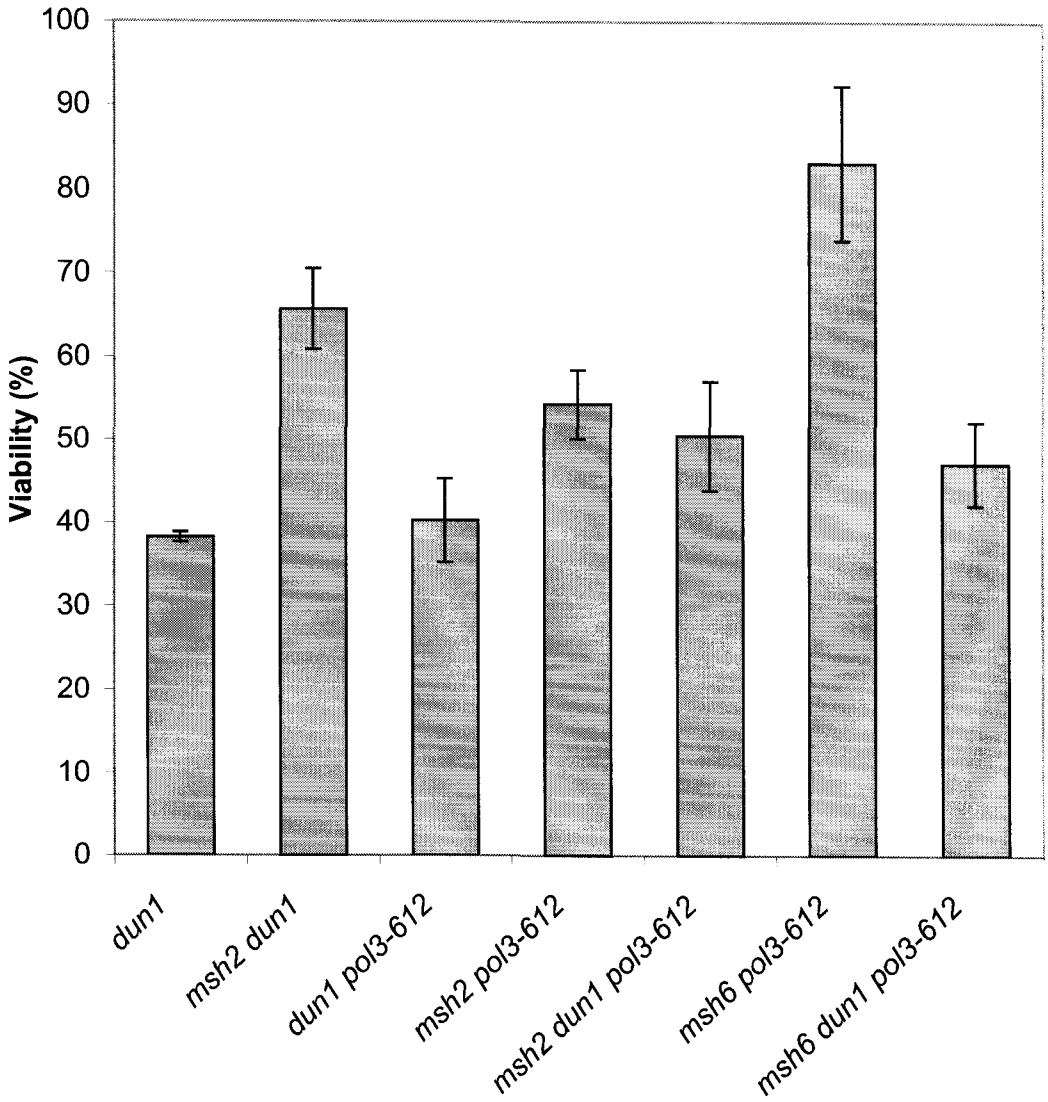

Figure 3.6.2 Viability of *dun1* and *dun1 pol3-612* cells in the presence and absence of MMR. Viability shown is the average of 3 to 8 independent isolates. Error bars indicate the standard error of the mean.

dun1 cells were viable, Figure 3.6.2). This was consistent with previous reports showing the importance of Dun1p in maintaining genome stability. Absence of Dun1p increases the rate of gross chromosomal rearrangements 200-fold (Myung *et al.*, 2001). Thus, one plausible explanation for observed viability loss of the *dun1* mutants may be increased genome instability. Little alteration in cell viability was seen when the *DUN1* gene was deleted in a *pol3-612* background (Figure 3.6.2). Considering L612M-DNA pol δ appeared to cause detrimental intermediates during replication, the absence of further viability loss in *dun1 pol3-612* compared with *dun1* suggested that genome abnormalities in *dun1* cells and that induced by the L612M-DNA pol δ could be subject to the same cellular surveillance mechanism.

It is also proposed that Dun1p plays a critical role in upregulating the dNTP pool concentration in response to DNA damage or replication inhibition (Zhao and Rothstein, 2002; Chabes *et al.*, 2003). The consequence of an inability to upregulate dNTP concentration may also account for viability loss in the strains with the *DUN1* deletion, because deletion of the *SML1* gene, which encodes an negative regulator controlling dNTP concentration, rescues lethality of the *mec1* or *rad53* null mutant, presumably due to recovered ability in increasing levels of dNTP pools (Zhao *et al.*, 1998; Zhao *et al.*, 2001). It is known that Dun1p directly phosphorylates Sml1p, which leads to the degradation of Sml1p (Zhao and Rothstein, 2002).

Interestingly, inactivation of the *MSH2* gene partially rescued the viability loss caused by the *DUN1* deletion (65% viability of *msh2 dun1 vs.* 38% viability of *dun1*, Figure 3.6.2). In addition, the *msh2 dun1* cells also seemed to be slightly less sensitive to PAA compared with *dun1* cells (Figure 3.6.1). Thus it appeared that inactivation of MMR might contribute to *dun1* cells survival. A possible mechanism for this rescue is discussed in Chapter 4.

3.7. The requirement of MMR in response to HU, UVC irradiation and *ts POL3* at restrictive temperature

3.7.1 MMR deficiency does not alter cellular sensitivity to UVC and HU

It is demonstrated here that the MMR system is required in the cellular responses to the PAA-inhibited L612M-DNA pol δ. To investigate whether or not the requirement for MMR also exists when DNA replication are disturbed by other factors, the *msh2 pol3-612* strain was examined for sensitivity to the DNA damaging agent UVC (254 nm) and replication inhibitor HU.

As shown in Figure 3.7.1, no difference in UVC sensitivity was detected between the MS71, *pol3-612*, *msh2* and *msh2 pol3-612* strains at both UVC doses, suggesting that MMR might not be required to protect cells from UVC damage. This was consistent with a previous report that inactivation MMR in yeast does not affect sensitivity to UVC irradiation (Durant *et al.*, 1999).

In the presence of 100 mM HU, the *msh2 pol3-612* cells showed slightly increased HU sensitivity as revealed by the smaller colony sizes compared to *pol3-612* cells (Figure 3.7.2a, on the HU plates). However, considering the slow growth phenotype of the *msh2 pol3-612* cells (Figure 3.7.2a, on the SD-complete plates), this slight difference in HU sensitivity was negligible. Because HU inhibition of DNA replication is reversible in *S. cerevisiae* (Kornberg and Baker, 1992), plates were kept for a longer time period to see reversibility. After 10 days, most of the smaller *msh2 pol3-612* cells seen earlier grew in a similar manner to the *pol3-612* cells, showing no defects in reversibility caused by MMR inactivation. In both cases, no difference was seen between MS71 and *msh2* strains. Taken together, these data indicated that MMR deficiency does not appear to alter the cellular sensitivity to HU and UVC irradiation.

3.7.2 MMR inactivation does not increase temperature sensitivity of ts POL3 mutants

Several temperature sensitive (*ts*) alleles were isolated during early genetic studies

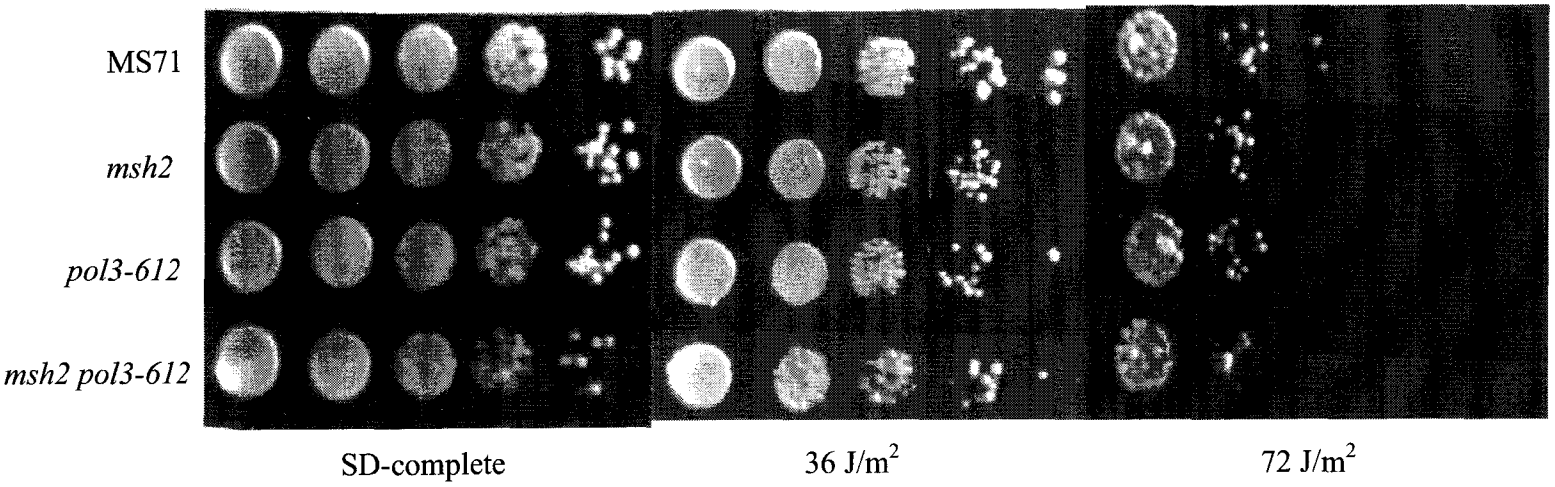

Figure 3.7.1 UVC sensitivity of *pol3-612* cells in the presence and absence of mismatch repair. Tenfold serial dilutions of each strain were spotted onto SD-complete plates subjected to UVC irradiation. Plates were kept in darkness at 30°C for 3 days.

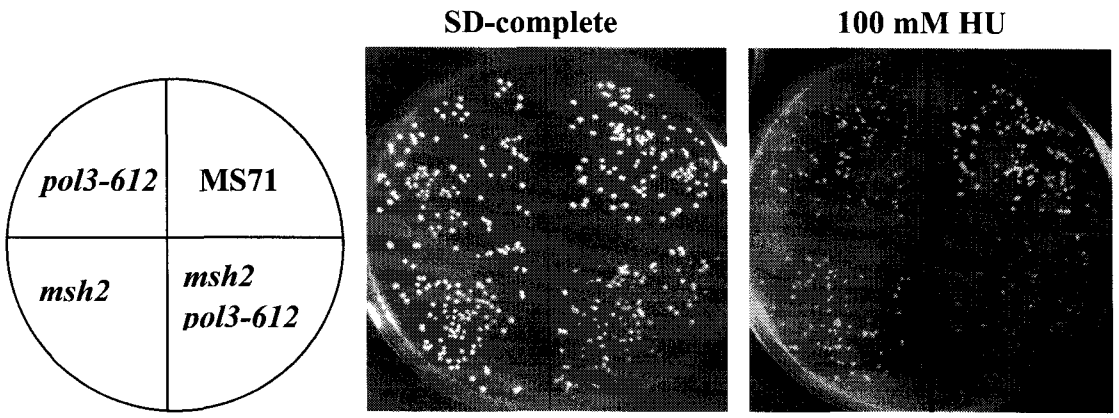

Figure 3.7.2 a

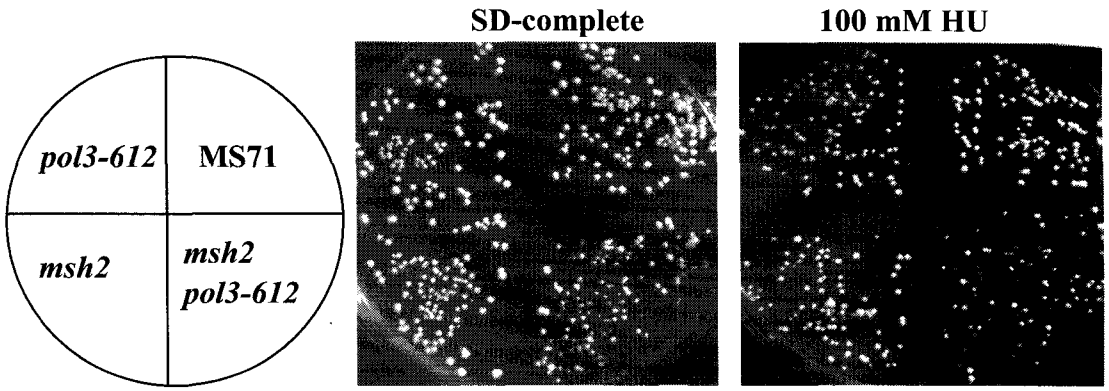

Figure 3.7.2 b

Figure 3.7.2 HU sensitivity of *pol3-612* cells in the presence and absence of MMR. Approximately 100 viable cells of each strain were plated onto SD-complete plates in the presence of 100 mM HU. Plates were incubated at 30°C for 3 days (a) to show HU-sensitivity and 10 days (b) to show reversibility.

on *S. cerevisiae* DNA pol δ (Culotti and Hartwell, 1971; Gordenin *et al.*, 1992). These mutant yeast strains displayed normal (or nearly normal) DNA replication at permissive temperature but aberrant replication profiles when shifted to restrictive temperature (Conrad and Newlon, 1983; Budd and Campbell, 1993; Jin *et al.*, 2001).

A *ts POL3* mutant, *pol3-t*, was chosen to investigate whether or not there is requirement for MMR system when replication forks are blocked at restrictive temperature. *pol3-t* has a single amino acid substitution D643N in DNA pol δ and displays growth inhibition at 37°C, but not at 30 °C (Tran *et al.*, 1997; Kokoska *et al.*, 1998). MMR was inactivated in the *pol3-t* strain by knocking out the *MLH1* gene. The *pol3-t* and *mlh1 pol3-t* strains were examined for differences in temperature sensitivity. As shown in Figure 3.7.3a, at 30°C the two strains displayed similar growth rates, but *mlh1 pol3-t* cells reached saturation with a lower titer (less than two fold). When switched to 37°C, both *pol3-t* and *mlh1 pol3-t* cells were completely inhibited for growth after 2 hours and started losing viability after 4 hours, which was consistent with previous studies on *ts* mutants of DNA polymerase in budding yeast (Budd and Campbell, 1987; Weinert and Hartwell, 1993). The magnitude of the decrease in viable cells at 37°C was very similar for both strains (Figure 3.7.2a), suggesting that inactivation of MMR did not enhance temperature sensitivity in the *pol3-t* cells. In support of this was the alteration in viability of the two strains after a switch to restrictive temperature. Both *pol3-t* and *mlh1 pol3-t* cells started losing viability after 4 hours at 37°C and no significant difference was observed between the severities of viability loss of the two strains (Figure 3.7.2c). Note that although MMR deficiency did not cause dramatic alteration in viability at 37°C, it did cause a moderate decrease in viability at 30°C. Viability of *mlh1 pol3-t* was about 80%, while *pol3-t* cells were 100% viable (Figure 3.7.2b), suggesting that the *pol3-t* product may not function properly even at 30°C and MMR may play a role in resolving the problems caused by the *pol3-t* gene product.

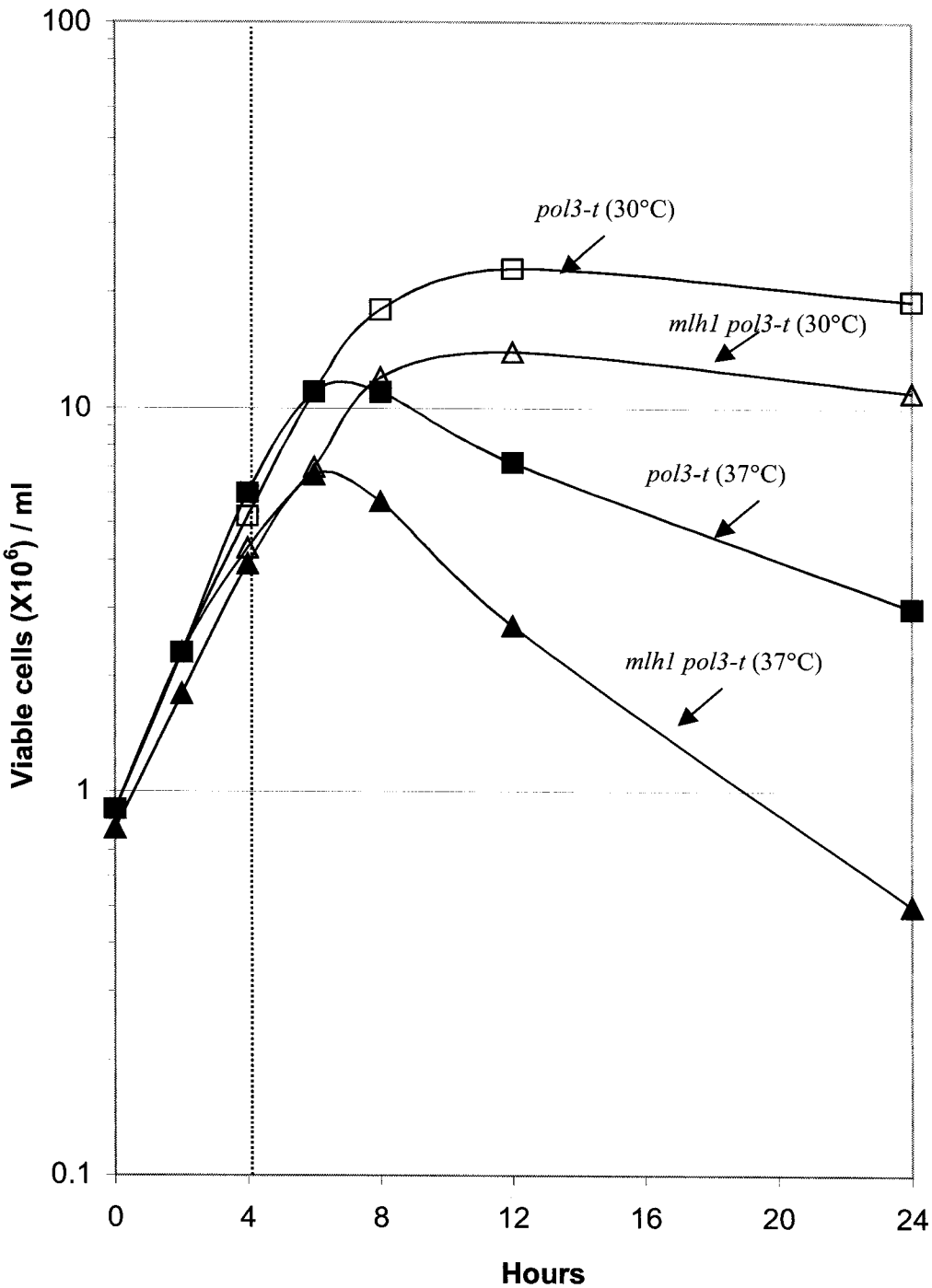

Figure 3.7.3 (a)

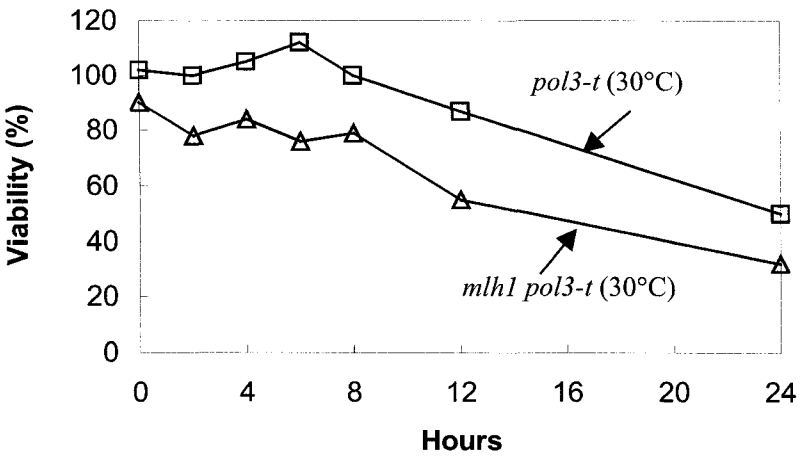

Figure 3.7.3 (b)

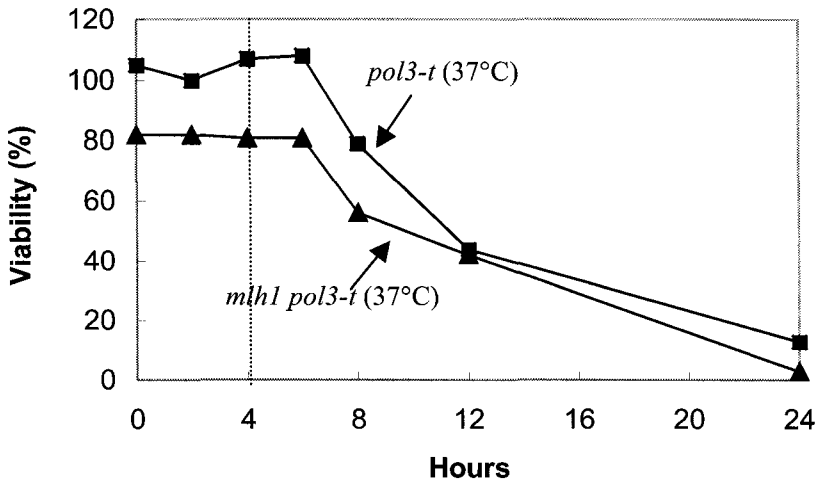

Figure 3.7.3 (c)

Figure 3.7.3 Cell growth of *pol3-t* and *mlh1 pol3-t* at permissive (30°C) and restrictive temperatures (37°C). Cells in logarithmic phase were inoculated in YPD broth. Each strain was inoculated in two flasks with approximately 10^6 cells/ml. All cultures were incubated at 30°C for four hours. One flask of each strain was shifted to 37°C (indicated by dashed line) while the other flask remained at 30°C. (a) Cells were titred at time indicated. Plates were incubated at 23°C for 5 days. (b) Cell viability of the two strains at 30°C is shown. (c) Cell viability of the two strains at 37°C is shown.

Taken together, the data above demonstrated that MMR deficiency did not alter cellular sensitivity to HU, UVC in the *pol3-612* background, neither did it increase temperature sensitivity of *ts POL3* mutant. In other words, the reliance on MMR might be unique to the PAA-response in the *pol3-612* cells.

CHAPTER 4 DISCUSSION

In this study, a novel *S. cerevisiae* DNA pol δ mutant strain, *pol3-612*, was constructed by using our knowledge on bacteriophage T4 DNA polymerase as a guide. The following points can be summarized based on the initial *in vivo* characterization:

(1). A methionine substitution of leucine at codon 612 in the Motif A of DNA pol δ conferred *S. cerevisiae* cells sensitivity to the antiviral drug PAA (Figure 3.1.3 and 3.1.5). This substitution also compromised replication fidelity of the enzyme (Table 3.2.1 and 3.2.2) and might cause other replication abnormalities (Figure 3.4.2).

(2). MMR appeared to play a critical role in L612M-DNA pol δ function both in the presence and absence of PAA (summarized in Table 4.1).

(3). The DNA recombination machinery also participated in the PAA-induced cellular response (Figure 3.5.1), suggesting double strand breaks might be generated when the L612M-DNA pol δ was inhibited by PAA.

(4). In the *pol3-612* background, the dependence on MMR might be unique to the PAA response. Inactivation of MMR in *pol3-612* cells did not alter cellular sensitivity to UVC radiation and HU treatment (Figure 3.7.1, 3.7.2), consistent with previous reports on the insensitivity of MMR-deficient *S. cerevisiae* strains to UVC irradiation (Durant *et al.*, 1999). Neither did MMR-deficiency affect temperature sensitivity of *ts* pol δ mutants (Figure 3.7.3).

4.1 PAA-sensitivity: selective inhibition of DNA pol δ

A long-standing question in eukaryotic DNA replication is what are the precise roles of DNA pol δ and ε at the replication fork. In order to address this question, DNA pol δ and ε need to be analyzed separately. Temperature-sensitive DNA polymerase mutants are informative in this respect. In fact much knowledge on DNA polymerases has been gained from *ts* alleles of *POL3* and *POL2* (for example, see Budd and Campbell, 1993;

Strain	Mutator phenotype rating	Viability	PAA-sensitivity rating
MS71	-	100%	-
pol3-612	1	100%	+
msh2	3	100%	-
msh2 pol3-612	5	50%	++++
mlh1 pol3-612	5	60%	++++
pms1 pol3-612	5	50%	++++
msh3 pol3-612	2	80%	+
msh6 pol3-612	4	80%	+++
msh3 msh6 pol3-612	5	50%	++++
exo1 pol3-612	2~3	100%	++++

Table 4.1 Correlation between mismatch repair deficiency and mutator activity, viability and PAA sensitivity in the *pol3-612* background. The ratings for mutator phenotype and PAA-sensitivity are qualitative. For mutator phenotype, 5 stands for the strongest mutator and 1 stands for the weakest. For PAA-sensitivity, ++++ represents the strongest and + for weak PAA-sensitivity. The wildtype MS71 strain is not considered as mutator. MS71 and *msh2* are not PAA-sensitive.

Budd and Campbell, 1995). But one of the problems of using *ts* mutants is that some *ts* DNA polymerase mutants show defects even at permissive temperature (Budd and Campbell, 1993; Kokoska *et al.*, 2000). Conventional *ts* mutants also can have leaky phenotypes at restrictive temperature (Hartwell *et al.*, 1970; Budd and Campbell, 1993). Another problem is that most *ts* DNA polymerase mutants lose viability at restrictive temperatures (Budd and Campbell, 1987; Weinert and Hartwell, 1993), making it difficult to study recovery mechanisms that restore DNA replication when restrictive conditions are removed. An additional concern with the use of *ts* DNA polymerase mutants is that normal metabolism may also be disturbed at restrictive temperatures.

Inhibitors of DNA polymerases and DNA replication are another commonly used tools for DNA replication studies. Biochemical studies of DNA polymerase inhibitors indicate that most inhibitors (such as aphidicolin and N-ethylmaleimide) have similar or only slight difference in inhibition on *S. cerevisiae* DNA pol α, δ and ε (Burgers and Bauer, 1988). While one inhibitor, BuPhdGTP, shows relatively large differences between DNA pol α and δ *in vitro*, this chemical cannot be used *in vivo* because of difficulties in transporting this drug into yeast cells. Another inhibitor, HU, targets the R2 subunit of ribonucleotide reductase (RNR), a critical enzyme for producing deoxyribonucleotides (reviewed by Elledge *et al.*, 1993). Therefore HU treatment lowers dNTP concentrations, which presumably inhibits all DNA polymerases, including DNA pol α, δ and ε.

Exonuclease deficient DNA pol δ and ε mutants have been used for probing the functions of these two enzymes as well. Studies on *pol3-01* and *pol2-4* (exonuclease deficient DNA pol δ and ε alleles, respectively) have revealed strand-specific mutation rates, implying that DNA pol δ and ε replicate opposite strands (Morrison and Sugino, 1994; Shcherbakova and Pavlov, 1996; Karthikeyan *et al.*, 2000). However, the significance of those discoveries remains questionable (see discussion in Karthikeyan *et al.*, 2000).

In present study, the antiviral drug PAA is selected as a DNA replication inhibitor. Wildtype *S. cerevisiae* cells are not sensitive to PAA at the tested concentration (Figure 3.1.5), so the observed inhibition of *pol3-612* cells suggests that PAA selectively inhibits the L612M mutant DNA pol δ, which is confirmed by the replacement experiment (Figure 3.1.4). Therefore, the "PAA and *pol3-612*" system provides a useful tool to probe the exact function of DNA pol δ.

It is not yet clear how PAA inhibited the L612M-DNA pol δ *in vivo*. Research in the T4 DNA polymerases demonstrates that PAA reduces translocation of the L412M-DNA polymerase along the template *in vitro*, while it hardly causes any effect to wildtype T4 DNA polymerase at the same concentration (Reha-Krantz and Nonay, 1994). Thus, it is possible that PAA also slows down DNA synthesis by the L612M-DNA pol δ *in vivo*, which may cause replication fork stalling or persistent single strand DNA regions during DNA synthesis.

4.2. The Interaction between MMR and DNA pol δ in *S. cerevisiae*

4.2.1 pol3-612 vs. pol3-01: a more informative mutant allele

Prior to this study, an interaction between DNA pol δ and MMR is known to result in synthetic lethality when *pol3-01* (a proofreading deficient mutant allele) and MMR deficiency are combined. In haploid cells, *pol3-01* is synthetic lethal when combined with *msh2, msh6, mlh1, pms1, exo1* null alleles, but not with *msh3* (Morrison *et al.*, 1993; Tran *et al.*, 1999a; Sokolsky and Alani, 2000; Argueso *et al.*, 2002). Error catastrophe is proposed to explain this lethality, i.e., due to extremely high mutational load with an error-prone DNA polymerase and inactivation of MMR, some essential genes would be inactivated, leading to cell death. In support of this hypothesis is the observation that none of the lethality is observed in homozygous diploid cells, possibly because in a diploid state there is a second genomic copy and the likelihood that both copies would be inactivated is very rare compared to the one copy haploid scenario (Morrison *et al.*, 1993;

Tran *et al.*, 1999a; Tran *et al.*, 1999b). In addition, in homozygous diploid cells, where mutation rates can be measured, strikingly strong mutator phenotypes are observed (Morrison *et al.*, 1993; Tran *et al.*, 1999a; Tran *et al.*, 1999b).

There are certain observations, however, that cannot be explained by the error catastrophe hypothesis. First, *pol3-01* is synthetic lethal with *rfc1* (a mutant allele of the *RFC1* gene encoding clamp loader), but *rfc1* is not a strong mutator (Xie *et al.*, 1999). Second, the synthetic lethality is also observed between *pol3-01* and *rad27Δ* (Kokoska *et al.*, 1998), even the *rad27Δ/rad27Δ, pol3-01/POL3* diploid is nonviable, which would not be predicted to have an excessive mutation rate because of the presence of the wildtype *POL3* (Gary *et al.*, 1999). Third, a temperature sensitive *MSH2* allele, *tsMSH2*-L910P, is defective in mutation avoidance at both permissive and restrictive temperatures, but is synthetic lethal with *pol3-01* only at a restrictive temperature (Sokolsky and Alani, 2000). Thus all observations above are not consistent with the error catastrophe hypothesis, suggesting other mechanisms may contribute to the observed synthetic lethality.

In addition to error catastrophe, an alternative hypothesis has been suggested to explain synthetic lethality. Sokolsky and Alani (2000) proposed that it is the DNA lesions made by *pol3-01*, which are recognized and/or corrected by MMR pathway, that causes cell death in the absence of MMR. A similar "DNA lesion" hypothesis has also been proposed to explain synthetic lethality in the *pol3-01 rad27Δ* strain (Jin *et al.*, 2001). But what precisely the DNA lesions are remains elusive. It is hypothesized that these DNA lesions might be double strand breaks (DSBs), since viability of *pol3*-EXO III *rad27-p* requires a functional DSB recombinational system (Jin *et al.*, 2001), while other reports exclude that possibility, because *pol3-01 rad52* is viable (Xie *et al.*, 1999). One important factor that hampers elucidation is the synthetic lethality of the *pol3-01* MMR-deficient cells.

The *pol3-01* and *pol3-612* alleles share certain similarities, for example, both

mutants may facilitate strand displacement synthesis and as a result, the 5'-flap formation. This activity of *pol3-01* is supported by biochemical studies (Jin *et al.*, 2003a). For *pol3-612*, this activity is hypothesized based on studies on T4 L412M-DNA polymerase (L.R-K., unpublished observations). Consistently, both *pol3-01* and *pol3-612* are synthetic lethal with deletion of the *RAD27* gene, a critical player in Okazaki fragment maturation. In addition, the high mutation rates of +1 frameshift observed for the *exo1 pol3-612* strain (Table 3.2.2) is also in line with the hypothesis that the L612M-DNA pol δ may produce an excessive number of 5'-flaps.

Despite the similarities, compared with *pol3-01*, *pol3-612* has one important advantage: it is viable when MMR is completely inactivated. Thus *pol3-612* provides a useful tool to explore the interaction between DNA replication and MMR. Comparison between *pol3-612* and *pol3-01* is listed in Table 4.2.

One example illustrating the utility of the *pol3-612* allele is in exploration as to what DNA lesions (or deleterious replication intermediates) the L612M-DNA pol δ might generate, which is also proposed as a reason for *msh2 pol3-01* lethality but remains unclear. Sokolsky and Alani (2000) excluded the possibility that *pol3-01* may, directly or indirectly, generate DSBs, because *rad52 pol3-01* double mutant is viable. This conclusion, however, may not be complete, because MMR is still functional in the *rad52 pol3-01* double mutant, which may "mask" the detrimental effects caused by aberrant replication intermediates made by *pol3-01*. The triple mutant *msh2 rad52 pol3-01* cannot be constructed, as *pol3-01* is lethal with *msh2*. This problem, however, can be addressed in a *pol3-612* background because the *msh2 pol3-612* strain was viable. Recombination does appear to be critical in *msh2 pol3-612* cells, because knocking out the *RAD52* gene reduces viability from approximate 50% to 20% (Figure 3.5.2). Considering the high mutation rates in *msh2 pol3-612* cells and extremely slow growth phenotype of *msh2 rad52 pol3-612* cells (Figure 3.3.1), the 20% survival might be due to the presence of second-site mutations. Significant loss of viability was also seen when the *RAD52* gene

	pol3-612	*pol3-01*
Amino acid substitution	Polymerase active center	Exonuclease active center[1]
Mutator phenotype	Weak	Strong[1,2,3]
***DUN1*-dependent mutator phenotype**	No	Yes[2]
PAA-sensitivity	Yes	No
Causes 5'flap	Yes (?)	Yes[6]
Viability with		
rad27	Lethal	Lethal
exo1	100%	Lethal[3]
msh2	~ 50%	Lethal[3]
pms1	~ 50%	Lethal[1]
mlh1	~ 50%	Lethal[4]
msh6	~ 80%	Lethal [5]
msh3	~ 80%	Viable[5]

Table 4.2. Phenotype comparisons between the *pol3-612* and *pol3-01* mutant strains. Data of the *pol3-01* strain is taken from 1.Morrison *et al.*, 1993, 2.Datta *et al.*, 2000, 3.Tran *et al.*, 1999b, 4.Argueso *et al.*, 2002, 5.Sokolsky and Alani, 2000, 6.Jin *et al.*, 2003a. The phenotype labeled with a "?" is deduced from indirect data.

was inactivated in *msh6 pol3-612* cells (Figure 3.5.2). Thus, as suggested by data presented here, DSBs could be (although maybe indirectly and in the absence of MMR) the consequence of deleterious replication intermediates generated by the L612M-DNA pol δ. A likely scenario is that the excessive number of 5'-flaps are produced by the L612M-DNA pol δ, which cannot be ligated by DNA ligase and may ultimately results in DSBs (see Section 4.3 for detailed discussions). Such detrimental replication intermediates (5'-flaps) may also be generated by *pol3-01*, considering similarities that are shared by *pol3-612* and *pol3-01* (see above). In both cases, MMR may prevent and/or resolve formation of an excessive number of 5'-flaps, thus alleviating the replication stress and maintaining normal cell viability.

A second advantage that the *pol3-612* mutant has, but the *pol3-01* doesn't, is the PAA-sensitivity, which provides a tool to study DNA pol δ specifically (see above). The insensitivity of the *pol3-01* strain also suggests important functional differences between these two polymerase mutants.

4.2.2 Requirement of MMR in the pol3-612 background

A surprising discovery for *pol3-612* MMR-deficient strains was the dramatic increase in PAA-sensitivity (Figure 3.3.2). Inactivation of *MSH2*, *PMS1*, *MLH1* and *MSH6* genes in the *pol3-612* background led to dramatic increases in PAA sensitivity, which was correlated to the magnitude of mutation rate increases, suggesting that the MMR system plays an important protective role in response to PAA. Moreover, as stated above, MMR also appears to provide protection against other replication abnormalities generated by the L612M-DNA pol δ in the absence of PAA, implying that MMR might play an important role in DNA pol δ function in addition to correcting misincorporations made by DNA polymerases. From the Table 4.1, it appears that there is a correlation between the mutator phenotype, viability and PAA-sensitivity in the *pol3-612* background, i.e., the less remaining MMR activity, the poorer viability and the more

severe PAA-sensitivity.

This correlation appears to apply to the *MSH2*, *MLH1*, *PMS1*, *MSH3* and *MSH6* knockouts. There is, however, an exception, i.e., the *exo1 pol3-612* strain, which has normal viability and only a moderate mutator phenotype, but very severe PAA-sensitivity that is comparable to the *msh2 pol3-612* strain (Figure 3.3.4). This normal viability and the moderate mutator phenotype could be explained by the possibility that exonuclease(s) functionally redundant to the Exo1p may exist in eukaryotic MMR and compensate the exonuclease loss caused by the *EXO1* deletion (Modrich and Lahue, 1996). In contrast to the minor role in MMR, the severe PAA-sensitivity of the *exo1 pol3-612* strain implies that Exo1p is indeed critical in response to the PAA-inhibited L612M-DNA pol δ.

The Exo1p and Msh2p appear to be in the same pathway in response to PAA treatment (Figure 3.3.4, no further increase in PAA-sensitivity is observed when deleting the *EXO1* gene in the *msh2 pol3-612* strain). The discrepancy observed for the *exo1 pol3-612* strain between the absence of PAA (normal viability and a moderate mutator phenotype) and presence of PAA (severe PAA-sensitivity) suggests that the genotoxic stresses generated by the L612M-DNA pol δ might be different in the presence compared to the absence of PAA and, different mechanisms might be used to alleviate this stress, although the MMR complex might be recruited in both cases. Exo1p is proposed to play a role in maintaining structural integrity of MMR complex in *S. cerevisiae* (Amin *et al.*, 2001). Thus, one possible scenario is that deletion of the *EXO1* gene may result in a MMR complex with impaired stability, which demonstrates only a minor defect in normal DNA replication, but a severe function loss in the presence of external interference of DNA replication, such as PAA inhibition of the DNA pol δ.

Since the major function of MMR is to correct replication errors, one possible explanation for the observed elevation in PAA-sensitivity together with MMR deficiency in *pol3-612* cells is error catastrophe, i.e., PAA induces numerous mutations that are

subject to MMR correction. In the absence of MMR, those mutations may inactivate essential genes and cause viability loss. However, this hypothesis does not appear to be correct, because PAA only slightly increased mutation frequencies (Table 3.3.1). Furthermore, for some mutagenic or DNA damaging agents, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU) and cisplatin, there are reports showing that loss of MMR in fact increases tolerance to these agents in human tumor cell lines (Drummond *et al.*, 1996; Duckett *et al.*, 1996), possibly due to elimination of MMR-mediated "excision-synthesis-excision" futile cycles of repair (Goldmacher *et al.*, 1986) or elimination of cell apoptosis (Hickman and Samson, 1999). In yeast cells, MMR deficiency does not affect sensitivity to mutagenic agents, such as MNNG and UVC irradiation (Xiao *et al.*, 1995; Bawa and Xiao, 1997; Durant *et al.*, 1999).

Another explanation for the requirement of MMR in PAA response is that MMR might be involved in sensing and/or signaling the stress caused by the PAA-inhibited L612M-DNA pol δ, which subsequently triggered the cell cycle checkpoint mechanisms. Consistent with this hypothesis, inactivation of MMR greatly impaired cell cycle arrest of *pol3-612* cells in presence of PAA (Figure 3.3.3). Moreover, the PAA-treated *msh2 pol3-612* cells started to lose viability after 4 hours and dropped to only 10% after 24 hours (Figure 3.3.3b), while no viability loss was observed in the PAA-treated *pol3-612* cells (Figure 3.1.5b).

The role of MMR in sensing/signaling genotoxic stress has been reported in mammalian cells. The MutSα complex (MSH2-MSH6), but not MshSβ, is required to signal the initiation of apoptosis induced by alkylating agents (Hickman and Samson, 1999). A similar study is also reported for cell survival in response to UVB, in which MSH2 may function as a sensor to trigger apoptosis after UVB damage (Peters *et al.*, 2003). Besides signaling in apoptosis, MMR is also proposed to trigger checkpoints and facilitate cell cycle arrest in response to ionizing radiation because MMR-deficient cells

show defects in activating S-phase checkpoint and altered G2/M arrest after ionizing radiation (Yan *et al.*, 2001; Brown *et al.*, 2003).

Recent studies reveal possible mechanisms underlying the roles of MMR proteins in the cellular responses to DNA damage. It has been reported that the MutSα complex can specifically recognize common DNA damage, such as O^6-methylguanine (O^6meG) and 8-oxo-guanine in mammalian cells (Duckett *et al.*, 1996; Colussi *et al.*, 2002), suggesting that MMR proteins may function as a sensor in response to DNA damage. Moreover, the BASC (BRCA1-associated surveillance complex) is identified at DNA damage sites (Wang *et al.*, 2000), which includes MMR proteins (MSH2, MSH6, MLH1), the DNA replication protein (RFC), DNA recombination proteins (RAD50-MRE11-NBS1) and checkpoint protein (ATM, ataxia telangiectasia mutated) as well as other proteins involved in DNA replication and repair (Wang *et al.*, 2000). Particularly noteworthy about the BASC complex is the coexistence of MMR proteins and ATM (a critical signaling molecule in checkpoint activation in mammalian cells), which is consistent with involvement of MMR in signal sensing and/or processing. Brown *et al.* (2003) reported direct interaction between MSH2 with CHK2, MLH1 with ATM at the damage site after ionizing radiation. Most recently, the interaction was demonstrated between human MSH2 and ATR (Ataxia- and Rad-related) proteins and it was proposed that MSH2-ATR could function as a signaling module in response to alkylation damage (Wang and Qin, 2003). Based on these observations, it has been proposed that the MMR complex may assemble at the DNA damage sites and subsequently recruit important players in checkpoint (for example, ATM and CHK2) and initiate a checkpoint cascade (for example, ATM may phosphate and activate CHK2 at the damage site) (reviewed by Bellacosa, 2001).

Alternatively, rather than direct recognition of DNA damage by MMR proteins, the intermediates of MMR process may function as signals to activate the checkpoint. For example MMR generates ssDNA that could be a few hundred nucleotides long

(reviewed by Modrich and Lahue, 1996). Zou and Elledge (2003) demonstrated that the ssDNA coated by RP-A proteins recruited the ATRIP (ATR-interacting protein) and facilitate the formation of ATR-ATRIP complex at ssDNA in human cells, which then activated downstream substrates in checkpoint pathways.

Compared with mammalian cells, much less is known how *S. cerevisiae* MMR proteins sense/signal genotoxic stress. Consistent with the observations in human cells, the Msh2p-Msh6p complex of *S. cerevisiae* also recognizes 8-oxo-guanine:adenine mispairs with high affinity and specificity (Ni *et al.*, 1999). In addition, the *S. cerevisiae* counterpart of human ATR-ATRIP complex, Mec1p-Ddc2p (*S. cerevisiae* homolog of ATR and ATRIP proteins, respectively), appears to be recruited by the RP-A proteins bound to the ssDNA region at the DSB sites in a manner similar to that in human cells (Zou and Elledge, 2003). Thus, these similarities between human and *S. cerevisiae* suggest that the MMR-dependent checkpoint may be activated through similar pathways in the two organisms.

In budding yeast, both the DNA damage checkpoint and replication checkpoint require Mec1p, the yeast homolog of ATR (Sanchez *et al.*, 1996), and Rad53p, the yeast homolog of CHK2 (Matsuoka *et al.*, 1998). It has been demonstrated that Mec1p is recruited to regions of stalled replication (Osborn and Elledge, 2003) and DSB sites (Kondo *et al.*, 2001). Interactions between MMR proteins and replication clamp PCNA have also been identified (Clark *et al.*, 2000; Flores-Rozas *et al.*, 2000). Therefore although hypothetical, it is possible that *S. cerevisiae* MMR proteins also recruit Mec1p and Rad53p at the stalled DNA replication forks (for example, by PAA or aberrant DNA structures) or at the mismatches generated by the L612M-DNA pol δ to start the cascade in checkpoint signaling pathway. Absence of functional MMR may lead to a defective checkpoint pathway in *pol3-612* cells.

Alternatively, the RP-A bound ssDNA produced in MMR process may function as a signal to trigger the S-phase checkpoint. As shown in this study, the L612M-DNA pol δ

induced lots of replication errors during genome replication. These mismatches were subject to correction by MMR (Table 3.2.1). Because DNA pol δ participates in DNA re-synthesis during the MMR process, the PAA-inhibited L612M-DNA pol δ might slow down the synthesis and a ssDNA region might be persistent. The RP-A coated ssDNA could recruit key transducers in checkpoint pathway and initiate the cascade as observed in mammalian cells (Zou and Elledge, 2003). Furthermore, the persistent ssDNA region could be converted to DSBs (Kuzminov, 2001), which is also bound by RP-A proteins that may recruit Ddc2p and facilitate the formation of Mec1p-Ddc2p complex in *S. cerevisiae* (Zou and Elledge, 2003). In the absence of functional MMR activities, the persistent ssDNA regions may not exist to serve as a genotoxic signal for the checkpoint activation.

Another explanation for the protective role of MMR in the PAA-treated *pol3-612* cells is that MMR may be required for replication restart. The supporting evidence is that in the presence of a functional MMR system, the PAA-treated *pol3-612* cells were able to resume growth after 6 hours, whereas *msh2pol-612* cells could not (Figure 3.3.3a). A possible experiment to test this hypothesis is presented in Section 4.6 Future directions.

4.2.3 Requirement of MMR: structural or functional?

Interactions between MMR proteins and DNA replication proteins have been well documented. Protein sequence analysis indicates that both Msh6p and Msh3p contain motifs specific for PCNA binding, which is also confirmed by functional analysis (Clark *et al.*, 2000; Flores-Rozas *et al.*, 2000). It is proposed that the interaction between PCNA and the Msh2p•Msh6p complex may help transfer this complex to mispaired bases in DNA (Lau and Kolodner, 2003). A larger and more comprehensive complex containing MMR and replication proteins, BASC, has been identified as well (Wang *et al.*, 2000). Thus, although evidence is still being collected, it seems plausible that MMR is part of the DNA replication complex during genome DNA replication. This association could

have the following functions: (1) correct mismatches that escape proofreading of DNA polymerase; (2) signal replication stress when DNA replication forks are stalled by DNA damage or replication inhibitor (such as HU and PAA); (3) participate in stabilization of stalled replication forks or facilitate the replication restart process. In all three scenarios, the role of MMR could be structural, i.e., the physical presence of MMR complex; or alternatively, functional, i.e., the activities of MMR proteins. These two possibilities cannot be differentiated by studies presented here, because all MMR deficient strains used were null (amorphic) alleles of the MMR genes.

At least two methods can be used to distinguish a structural requirement from a functional requirement for MMR in the PAA-responses. One method is to use hypomorphic alleles of MMR genes to replace the amorphic alleles, followed by examination of alterations in PAA response in the *pol3-612* background. There are some well-documented alleles that can be examined, for example, the G693A allele of *MSH2*, which has an alanine substitution at an invariant glycine residue (Studamire *et al.*, 1998; Drotschmann *et al.*, 1999). The G693A-Msh2p is expressed in a similar pattern as wildtype Msh2p, but has a functional defect as revealed in assessment of replication fidelity, presumably due to disruption of the ATPase activity of Msh2p required during MMR. A similar observation is also reported for the *msh2*-G693D allele (Studamire *et al.*, 1998). Therefore, mutant *msh2* alleles with mutation at G693 residue may be useful in dissecting either functional role or structural role that Msh2p plays in PAA-response. If the combination of *msh2*-G693A with *pol3-612* also leads to dramatic enhancement of PAA-sensitivity, then it would suggest it is MMR function in error correction that is most relevant to PAA-response. Otherwise, there are two possibilities to explain the disappearance of PAA-sensitivity increase: (1) the physical existence of MMR and its connection with DNA replication complex may be important. This possibility can be further confirmed by using an *msh6* mutant that is defective in interacting with PCNA, disrupting the interaction between MMR and the DNA replication machinery

(Flores-Rozas *et al.*, 2000); (2) it is the role of Msh2p in signaling DNA damage that protects *pol3-612* cells in the presence of PAA. This possibility is based on a study in the $Msh2^{G674A}$ (the mouse counterpart of *S. cerevisiae msh2*-G693D allele) mutant mice, which displays a mutator phenotype but retains signaling function in response to DNA damaging agents such as cisplatin and MNNG (Lin *et al.*, 2004). Isolation of the "separation-of-function" mutant alleles of the *MSH2* gene may be helpful to differentiate the above two possibilities (see below).

In addition to using hypomorphic MMR alleles, one could also take advantage of chemical reagents that inactivate MMR function. Most recently, it was reported that MMR could be functionally inhibited by cadmium (Jin *et al.*, 2003b). Thus, applying cadmium to *pol3-612* cells (which have a functional MMR system) may mimic the *pol3-612* MMR-deficient cells without physically removing MMR proteins. Studies on how cadmium-treated *pol3-612* cells respond to PAA would help to elucidate if the requirement for MMR is functional or structural in a *pol3-612* background. Another advantage using cadmium is that MMR can be inactivated only when needed, thus minimizing the possibility of the occurrence of additional mutations in the yeast genome, a concern in *pol3-612* MMR-deficient cells due to the strong mutator phenotype.

These two strategies outlined above are based on the known major function of MMR, i.e., mutation prevention. However, additional activities of MMR proteins have been reported. For example, Msh2p and Msh3p, but not Mlh1p or Pms1p, are required for single strand annealing during repair of double strand breaks (Sugawara *et al.*, 1997). "Separation-of-function" mutations in the *MSH2* gene have been isolated, which confer mismatch repair defects but do not affect single strand annealing (Studamire *et al.*, 1999). Another example of the "separation-of-function" mutants is the $Msh2^{G674A}$ mutant allele of mouse *Msh2* gene, which confers deficiency in the repair process while does not affect signaling in apoptosis (see above) (Lin *et al.*, 2004). Thus it is possible that other unknown activities of the MMR proteins may underlie the observations shown in

PAA-response. Isolation and use of "separation-of-function" mutant alleles of MMR genes may provide a useful tool in further investigation on the roles that MMR plays in DNA replication.

4.2.4 Why does inactivation of MMR partially rescue viability loss in the dun1 strain?

One interesting observation in this study was that MMR deficiency partially rescued viability loss in *dun1* cells (Figure 3.6.2). Inactivation of MMR also partially alleviated PAA-sensitivity of *dun1* cells (Figure 3.6.1). It is proposed that Dun1p plays a critical role in upregulation of dNTP concentration (Zhao and Rothstein, 2002), thus deletion of *DUN1* would result in lower dNTP concentration, consistent with the fact that the *dun1Δ* mutant has a longer S-phase. Another consequence of inactivating *DUN1* is genome instability. Compared with wildtype cells, the *dun1* mutant has more than a 200-fold increase in gross chromosomal rearrangements (Myung *et al.*, 2001b), which also presumably increases the likelihood of homeologous recombination between similar but not identical sequences. MMR is well known to suppress homeologous recombination. Although the molecular basis of the anti-recombination activity exerted by MMR proteins is unclear, it is proposed that *de novo* DNA synthesis is involved in the suppression process, which includes DNA synthesis after removal of mismatches (reviewed by Harfe and Jinks-Robertson, 2000). Because gaps in the eukaryotic MMR process could be a few hundred nucleotides long (Fang and Modrich, 1993), consumption of dNTPs during the MMR process may further exacerbate the restrictive dNTP concentration in *dun1* cells, which already have reduced dNTP levels. Thus the observed partial rescue of viability loss and PAA-sensitivity in the *msh2 dun1* strain compared with *dun1* strain could be due to alleviation of the low dNTP concentration crisis by inactivation of MMR.

Deletion of the *SML1* gene, which encodes a negative regulator of dNTP levels and is a target of Dun1p for regulation, can be used to test the above hypothesis. Inactivation

of the *SML1* gene suppresses lethality of *mec1Δ*, *rad53Δ* mutants and increased petite formation in *dun1* cells, presumably due to restored dNTP levels (Zhao *et al.*, 1998; Zhao and Rothstein, 2002). It is interesting to examine the effects by deletion of *SML1* in *dun1* and *dun1 msh2* strains. If the hypothesis above is correct, then it is expected that suppression of viability loss and PAA-sensitivity would be seen in both strains when the *SML1* gene is deleted.

4.3. The role of DNA recombination in the *pol3-612* MMR-deficient strains

In addition to MMR, DNA recombination also appeared to play a role in the PAA-response in *pol3-612* cells (Figure 3.5.1b). But compared to MMR, recombination seemed to play a less important role, since the PAA-sensitivity of the *pol3-612* recombination deficient strains was much less than that of the *pol3-612* MMR-deficient strains (compare Figure 3.5.1b with Figure 3.3.2). In the absence of PAA, abolishment of recombination activity in *pol3-612* strain did not cause viability loss until MMR was inactivated (Figure 3.5.2).

In *E. coli*, DNA recombination is the major means by which stalled DNA replication forks are rescued (reviewed by Cox *et al.*, 2000). In yeast cells, recombination is the main pathway for repairing DSB (reviewed by Haber, 2000). In addition, recombination deficient *S. cerevisiae* mutants (*rad51*, *rad52*) show sensitivity to DNA damage and HU (Sung and Stratton, 1996; Kokoska *et al.*, 2000), suggesting that in yeast replication forks stalled by DNA damage or HU may also lead to DSB formation, as observed in *E. coli* (Kuzminov, 1995; Kuzminov, 2001).

In this study, L612M-DNA pol δ might produce deleterious replication intermediates. For example, compared to cells with wildtype DNA pol δ, L612M-DNA pol δ may promote displacement synthesis during lagging strand replication and cause excessive 5'-flap formation (see above), which cannot be ligated by DNA ligase and results in nicks in the DNA. Those nicks may increase the likelihood of DSBs formation,

since it has been demonstrated that single strand interruptions can lead to DSBs in *E. coli* (Kuzminov, 2001). Furthermore, in the next round of replication, unligated Okazaki fragments may also cause DSB when DNA replication forks encounter nicks (Jin *et al.*, 2001). In the presence of PAA, DNA replication forks may be stalled because of PAA-inhibited L612M-DNA pol δ. This stalled replication fork could lead to DSB (Kaliraman *et al.*, 2001). In addition, the PAA-inhibited L612M-DNA pol δ may slow down DNA synthesis and cause persistent ssDNA regions, which increases the likelihood of DSB formation. In all scenarios described above, DSB may be formed due to L612M-DNA pol δ, either directly or indirectly, which is dependent upon the recombination machinery to repair. However, prior to the participation of recombination, MMR appears to be the primary mechanism in response to stress induced by the L612M-DNA pol δ.

4.4 MMR requirement: not a universal response to replication stress?

Despite the critical role that MMR could play in the PAA-response, it appeared that MMR played no role when the DNA replication is disturbed by HU treatment or UVC damage in *pol3-612* cells (Figures 3.7.1 and 3.7.2). Similar results were observed for MMR in the *ts POL3* mutant at restrictive temperature (Figure 3.7.3). Thus from the preliminary analysis it appeared that requirement of MMR was restricted to PAA responses in *pol3-612* cells.

The above insensitivity of MMR inactivation could reflect the independence of MMR in response to replication stress. In *S. cerevisiae* it has been reported that inactivation of MMR does not affect sensitivity to alkylating agents (Xiao *et al.*, 1995; Bawa and Xiao, 1997) and UVC irradiation (Durant *et al.*, 1999). The *msh2 pol3-612* strain was not sensitive to HU (Figure 3.7.2), but extremely sensitive to PAA (Figure 3.3.1, 3.3.2), suggesting that MMR might not be required when replication forks were stalled by HU, but was required in the presence PAA, since *msh2 pol3-612* cells did not appear to arrest in the presence of PAA and lost viability (Figure 3.3.3). Experiments

done by lab coworker K. Murphy also demonstrated that *msh2 pol3-612* cells were arrested in the presence of HU and retained viability (data not shown).

The differences between sensitivity to HU and PAA of *msh2 pol3-612* cells could be due to a threshold in S-phase checkpoint activation (Shimada *et al.*, 2002), i.e., in *msh2 pol3-612* cells the HU treatment produced signal strong enough to trigger the checkpoint, while PAA treatment did not. HU targets the R2 subunit of ribonucleotide reductase (RNR) and lowers the dNTP concentration (Elledge *et al.*, 1993), which presumably inhibits all DNA pol α, δ and ε. It has been reported that DNA pol ε may directly sense replication blocks generated by HU or MMS and trigger checkpoint responses (Navas *et al.*, 1995; Dua *et al.*, 1998). PAA, however, specifically inhibits the L612M-DNA pol δ but has little effect on DNA pol α and ε at the tested concentration (0.5 mg/ml). Therefore the PAA-induced replication fork stalling might not be "sensed" by the surveillance mechanism in the absence of MMR and generated signals strong enough to activate the S-phase checkpoint as HU did. Moreover, in *pol3-612* cells that have functional MMR, viability loss was observed when the HU-triggered checkpoint, but not when the PAA-triggered checkpoint, was overridden by caffeine treatment (K. Murphy), suggesting that there could still be sufficient cellular repair ability in response to the PAA-induced replication block even when the checkpoint mechanism is impaired. One possible explanation is that, in the PAA-treated *pol3-612* cells, DNA pol ε still has the potential to replace the L612M-DNA pol δ, which is impossible in the HU-treated cells since all essential DNA polymerases are inhibited. To further compare the HU-induced and the PAA-induced checkpoint pathways, we need to examine the components required in each pathway, for example, by inactivation of different transducer genes or examination of the phosphorylation status of those transducers. It will also help to elucidate the difference between the cellular responses to PAA and HU by monitoring the DNA synthesis through techniques such as FACS (fluorescence-activated cell sorter) analysis and two-dimensional gel electrophoresis.

Another explanation for the absence of phenotype after MMR inactivation could be due to the use of an inappropriate DNA pol δ allele. For example, inactivation of MMR in wildtype *POL3* background did not cause PAA-sensitivity. A dramatic increase was only observed when *pol3-612* and *msh2* were combined. In the UVC and HU sensitivity test, the *pol3-612* mutant did not appear to be sensitive in either situation (Figures 3.7.1 and 3.7.2). Dependence on MMR might not be observed unless the *POL3* mutant allele itself already shows appreciable sensitivity. A UV-sensitive *POL3* mutant allele (for example, *pol3-13* (Giot *et al.*, 1997) or a HU-sensitive *POL3* allele may need to be examined to further confirm the unique role of MMR in PAA-sensitivity in the *pol3-612* strain.

4.5. Functional conservations between T4 DNA pol and *S. cerevisiae* DNA pol δ

Despite divergent protein sequences, DNA polymerases appear to share conserved motifs and amino acid residues in their catalytic domains (Delarue *et al.*, 1990). This conservation implies functional importance of those motifs and residues in maintaining enzyme activities. Thus investigations on those motifs and key residues will be critical to our understanding of how DNA polymerases function as the central player of DNA replication.

Motif A is proposed to exist in all DNA polymerases (Delarue *et al.*, 1990). Mutational analysis on the Motif A of Family A DNA polymerases, *Thermus aquaticus* DNA polymerase I (*Taq* pol I) and *E. coli* DNA polymerase I demonstrate that this motif is highly tolerable to mutations, i.e., multiple amino acid substitutions (except for the catalytic Asp residue) still allow cell growth and preserve polymerase activity similar to the wildtype enzyme (Patel and Loeb, 2000; Shinkai *et al.*, 2001). This is surprising because the primary structure of Motif A in Family A DNA polymerase is highly conserved. A hypothesis was made to explain the seemingly contradiction: the high plasticity of Motif A in Family A DNA polymerases may be used to promote cell

tolerance to the changing environmental stress (Patel and Loeb, 2000; Shinkai *et al.*, 2001).

Unlike observations in *Taq* pol I and *E. coli* pol I, Motif A of *S. cerevisiae* DNA pol δ does not appear to be tolerable for mutations: substitution of the tyrosine residue with alanine in Motif A of DNA pol δ leads to lethality (Pavlov *et al.*, 2001). However, other than the essential requirement for a tyrosine residue, the function of Motif A in eukaryote pol δ remains poorly addressed.

Both *S. cerevisiae* DNA pol δ and T4 DNA polymerase are in Family B DNA polymerases and both are genome-replicating enzymes. Functional conservation on Motif A may exist between T4 DNA polymerase and *S. cerevisiae* DNA pol δ due to their striking similarities on the Motif A sequence (Figure 1.1). This hypothesis was confirmed by present study. Like T4 phage, an amino acid substitution in Motif A conferred cells sensitivity to PAA and greatly compromised replication fidelity of the DNA polymerase, suggesting that the Leu612 residue and Motif A are critical factors for ensuring replication accuracy in *S. cerevisiae* DNA pol δ. Studies on T4 DNA polymerase demonstrated that Motif A plays an important role in determining the fidelity of nucleotide incorporation as well as the balance between the polymerase activity and the exonuclease activity of the enzyme, both of which contribute to the overall replication fidelity of DNA polymerase (Reha-Krantz and Nonay, 1994; Stocki *et al.*, 1995; Fidalgo da Silva *et al.*, 2002). The L412M-T4 DNA polymerase displays defects in both aspects as revealed by biochemical analysis (Fidalgo da Silva *et al.*, 2002). Considering the sequence conservation of Motif A between T4 DNA polymerase and *S. cerevisiae* DNA pol δ, the L612M substitution may also impair DNA pol δ function in a similar way.

The PAA-sensitivity of T4 DNA polymerase mutants can be suppressed by second site mutations in the gene. Two second-site suppressors, I417V, S411T, are found to suppress L412M-conferred PAA-sensitivity (Reha-Krantz and Nonay, 1994), while two

other second-site suppressors, *sup2* (cysteine substitution for arginine at codon 335) and *sup4* (phenylalanine substitution for serine at codon 345), suppress PAA-sensitivity of the *tsP36* T4 DNA polymerase mutant that has a aspartate residue insertion following Asp863 residue (Reha-Krantz, 1989; Reha-Krantz and Wong, 1996). In present study, similar suppression was also identified in yeast DNA pol δ. In selecting for spontaneous suppressors of the PAA-sensitivity conferred by the L612M substitution in Motif A, a second-site mutation was isolated in the *POL3* gene, which encodes the V758M substitution in Motif C (Figure 3.3.6, 3.3.7). Structure studies on RB69 DNA polymerase, an enzyme closely related to T4 DNA polymerase, demonstrated that both Motif A and Motif C are located in the polymerase active center (Franklin *et al.*, 2001). The fact that the PAA-sensitivity conferred by L612M in Motif A could be suppressed by V758M in Motif C suggests functional interactions between these two motifs in DNA replication.

Selection for second-site suppressors can also be used as a tool to identify antimutator T4 DNA polymerase (Reha-Krantz and Wong, 1996). When isolated individually, second-site suppressors I417V, *sup2* and *sup4* all display antimutator phenotypes for base substitution. Although there is no report for replication fidelity of S411T mutant alone, the double mutant, S411T + L412M, still has an antimutator phenotype (Reha-Krantz and Nonay, 1994), implying that the S411T mutation may also increase replication fidelity. Similar results were observed for mutation rates of the V758M-DNA pol δ in yeast as well. As shown by the preliminary results, an approximately 5-fold increase in replication fidelity was seen using the *ade5-1* reporter that detects the base substitutions, while no difference was seen for the *his7-2* reporter that detects frameshift mutations and 3-fold decrease was seen for the *trp1-289* reporter that detects base substitutions (Table 3.3.2). The fact that replication fidelity is not increased against all base substitutions mutations *in vivo* are also reported in the T4 antimutator studies (Drake 1969; Ripley, 1975). Thus it is important to find appropriate reporters for replication fidelity measurement of antimutator DNA polymerases. The

V758M studies presented are only preliminary due to leakiness of the *ade5-1* reporter (L R-K and L Li, unpublished observations, T Petes, personal communications). Therefore, a more appropriate reporter for detecting base substitution should be identified and used for any further characterization of the antimutator DNA pol δ mutants.

Data presented here demonstrate functional conservation between T4 DNA polymerase and yeast DNA pol δ. Functional difference, however, may also exist between these two DNA polymerases as revealed by previous studies in our laboratory. A different DNA pol δ mutant, *pol3-447* was constructed as the yeast counterpart of the T4 G255S DNA polymerase mutant (Hadjimarcou *et al.*, 2001). Unlike the L412M substitution, which is in the polymerase active center, the G255S substitution is in the exonuclease domain. *In vitro* studies indicate that the G255S mutant is defective in strand separation and transfer of the primer-terminus to the exonuclease center for proofreading (Marquez and Reha-Krantz, 1996). Consistently, the T4 G255S mutant displays strong mutator phenotype for base substitutions (Hadjimarcou *et al.*, 2001). The yeast G447S mutant, however, does not appear to be a mutator for base substitution, even in the absence of MMR (Hadjimarcou *et al.*, 2001), suggesting functional differences may exist between these two enzymes.

4.6 Future directions

The observation of a PAA-sensitive phenotype in *pol3-612* cells opens the door to many interesting research projects. There are many important questions that remain to be answered.

4.6.1 Cell cycle and checkpoint studies

One critical question about the *pol3-612* MMR-deficient strains is whether checkpoint activation is defective. This study did not provide a direct answer. For further clarification, useful markers acting as a hallmark of checkpoint activation should be used.

For example, it has been well accepted that phosphorylation of Rad53p is an essential step in checkpoint activation (Allen *et al.*, 1994; Sanchez *et al.*, 1996). Examination of the status of Rad53p in PAA-treated and untreated *msh2pol3-612* cells may therefore provide useful information regarding checkpoint activation. In addition to Rad53p phosphorylation, measurement of the mitotic spindle together with nuclear staining is also a commonly used method that can be employed to assess checkpoint activation in budding yeast (for example, see Allen *et al.*, 1994; Osborn and Elledge, 2003).

Secondly, if MMR is involved in the checkpoint pathway, then most likely it should act in S-phase. There are two interconnected but also distinct checkpoint pathways in S-phase: the DNA replication checkpoint and the DNA damage checkpoint (reviewed by Kolodner *et al.*, 2002). Both pathways require Mec1p and Rad53p as key transducers for signaling, but they may also have different transducers in the pathway: *RAD9* in DNA damage checkpoint and *MRC1* in DNA replication checkpoint (Weinert and Hartwell, 1988; Alcasabas *et al.*, 2001). At present it is not clear which pathway MMR is involved in, but this can be dissected by examining the consequence of inactivating the *RAD9* and *MRC1* genes in the *pol3-612* and *msh2 pol3-612* background in terms of PAA-sensitivity and viability. Studies on *MRC1* are particularly intriguing, because it is involved in checkpoint activation in the HU response. Presumably, PAA induces cell cycle arrest in a manner similar to HU, rather than DNA damage. In addition, Mrc1p is found as a component of the replication fork and forms a stable pausing structure after HU treatment (Katou *et al.*, 2003; Osborn and Elledge, 2003). It is proposed that in the presence of replication blocks, Mec1p is recruited to stalled replication forks and subsequently phosphates Mrc1p, which is required for Rad53p activation (Osborn and Elledge, 2003). Considering the possible interaction between MMR proteins and Mec1p, Rad53p (see Section 4.2.2), it is plausible that the MMR proteins may form a scaffold for assembly checkpoint proteins and initiate a phosphorylation cascade at the stalled replication fork. Analysis of the interactions between the MMR proteins, Mec1p, Mrc1p

and Rad53p will be valuable in this respect.

Thirdly, it is not clear whether MMR is involved in establishing a checkpoint and stabilization of the stalled replication fork, or facilitating replication restart, or both. To distinguish between these alternatives, a mutant strain can be constructed in which the *MSH2* gene expression can be controlled. For example if *MSH2* expression is under the control of a *GAL* promoter, so that gene expression can be turned on or off by switching between media with different carbon sources (Tercero *et al.*, 2003), then *pol3-612* cells treated with PAA can be analyzed for viability with and without the *MSH2* gene expression. If cells are viable when *MSH2* is expressed after PAA-treatment, then it implies that MMR may be required for replication restart. Otherwise, it suggests that MMR may be required to trigger a checkpoint and stabilize replication forks when treated with PAA, the absence of which leads to irreversible inhibition.

4.6.2 Other pathways involved in PAA-response

This study demonstrated the requirement of MMR and recombination in response to PAA as well as the presumably L612M-DNA pol δ-induced deleterious replication intermediates. Since defects in MMR and DNA recombination modulate PAA-sensitivity in *pol3-612* cells, then alteration in PAA-sensitivity can be used as a tool to pinpoint the interaction between DNA replication and other cellular pathways and processes. Recently, lab coworker K. Murphy demonstrated that the *RAD6*-dependent pathway was also involved in the PAA-response, which was independent from MMR. The *RAD6* product participates in various activities in the cell with the best characterized role being in post-replication repair (PRR) (reviewed by Broomfield *et al.*, 2001). The *RAD6*-dependent PRR includes error-prone and error-free pathways (Xiao *et al.*, 2000). Inactivation of the *RAD5* gene, a component of the error-free pathway, did not affect PAA-sensitivity both in the wildtype and in the *pol3-612* background (K. Murphy), nor did the error-prone pathway (DNA pol ζ) as shown in this study, thus it appeared that

others aspects of the *RAD6*-dependent mechanisms account for its role in the PAA-response.

4.6.3 Isolation of second-site suppressors

As shown in present study, a second-site mutation in the *POL3* gene suppressed the PAA-sensitivity conferred by the L612M substitution. Suppression of PAA-sensitivity implicates functional interactions between different amino acid residues of DNA pol δ. So far there is no crystallography structures available for eukaryotic DNA pol δ, thus isolation of second-site suppressors assists in genetic characterization of eukaryotic DNA pol δ. Furthermore, based on preliminary results, selection of second-site suppressors of PAA-sensitivity appears to be a useful strategy for isolation of antimutator DNA pol δ mutants in *S. cerevisiae*. Antimutator DNA polymerases provide insight on mechanisms by which high replication fidelity is achieved (reviewed by Reha-Krantz, 1995; Schaaper, 1998).

In summary, a novel phenotype of *S. cerevisiae*, PAA-sensitivity, was generated by engineering a mutant strain that expresses the L612M-DNA pol δ. This phenotype provides a means to study DNA pol δ separately from other essential DNA polymerases. The MMR system appears to play an important role in DNA pol δ function in addition to correcting misincorporations. Because in the *pol3-612* background, the severity of PAA-sensitivity can be modulated by second mutations in genes that are involved in MMR, DNA recombination, and the *DUN1*-dependent checkpoint pathway, therefore PAA-sensitivity is also a valuable tool to investigate interactions between different important cellular processes, such as DNA replication, post-replication MMR, recombination, and checkpoint activation. Since most cancer cells display genome instability, aberrant DNA recombination and defective checkpoint activation, data presented here and use of this model in further analysis may be helpful in understanding cancer development in human.

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