ISOLATION AND CHARACTERIZATION OF DNA POLYMERASE EPSILON MUTANTS AFFECTING DNA REPLICATION, REPAIR AND CELL CYCLE CHECKPOINTS IN *SACCHAROMYCES CEREVISIAE*.

THESIS

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Natashya J. Waring, BSW

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TABLE OF CONTENTS

LIST OF TABLES

Page

TABLES

LIST OF FIGURES

PAGE

FIGURE

CHAPTER 1

INTRODUCTION

Saccharomyces cerevisiae, baker's yeast, is widely used as a model system for studies of cellular processes in eukaryotic organisms. *S. cerevisiae* cells are more complex than bacteria but share many of the technical advantages associated with the study of bacteria. Properties that make yeast ideal in biological studies include rapid growth, a budding pattern resulting in easily monitored cell cycle phases, ease of replica-plating and mutant isolation, a well-defined genetic system and a highly versatile DNA transformation system (1, 2). Yeast is a non-pathogenic organism and therefore can be handled with few precautions. Strains have both a stable haploid and diploid state and are viable with a great many available genetic markers (1, 2). Improved DNA transformation protocols have made yeast more receptive to gene cloning and genetic engineering techniques. Development of techniques for the direct replacement of genetically engineered DNA sequences into normal locations has allowed normal (wildtype) genes to be replaced with altered and disrupted alleles (1, 2). Identification of phenotypes arising after alteration or disruption has allowed for the study of proteins in cells. Due to these characteristics, there

has been an explosion in the amount of information and understanding of the mechanisms and proteins involved with DNA replication and the cell cycle in eukaryotes (1, 2, 3).

The cell cycle is an ordered series of events during which a cell's chromosomes are duplicated and cytoplasmic contents are redistributed. One copy of each duplicated chromosome segregates to each of two daughter cells. That each daughter cell receives a duplicate copy with minimal errors in the DNA sequence is especially important for maintenance of cell viability and proper functioning of specialized tissues. The cell cycle can be subdivided into Gap phase I (G_1) which is the interval following mitosis, S phase during which DNA replication occurs, Gap phase II (G_2) which follows DNA synthesis and mitosis during which the contents of the cell are segregated (4, 5).

Cell cycle events are regulated such that each phase occurs at the appropriate stage in the cycle to insure that each daughter cell receives the correct duplicate of genetic material. In addition, some cell cycle events are linked in dependent pathways. At certain points in the pathway "checkpoints" arise that prevent the cell cycle from proceeding if there is a problem (3, 8, 9). For example, failure to complete DNA replication causes an arrest in the S phase, DNA damage typically causes an arrest in G_2 phase and improper spindle formation causes arrest in mitosis (3, 8).

DNA replication is the cellular process in which copies of the original parental strands of DNA are produced (11). The process is semi-conservative, meaning that each daughter molecule contains one strand of parental DNA and one newly synthesized strand (11). Replication begins at specific points along the chromosome called origins. In yeast these origins are referred to as autonomously replicating sequences or ARS elements (5). The complete process of replication occurs in three stages: initiation,

elongation and termination (11). Most research has involved the study of elongation. The proteins involved in replication include DNA polymerases, single-strand DNA binding proteins, helicases, primase, topoisomerases, DNA ligases and accessory proteins (11).

DNA polymerases are enzymes whose main function is the catalysis of the chemical reaction of DNA replication. They create the phosphodiester bonds between deoxyribonucleotides in a DNA strand (11). In these reactions, the 3' hydroxyl group at the end of a growing DNA strand carries out a nucleophilic attack on the α phosphate of an incoming deoxyribonucleotide triphosphate (dNTP) (8, 11). The dNTP is positioned for incorporation into the growing strand by hydrogen bonding with the appropriate nucleotide in the template strand according to the normal rules of base-pairing, A:T and G:C. DNA polymerases catalyze DNA chain growth in only the 5'→ 3' direction (8, 11).

There are several polymerases involved in DNA replication in yeast of which three are essential. The essential polymerases are DNA polymerase alpha (α) which is involved in replication, DNA polymerase epsilon (ε) which is involved in replication and repair and DNA polymerase delta (δ) which is involved in replication and repair. DNA polymerases alpha, epsilon and delta have proven to be essential for cell growth (12, 13, 14).

S. cerevisiae DNA polymerase epsilon was first reported in the early 1970's as a polymerase activity in mitochondria-free cell extracts that was chromatographically distinct from DNA polymerase alpha and associated with an exonuclease activity (13, 15, 16). DNA polymerase epsilon received far less attention than the other major replicative polymerases until studies showed that disruption of the *POL2* gene, encoding one component of the DNA polymerase epsilon complex, affected cell viability (14, 25, 26).

DNA polymerase epsilon is thought to play important roles in DNA synthesis, recombination and DNA repair. Analysis of temperature-sensitive mutants defective in *POL2* have shown that it is involved in elongation. It is also believed that DNA polymerase epsilon is vital for progression through S phase in budding and fission yeast (14, 18, 19). Previously characterized temperature-sensitive mutants of *S. cerevisiae POL2* have greatly elongated S phases and arrest at the restrictive temperature with a cell division cycle phenotype characteristic of DNA replication mutants (5, 14). DNA polymerase epsilon is highly accurate. For example, it has been shown to have a very low rate of substitutions that revert a termination codon in a *lacZ* gene (30). DNA polymerase epsilon is also believed to be involved with nucleotide excision repair (NER) and base excision repair (BER) (20). NER is the process by which DNA lesions are removed by excising an oligonucleotide containing the lesion. This leaves a single-stranded DNA gap of ~30 nucleotides and evidence suggests that DNA polymerase epsilon and delta are polymerases that fill this gap (20). In the BER pathway damaged bases are first removed by a DNA glycosylase generating an abasic site (20). In some cases a polymerase may perform limited displacement synthesis to incorporate 2-13 nucleotides generating a single-stranded DNA flap that is removed by the Fen1 flap endonuclease (20). This is referred to as long patch base excision repair. DNA polymerase epsilon, along with delta and beta, has been shown to perform DNA re-synthesis during this repair pathway (20). DNA polymerase epsilon has been isolated from a number of sources including *Schizosaccharomyces pombe*, the silkworm *Bombyx mori* and HeLa cells (31).

The purified DNA polymerase epsilon complex has several distinguishing properties. It co-purifies with a 3'→ 5' exonuclease activity, which is intrinsic to its catalytic subunit

(5). It has a preference for primer templates with long stretches of single strand templates (5). DNA polymerase epsilon also is comparatively resistant to BuPdGTP, a nucleotide analog (5).

All polymerases are put into a family based on amino acid sequence similarities. DNA polymerase epsilon is a class B polymerase (12, 13, 14, 20). Class B polymerases are characterized by a series of conserved regions that occur in the same linear order in all members (12-14). DNA polymerase epsilon's catalytic subunit, Pol2p, shares amino acid sequence similarity to metazoan alpha and epsilon polymerases and is sensitive to aphidicolin. Aphidicolin is an inhibitor derived from fungi with a steroid-like structure that inhibits replicative DNA synthesis specifically in eukaryotic cells. Figure 1 shows conserved regions shared with different yeast DNA polymerase regions. Catalytic subunits of yeast DNA polymerase alpha (1), epsilon (2), delta (3) and Rev3 proteins are schematized as horizontal lines with their N-terminus to the left and C-terminus to the right. Boxed regions represent conserved regions (13). Solid boxes I-VI represent conserved regions defined by Wary *et al.* and VII-VIII are 2 additional conserved regions (13). Open boxes labeled Exo I, Exo II and ExoIII represent conserved motifs containing 3' → 5" exonuclease site residues. DNA polymerase ε and DNA polymerase δ appear to be the only candidates capable of exonucleolytic editing during DNA replication (12, 13, 21-25). Gray boxes labeled Cys represent cysteine-rich regions (13). Region VII contains several basic residues and a tyrosine and is conserved in many Class B DNA polymerases. It is also characterized by the KKRY motif. Region VIII is characterized by the DXXYY motif and is not clearly conserved in Class B polymerases.

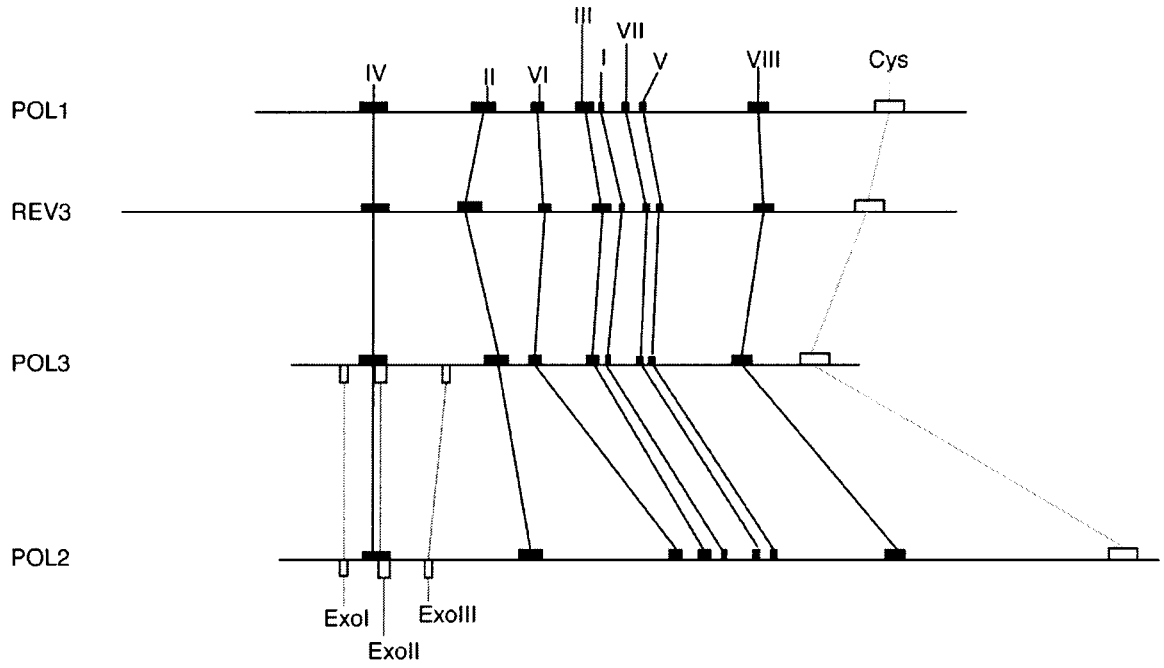


Figure 1 Conserved Regions of Pol1p, Pol2p, Pol3p and Rev3p

DNA polymerase epsilon is a multimeric holoenzyme consisting of Pol2p and at least three other subunits: Dpb2p, Dpb3p, and Dpb4p (12-14). It also requires proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) to catalyze processive DNA synthesis on singly primed single-stranded viral DNA (13). It also appears that the protein Dpb11p and a protein encoded by the *SLD2* gene are also involved with the pol epsilon complex (31). However, by itself it is a highly processive enzyme complex (13).

The *POL2* gene encodes the catalytic subunit, Pol2p, which is essential in yeast and is located on *S. cerevisiae* chromosome XIV (12, 15, 17). It has a mass of 256 kDa and displays sequence similarity to the class B DNA polymerases in its N-terminal half (see Figure 1) (12-14). Figure 2 shows the different domains encoded by the *POL2* gene. The N-terminal region of Pol2p contains proofreading 3'→5' exonuclease motifs called EXO

I, EXO II and EXO III and a catalytic domain. These sites are based on alignments with amino acid sequences within the *E. coli* DNA polymerase I Klenow fragment (13, 21). Studies have shown that mutations in the catalytic region block polymerase function. The C-terminal half of Pol2p includes a long region of amino acids that is conserved in other eukaryotic Pol2p proteins, but is not found in any other Class B polymerase (13). The polymerase domain of Pol2p is similar to conserved regions within other polymerases. The C-terminus also contains an "interacting" domain thought to be important for subunit associations and contains a cysteine-rich region that comprises the zinc-finger DNA–binding domain that is important for cell cycle checkpoint functions. (13, 21). Evidence suggests that a specific function of the C-terminal half is to hold dpb2p at the site of action of DNA polymerase II.

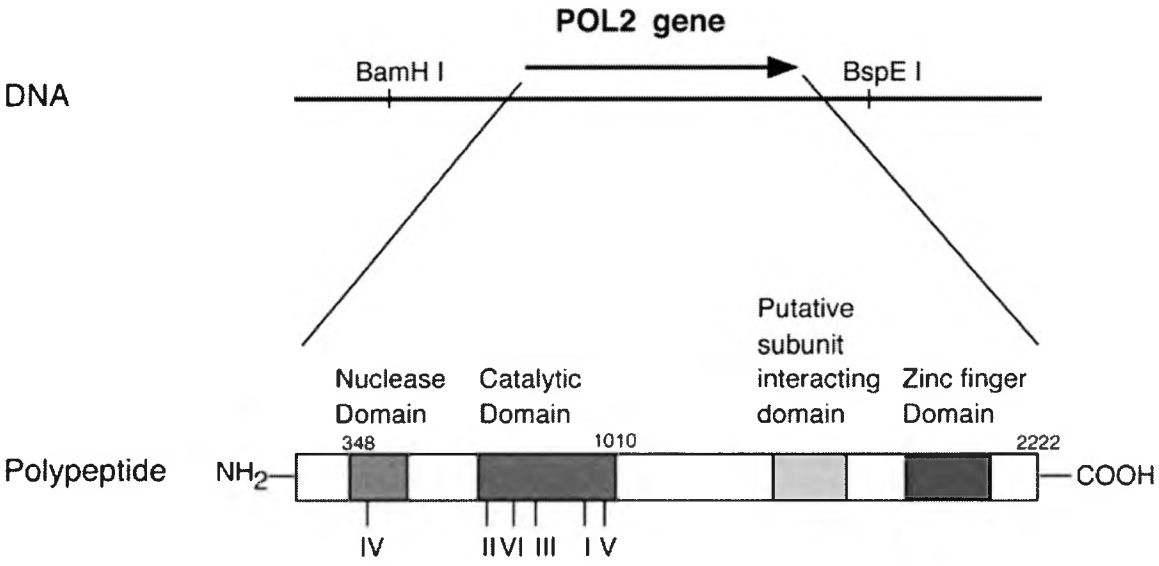


Figure 2 **Amino acid domains of *POL2***

The C-terminal half and the C-proximal unique region are not required for DNA polymerase II function either *in vivo* or *in vitro* (13, 21). Mutations conferring altered

sensitivity to nucleotide analogues were mapped to positions in and around polymerase domain regions II-V and the mutation of specific residues in regions I and II eliminated DNA polymerase activity (see Figure 2) (12, 13).

The second subunit of DNA polymerase epsilon is Dpb2p and is encoded by the *DPB2* gene (13,14). It is 80 kDa in size and is essential for yeast cell growth and required for chromosomal DNA replication (13,14). It copurifies with the Pol2p catalytic subunit and the two polypeptides appear to be maintained in a 1:1 stoichiometric ratio in purified preparations (13,14). The *POL2* and *DPB2* genes appear to share a common cell cycle regulation and their temperature-sensitive and/or disruption mutants exhibit the same phenotypic consequences of cessation of genomic DNA synthesis including arrest of the cell cycle at the dumbbell morphological stage (G_2 phase) and cell death (12, 13, 21, 23).

The *DPB3* gene encodes the third subunit. The products of this gene are two polypeptides with masses of 34 kDa and 31 kDa (13). Deletion of the *DPB3* gene has no appreciative effect on cell growth. Experiments with *dpb3* mutants have shown a slight increase in mutation rate (13).

The *DPB4* gene encodes the fourth subunit, Dpb4. Not much is known about this subunit. However, it is not essential for cell growth (13).

Many polymerases share a common catalytic structure and mechanism. This structure was first defined by studies of the Klenow fragment of *E. coli* DNA Polymerase I (8, 11). The Klenow fragment resembles a closed right hand with domains identified as "palm", "fingers" and "thumb" (8, 11). Figure 3 depicts the Klenow fragment as it slides along a strand of DNA. The picture on the left shows DNA elongation and the picture on the right shows how the strand is flipped to the exonuclease region for correction (8, 11).

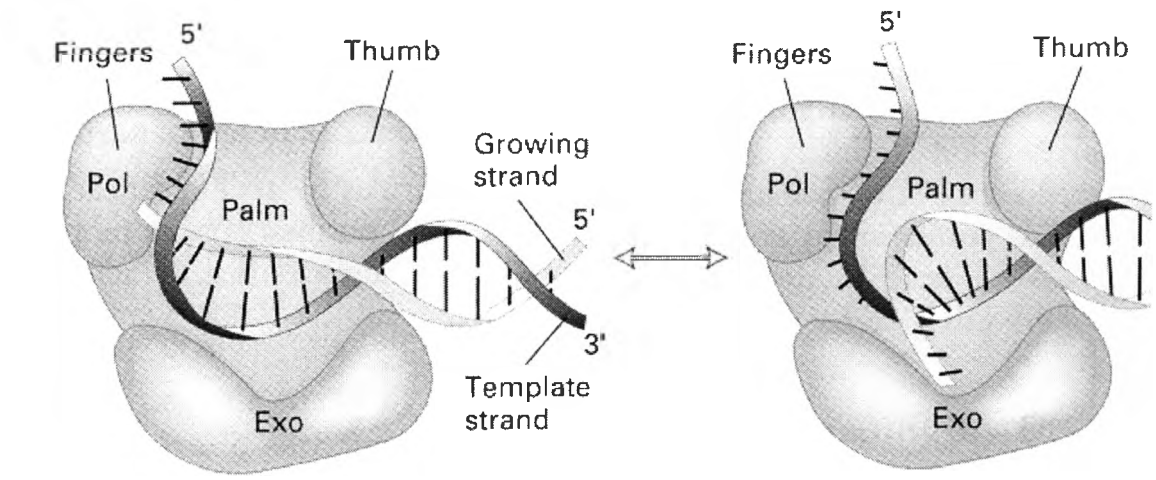


Figure 3 **Klenow fragment**

The polymerase active site lies in the palm domain and the 3' exonuclease site lies at the base of the palm (11). Based on the structure of the T7 phage Klenow fragment, Thomas Steitz proposed a general mechanism for polymerization (11). In this mechanism there are two metal ions, one metal ion polarizes the hydroxyl group at the 3' primer terminus causing subsequent nucleophilic attack of that moiety upon the α-phosphate of the dNTP substrate (11). Another metal ion is bound to conserved aspartate residues known to be essential for catalysis (11). Both metals stabilize a pentacovalent transition state, in which the α-phosphorous is linked to five oxygens, and the second metal facilitates the leaving of the pyrophosphate (11).

There has been increasing progress in the identification and characterization of proteins involved in DNA replication in yeast. Several of these proteins have proven to be homologous to replication proteins from other eukaryotes. Very few DNA polymerase epsilon structure-function studies have been done and there has been little characterization of temperature-sensitive mutants of DNA polymerase epsilon. This is

due in part to the extremely large size of the *POL2* gene, which is significantly larger than other polymerase genes. Furthermore, when cloned into *E. coli*, the product of *POL2* causes cell death. This makes it hard to isolate intact copies of the gene. The experiments outlined in this proposal were designed to address this paucity of information about the functions of Pol2p and the polymerase epsilon complex. Historically, the creation and characterization of temperature-sensitive mutants has proven to be a valuable tool for analyzing genes that are essential for cell growth. The major goal of this work was to generate, isolate and study new temperature-sensitive mutants of the *S. cerevisiae POL2* gene. The locations of the mutations in the *POL2* gene were determined and each mutant was further characterized for potential impacts on important aspects of DNA metabolism including mutation rates and resistance to DNA damage. Information obtained in these studies should advance our understanding of the roles of this conserved DNA polymerase in replication and repair in both yeast and higher eukaryotic cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 General Reagents

Ammonium sulfate (granular) and sodium chloride were purchased from Mallinckrodt AR (Paris, Kentucky). Agarose and ethidium bromide were purchased from Shelton Scientific, Incorporated (Shelton, CT). Hydroxyurea (HU) and 5-fluoroorotic acid (5-FOA) were obtained from US Biological (Swampscott, MA). Methyl methanesulfonate (MMS), lithium acetate dihydrate, calcium chloride, 99% glycerol and magnesium chloride were purchased from Sigma Chemical Company (St. Louis, MO). Tris base was purchased from Invitrogen Life Technologies (Carlsbad, CA). dNTP solution (100 mM) was purchased from Stratagene (La Jolla, CA).

2.1.2 Bacteriological and yeast media

All amino acids, plate agar, D-(+)-glucose, polyethylene glycol, glycerol, 5-azacytidine, L-canavanine, 2-aminopurine and ampicillin were purchased from Sigma Chemical Company (St. Louis, MO). Difco bacto peptone, bacto yeast extract, bacto tryptone and bacto yeast nitrogen base without amino acids and ammonium sulfate were from Becton Dickinson Microbiological Systems (Sparks, MD).

2.1.3 Yeast Strains and Plasmids

Plasmid p173 was constructed by subcloning the BamHI-BspEI fragment of *POL2* from YCpPol2 into the BamHI-AvaI sites of pFL34* (12). The BamH1-BspE1 fragment contains nt 2362 to nt 7903 of the *POL2* gene sequence on chromosome XIV. A strain was constructed from the parent strain CG379 (MATα *ade5-1 his7-2 leu2-3 112 trp1-289 ura3-52*) that contained *pol2-4*. The *pol2-4* allele has a point mutation in the exonuclease domain of the *POL2* gene resulting in the loss of 3'→5' proofreading exonuclease activity. The strain also has a modified *insE* insert in the chromosomal *LYS2* gene, where the wildtype A_4 run was changed to A_{12}. Thus, the strain is $Lys^{(-)}$, but can revert to $Lys^{(+)}$ by introduction of +1 slippage events (25).

2.1.4 Enzymes

Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Taq Plus Long was purchased from Stratagene (LaJolla, CA).

2.2 Culture solutions and media

E. coli cells were grown on LB medium (10 grams Bacto-tryptone, 5 grams bacto-yeast extract, 10 grams NaCl, 20 grams bacto agar per liter). In addition, 100 μg/ ml ampicillin was added for selection of plasmids.

Yeast cells were grown on YPAD medium (1% bacto yeast extract, 2% bacto peptone, 2% glucose, 0.004% adenine sulfate, 2% bacto agar). For assessment of mitochondrial function, yeast cells were grown on YPG medium (1% bacto yeast extract, 2% bacto peptone, 3%(v/v) glycerol, 2% bacto agar). Synthetic complete medium with drop-out mix contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.2% drop-out mix, 2% glucose, 2% bacto agar and 0.002%

adenine sulfate. Canavanine medium contained 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% -arginine drop-out mix, 2% glucose, 2% bacto agar 0.002% adenine sulfate, 60 mg/liter final concentration of canavanine. Hydroxyurea plates were prepared using YPAD medium + 1M HU solution to a final concentration of 200 mM. Methyl methanesulfonate plates were prepared by combining YPAD medium with MMS to a concentration of 2 mM. The 5- FOA medium was prepared by combining synthetic complete drop out media with 5-FOA to make a final concentration of 5.7 mM.

2.3.1 E. coli transformation

Competent DH5α *E. coli* strains were transformed with the plasmid p173 based on the method of Chung and Miller (1988) as follows: Approximately 2 μg DNA was added to 100 μl KCM solution and placed on ice. Competent DH5 α *E. coli* cells, 100 μl were added to DNA/KCM solution and gently mixed. The mixture was then placed on ice for 10-20 minutes, heat shocked at room temperature for 10 minutes, and 50-200 μl of transformation mixture was then spread on to LB + ampicillin plates to isolate colonies transformed with plasmid and grown overnight at 37° C. Single colonies were isolated and then streaked for single colonies again on LB + ampicillin plates and grown overnight at 37° C.

2.3.2 Yeast transformation

Yeast cells were transformed with DNA based on the High Efficiency Transformation Protocol by Agatep *et al* (26).

2.3.3 Random Mutagenesis with 5-Azacytidine

A single colony of *E. coli* transformed with p173 (Amp^r, *pol2*) was added to 4.5 ml of LB+Amp liquid and vortexed thoroughly. A 0.5 ml sample of culture was added to another 4.5 ml of LB + Amp liquid in a test-tube and placed in a 37°C incubator, shaking, overnight. Ninety five milliliters of LB-amp was added to a 500 ml Erlenmeyer flask and 5 ml of overnight culture was added and then placed in a 37°C shaking incubator for three hours. A 5-azacytidine solution was added to give a final concentration of 0, 0.1, 1 or 10 µg/ ml and the mixture was shaken at 37°C for four hours. Cells were harvested and washed twice with LB liquid. A sample of each concentration was used to perform a cell viability count and the rest was frozen. LB-amp plates were spread with a 10^{-1} dilution of the 0, 0.1, 1 and 10 µg/ ml and grown overnight at 37°C. The concentration yielding a moderate to high amount of growth relative to unmutagenized cells was used to isolate mutagenized p173 (27).

2.3.4 Random Mutagenesis with 2-Aminopurine

A single colony of *E. coli* transformed with p173 was added to 10 ml of LB + Amp and shaken at 37°C. A 0.5 µl sample of the culture was added to 500 µl of sterile water. Sterile LB broth was mixed with 2-aminopurine to bring to a final concentration of 5.2 mM 2-aminopurine. Six milliliters of LB/2-aminopurine broth was inoculated with 100 µl of diluted culture in a sterile test-tube and placed in a 37°C incubator shaking for 48 hours. Cells were harvested, spun down and frozen (28).

2.3.5 Plasmid DNA Isolation

Plasmid DNA was isolated from mutagen-exposed *E. coli* cells based on the methods of Sambrook, *et al.* (1989) using the HiSpeed Plasmid Purification Kit (Qiagen Co)

according to the manufacturer's instructions. Plasmid DNA concentration was then quantitated using a Perkin-Elmer flourospectrophotometer (Wellesley, MA) and Picogreen dsDNA Quanitation Reagent from Molecular Probes, Inc., (Eugene, OR).

2.3.6 Restriction Enzyme Digest

p173 was cut using the restriction enzyme AgeI as follows. In a 1.5 ml microcentrifuge tube the following were added in order: 1µl of AgeI 10x buffer, 7.5_l sterile water, 1µl (0.391 µg) DNA in 1x TE and 0.5 µl of AgeI enzyme. The mixture was microcentrifuged to remove air bubbles for 3 seconds at 2000 r.p.m. and incubated at 37°C for one hour. A sample was removed and run on 0.7% agarose gel in 0.5X TAE solution. The remaining was frozen until used in transformation reactions.

2.3.7 Polymerase Chain Reaction

The following were added to a 200 µl microcentrifuge tube to amplify a fragment of the p173 plasmid. Thirty six microliters of sterile water, 1 µl of wildtype p173 plasmid, 5 µl of forward primer, 5 µl reverse primer, 5 µl of 10x Taq Plus Long High Salt Buffer, 5 µl dNTP's 100 mM each and 1µl Taq Plus Long. Two microliters of each polymerase chain reaction sample was then fractionated on a 0.7% agarose gel in 1x TAE buffer using an Owl Separation Systems Easycast Horizontal Electrophoretic Apparatus (Portsmouth, NH). The gel was then soaked in ethidium bromide solution and observed using a Kodak fluorescent detector. Primer sequence and PCR parameters are defined in Table 1 and Table 2.

Table 1. *POL2* PCR Primers

Forward		Reverse	
Primer	Sequence	Primer	Sequence
+5947	5'-GAATTCAGGGACCAAAGACCTACCC-3'	-1000	5'-TTTCGGTGTATACTCAAAGTC-3'
+2948	5'-GGCTACTTGCTGAACTGAAAGG-3'		

Table 2. PCR Amplification Parameters

Primer Pair	Template	Denaturation 94°C	Annealing	Extension 72°C	Cycle #
-1000, +5947	p173 plasmid	20 sec	44°C - 30 sec	4 min 30 sec	35
-1000, +2948	p173 plasmid	15 sec	46°C - 25 sec	3 min 30 sec	35

2.3.8 Cell Cycle Arrest Assay

Four milliliters of YPAD liquid was inoculated with yeast cells and shaken overnight at 30°C. Two test-tubes each with 5 ml of fresh YPAD liquid were inoculated with 0.25 ml of overnight culture and shaken at 30°C for one hour. A 0.5 ml sample was removed from each culture, diluted, sonicated and 12 µl was loaded into a hemocytometer to count the number of unbudded, small-budded and large-budded cells. One tube was placed back at 30°C shaking and one tube was placed at 42°C shaking. After two hours another

sample was removed, counted and placed back in the incubators. Counting was repeated twice more at 2-hr intervals for a total of eight hours.

2.3.9 Cell Viability Assay

Five milliliters of YPAD was inoculated with yeast cells and shaken overnight at 30°C. Two tubes each with 5 ml of fresh YPAD were inoculated with 250 µl of the overnight culture per sample and shaken at 30°C for 1-2 hours. A 200 µl sample was removed from each, added to 400 µl of water, sonicated and the number of total cells per ml was counted with a hemocytometer. Another sample was removed from each test tube diluted and spread onto YPAD plates. The test tubes were shaken at 42°C for four hours. At this time cells were sonicated and the number of total cells counted again. Cells were diluted in water and plated. The tubes were shaken for another 4 hours and a sample of each wildtype strain was diluted 1/10 and the mutants were diluted 1/4. Cells were sonicated and the number of total cells per ml was counted as before. Another aliquot of the cells was diluted and plated. The tubes were again shaken at 42°C for 16 more hours for a total of 24 hours at 42°C. A sample of the wildtype and mutants was removed and diluted with water, sonicated and counted. Another aliquot was diluted with water and spread onto YPAD plates.

2.3.10 Dilution Pronging Survival Assay

In a sterile 96-well microtiter plate, six wells with 220 µl of YPAD liquid per well were inoculated with cells and incubated overnight at 30°C. One hundred sixty microliters of sterile water was added to the next five wells for each strain. Forty microliters of the overnight culture was added to the following well and 40 µl was added to each following well from the previous dilution. The samples were then pronged onto

plates containing either 2 mM MMS, 200 mM HU or no additions and incubated at 30°C. For temperature-sensitive experiments, YPAD plates were incubated at 30°C, 35°C, 37°C and 42°C for 2-3 days. Pictures of plates were obtained using a Canon Powershot G3 digital camera and images saved as high .JPG files.

CHAPTER 3

RESULTS AND DISCUSSION

Mutagenesis with 5-azacytidine and 2-aminopurine

The goal of this work was to generate and characterize new mutant alleles of *POL2* that are temperature-sensitive for growth and defective in DNA repair and mutagenesis. To generate yeast strains containing a mutated *POL2* gene, *E. coli* cells were first transformed with the plasmid p173. p173 contains all of *POL2* except the exonuclease (proofreading) domains encoded at the 5' end. It contains an AgeI restriction enzyme site that cleaves in the 5' portion of the *POL2* gene (12, 24, 25). This construct was chosen for the mutagenesis in order to ensure that new mutations would be outside the proofreading domain. p173 also has Amp^r and *URA3* genes that serve as selectable markers to identify colonies that have picked up the plasmid and the *POL2* gene. Figure 4 below shows the structure of p173 (12, 24, 25).

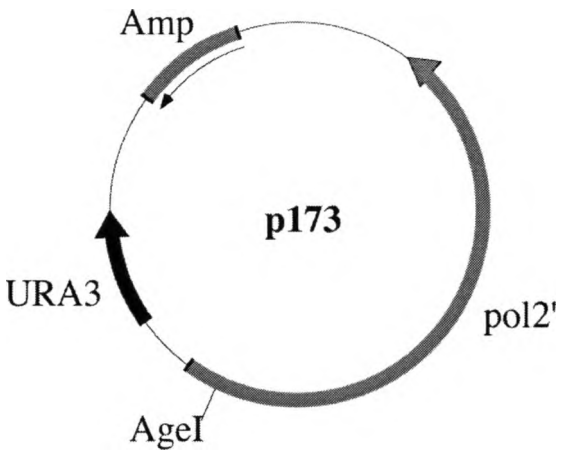


Figure 4 Structure of p173

Colonies were grown in media containing either 5-azacytidine or 2-aminopurine. The chemical 5-azacytidine is a nucleoside analog that inhibits cytosine methylation as a result of the formation of a stable covalent complex with DNA methyltransferases (24). The chemical 2-aminopurine is a highly mutagenic base analog that is known to cause increased frequency of G:C > A:T and A:T > G:C transitions by preferentially base-pairing with thymine (9). Plasmid DNA was extracted from *E. coli* cells using a Qiagen Kit and analyzed by gel electrophoresis. Figure 5 is a picture of the isolated plasmid compared to a standardized 1kb ladder run on a 0.7% agarose gel. The gel shows the plasmid in its circular form in lane 2 and linearized (AgeI-cut) form in lanes 3-5.

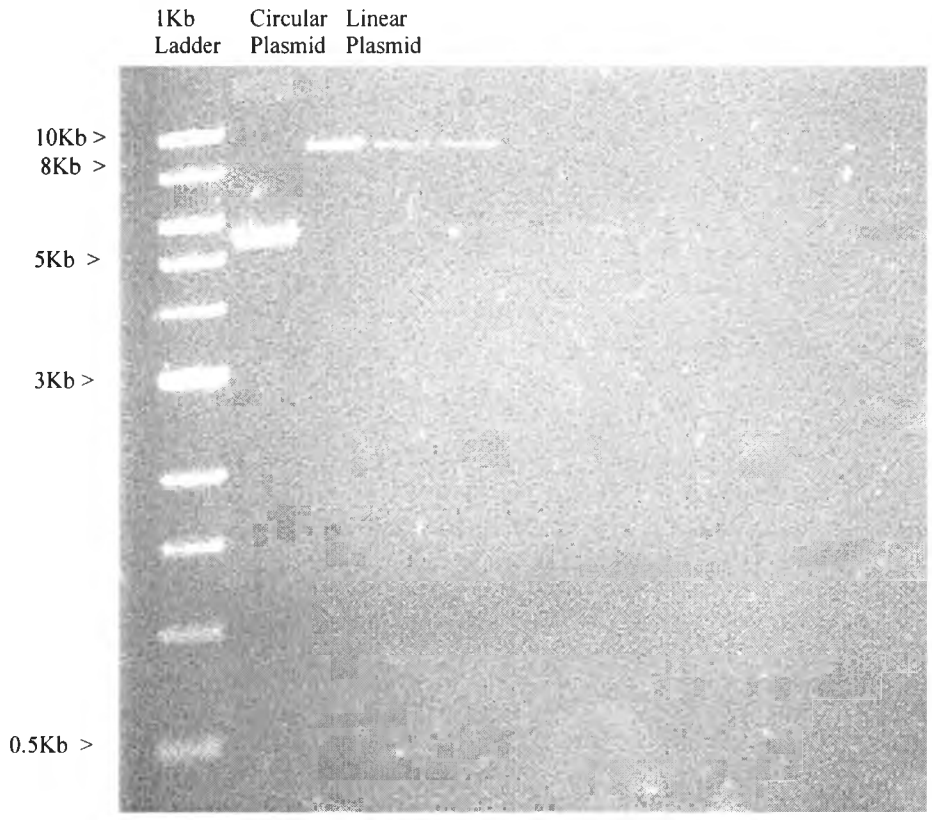


Figure 5 p173 plasmid

Mutated and wildtype p173 plasmids were transformed into the yeast strain S1-12A *pol2-4* (exo$^-$) (12). This strain has a substitution within the exonuclease domain (*pol2-4),* that causes a spontaneous mutation rate increase of 10-20 fold (12). This *pol2-4*-containing host strain was chosen because it increased the potential for identifying new mutator alleles of *POL2*. The effects of multiple mutations within a gene are often synergistic, providing a more easily scorable phenotype than either mutation alone. p173 was cut and linearized with the restriction enzyme AgeI in order to facilitate integration into the yeast genome by homologous recombination. The p173 plasmid lacks an ARS element and cannot replicate on its own, so all *URA3* transformants arise by integration

of the plasmid. Figure 6 illustrates the integration scheme of p173 into its complementary region.

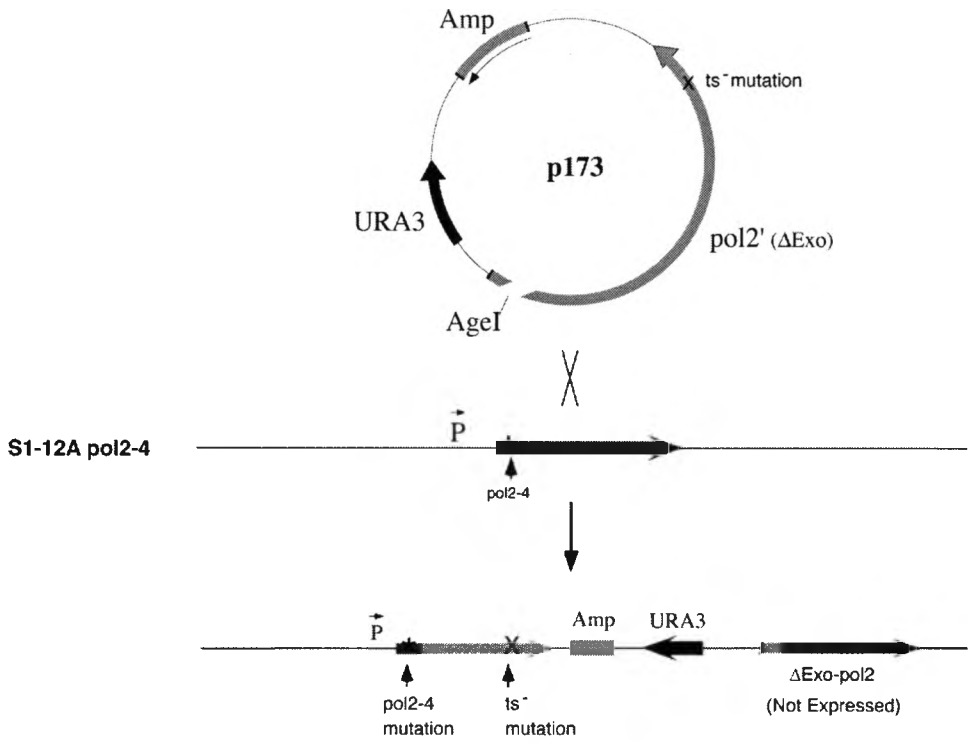


Figure 6 **Integration of p173 into POL2 gene**

To isolate colonies that have taken up the plasmid, yeast cells were plated onto synthetic-complete drop-out media lacking the nucleotide base uracil and grown at 30°C, the normal growth temperature for yeast cells. A total of 1,787 colonies with integrated mutagen-treated plasmids were isolated. Recombination is very efficient in yeast cells and most of these transformant cells have integrated the mutagenized *POL2* plasmid into the *pol2*-4 locus on chromosome XIV. The integration event produces a duplication (see Figure 6). The upstream allele contains the 5' *pol2*-4 mutation plus additional mutation(s) present in the original mutagenized plasmid. Only this upstream allele is fused to the

POL2 gene promoter and is expressed inside the cells. As a control, we transformed yeast cells with non-mutagenized p173 and analyzed 120 colonies. Each of the colonies exhibited normal *POL2* function and was not temperature-sensitive.

Isolation of temperature-sensitive mutants

Colonies with mutagenized plasmids were replica-plated onto media lacking uracil and grown at 42°C to identify strains that were temperature-sensitive for growth. Colonies were also tested on 3% YPG plates to remove mutants with impaired mitochondrial function (petite strains). Mutants with impaired mitochondria cannot utilize glycerol as a carbon source and will not grow on YPG plates.

The chemical 5-FOA was used to isolate colonies containing only the *POL2* gene with the new *pol2* mutation. The gene products of *URA3* and *URA5* catalyze the conversion of 5-FOA to 5-fluoro-uridine monophosphate (5-FUMP). Following this conversion, flourodeoxyuridine is formed which is a potent inhibitor of thymidylate synthetase and therefore quite toxic to cells (1). Only strains lacking *URA3* gene function can survive on media containing 5-FOA. This assay was performed because closely spaced direct repeat sequences such as the duplicated *POL2* alleles in most transformant strains recombine with each other at a rate of about one in ten thousand (10^{-4}). In such recombination events, one of the duplicated alleles and the intervening DNA between the repeats is lost. Thus, these cells form Ura^- ($5\text{-}FOA^r$) cells at high frequency and almost all of them have retained only one of the *pol2* alleles. Either the upstream or downstream allele is lost and colonies formed on 5-FOA plates retain either one allele or the other. A total of 24 strains

were isolated that were 5-FOA^r (without the duplicated *pol2* alleles), YPG$^+$ and 42°C ts$^-$. Figure 7 shows five of these mutants and their ts$^-$ phenotype.

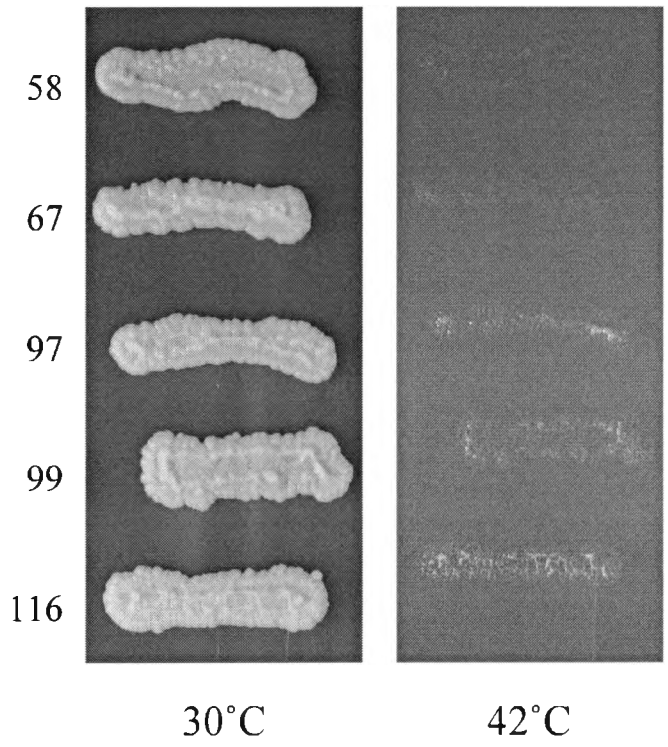
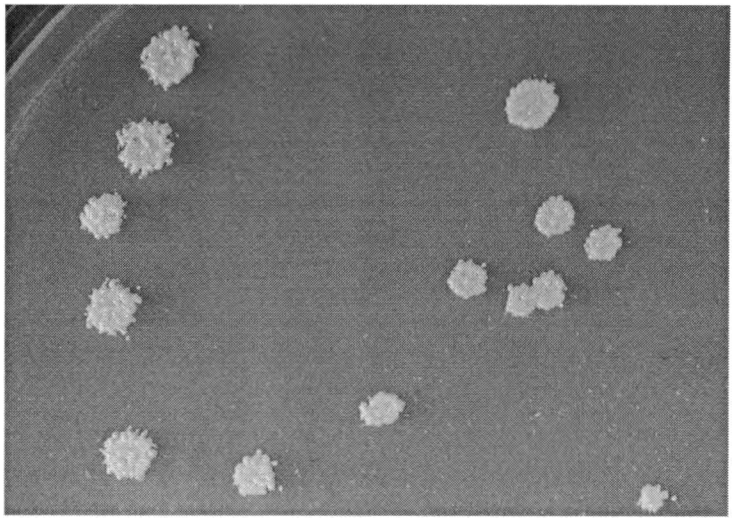


Figure 7 Temperature-sensitive mutants

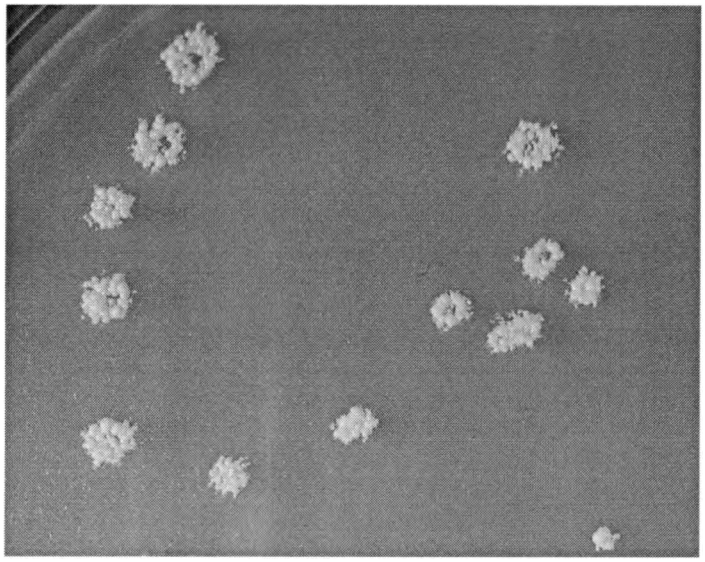
Identification of transformant strains containing a mutation in the POL2 gene

The collection of new ts$^-$ mutants includes strains that are ts$^-$ because of mutations introduced into the *POL2* gene and other strains that happen to have mutations in another yeast gene that produce a 42°C ts$^-$ phenotype. In order to identify the subset of mutants with a ts$^-$ mutation specifically in the *POL2* gene, a complementation assay was done. The unmutated p173 plasmid containing all of *POL2* except the exonuclease domains

was cut with AgeI and transformed into each mutant. Transformants were spread onto -uracil plates and grown at 30°C. Plates were then replica-plated to 42°C. Ts^- mutants that had reverted to 42°C ts^+ were isolated which was an indication that wildtype *POL2* gene sequence had been integrated into the chromosomal *pol2* ts^- allele. Since the ts^- phenotype in these mutants was reverted to ts^+, the mutation generating the ts^- phenotype must have been in the *POL2* gene. Figures 8a and b depict the results for mutant 67 with the integrated wildtype p173 plasmid incubated at normal growth temperature and at 42°C.



a. 67 With p173 wt^{+} 30°C



b. 67 With p173 wt^{+} 42°C

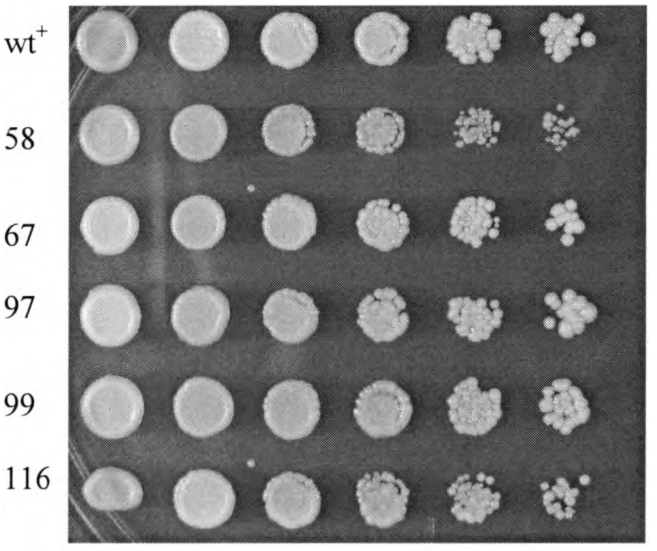
Figure 8a and b Mutant strain with wildtype p173

Mutant strains that did not become 42°C resistant were removed from the mutant collection because that meant there was a mutation in another gene. A total of 5 final *pol2* mutants were isolated. Four had been exposed to aminopurine and one had been exposed to azacytidine. The 5 isolates were designated 58, 67, 97, 99 and 116.

For further confirmation that the mutation occured in the *POL2* gene, the five mutants containing integrated *POL2* plasmids were spread onto 5-FOA plates to get Ura$^-$ colonies. As before, Ura$^-$ cells should form by recombination between the duplicated *POL2* alleles upstream and downstream to delete the intervening *URA3* gene. In a fraction of cells all that will remain is the allele containing the 42°C ts$^-$ mutation. Thus a true *pol2* ts$^-$ mutant will produce a mixture of 42°C ts$^-$ colonies and 42°C resistant colonies. Each of the 5 *pol2* mutants produced such mixtures (data not shown) confirming their genotypes.

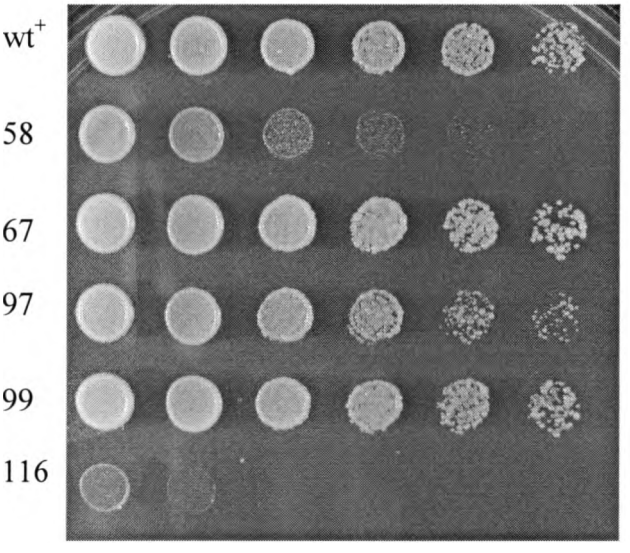
Semi-permissive and non-permissive temperature assay

Figure 9 (a-d) shows results of 5-fold dilution pronging of mutant cells onto plates that were incubated at different temperatures. Wildtype cells showed no growth impairment at the permissive temperature of 30°C and all other temperatures. Mutant 58 showed no difference at 30°C but did show growth inhibition at 35°C, 37°C and 42°C (Figure 9b, c and d) when compared to wildtype. Mutant 67 exhibited no difference from wildtype cells at 30°C, 35°C or 37°C, but showed an increase in growth inhibition at 42°C (Figure 12b, c and d). Mutant 97 exhibited no difference from wildtype at 35°C but showed growth inhibition at 37°C and 42°C (Figure 9b, c and d). Growth of mutant 99 was not affected at 30°C or 35°C but was inhibited at 37°C and 42°. Mutant 116 exhibited strong growth inhibition at 35°C, 37°C and 42°C.



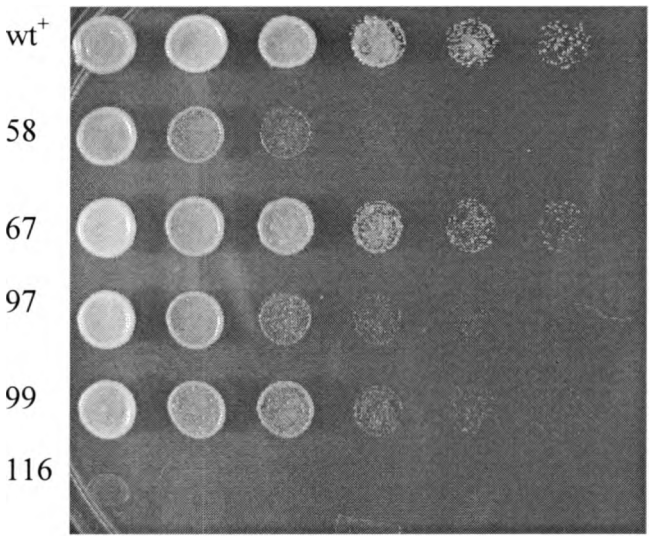
30°C

a



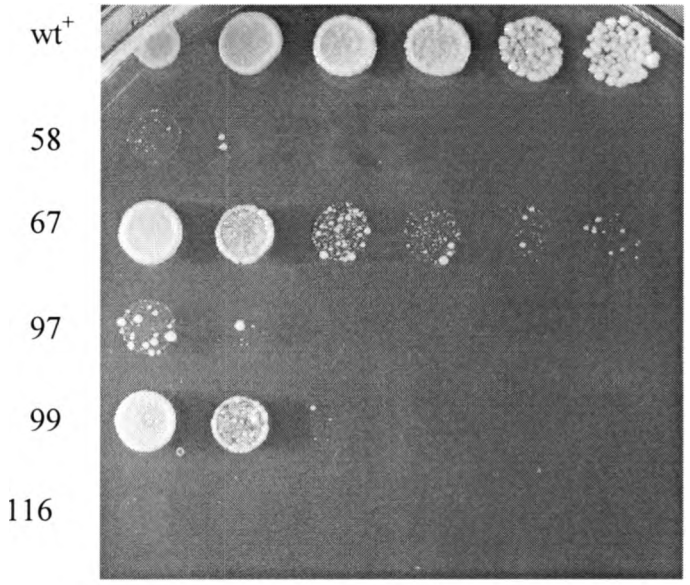
35°C

b



37°C

c



42°C

d

Figure 9a-d **Temperature sensitivity assay**

Cell viability Assay

The specific replication defect of each mutant might produce either rapid or slow loss of cell viability after transfer to the restrictive temperature. Figure 10 shows the results of cell viability assays for wildtype cells and the 5 mutant strains. Each mutant was grown at the non-permissive temperature and checked at 4 hour intervals for up to 24 hours. At 8 hours loss of viability became apparent in mutants 58, 97 and 116, but mutants 67 and 99 could still be rescued efficiently after transfer to plates and incubation at 30˚C. After 24 hours survival was reduced 3-4 orders of magnitude in mutants 58, 67, 97 and 116. Mutant 99 was distinct from the others as it showed only approximately 30-fold loss of viability after 24 hours at 42˚C.

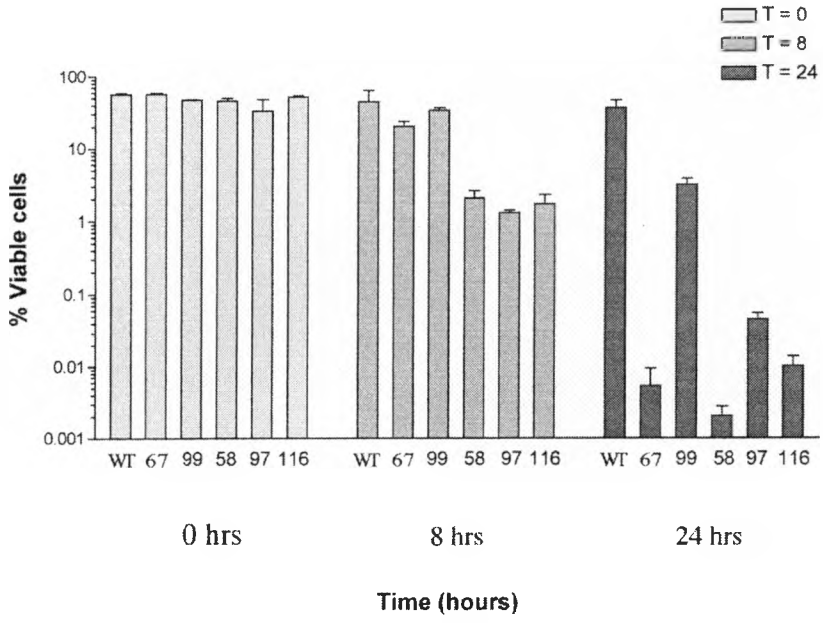


Figure 10 Cell Viability Assay

Impact of pol2 mutations on cell cycling

As described earlier, inhibition of DNA replication elongation leads to a lengthening of the S phase of the cell cycle. Past studies of temperature-sensitive alleles of genes encoding replication fork complex proteins found that these mutations allow the initiation of the anaphase stage of cell division before DNA replication has been completed at normal temperatures (3). Thus, mutant cells attempt to segregate incompletely replicated genomes, which leads to DNA fragmentation, unequal segregation and cell death. These checkpoint-defective alleles included mutations in *POL2* and *DPB11* (3). Mutations in replication fork complex proteins that cause checkpoint defects reinforce the hypothesis that the replication fork is the origin of the checkpoint signal and that one or more of these components transmit the signal to a downstream checkpoint protein (3).

Figure 11 is a picture of the yeast cells as seen through a visible light phase contrast microscope. Each cell can be classified visually as unbudded, small-budded and large-budded.

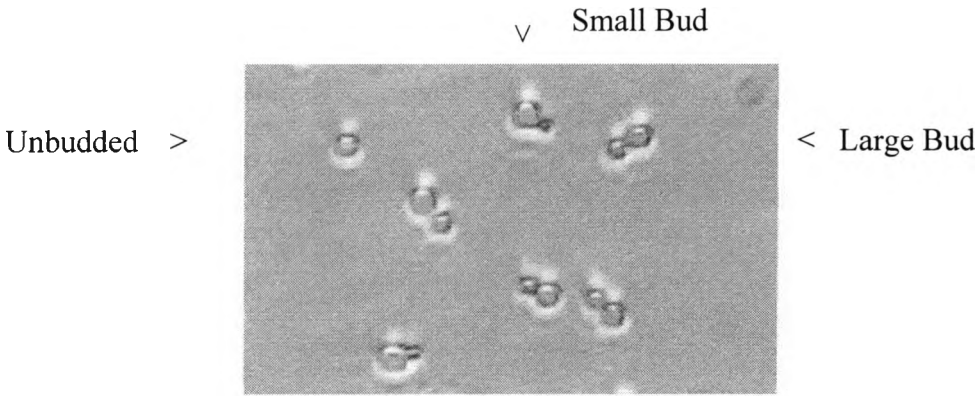


Figure 11 **Individual yeast cells**

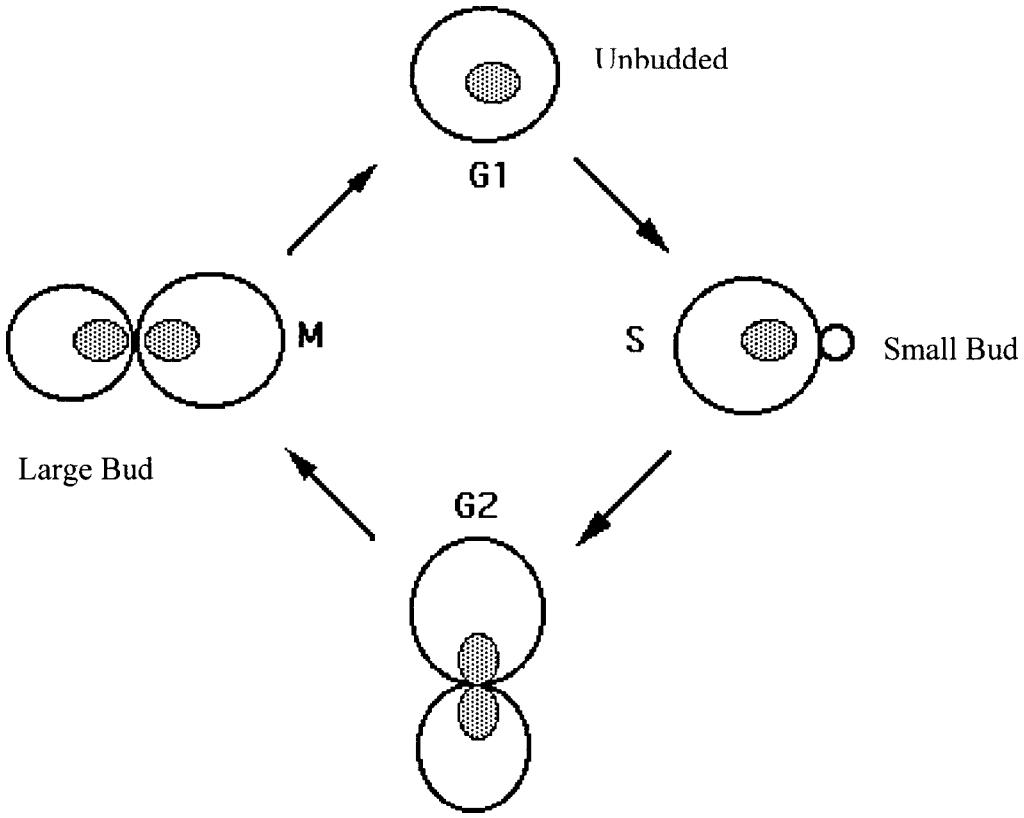


Figure 12 Yeast cell morphology and cell cycle

Figure 12 shows the morphology of yeast cells at each phase of the cell cycle. Cells in the G_1 phase, under a light microscope, give the appearence of having no buds. Cells undergoing S phase appear to have a tiny bud growing from the mother cell. The G_2 and M phases are classified as a single group because under a light microscope cells in either stage appear to have a large bud protruding. However, most of these cells are in G_2 phase. Special equipment and dyes are required to distinguish between M and G_2 phases by looking at the structure and localization of the nucleus. Normal yeast cells show a distribution of about 1/3 in G_1, 1/3 in S and 1/3 in G_2/M during log phase growth. Cells

experiencing DNA damage pause in the cycle with 60-90% arresting as G_2/M cells. This arrest serves as a protective function as it allows the cells time to repair damage before resuming cycling. Cells with impaired replication machinery that slows elongation show an extended S phase (when DNA synthesis takes place). Such cell populations contain an elevated proportion (50-70%) of S phase cells because the replication machinery is stalled during replication. Cell cycle phase distributions were analyzed in each of the new mutant strains. Table 3 shows the percentage distribution of cells in each cell cycle phase.

None of the mutants in this assay displayed an excess number of S phase cells at 30°C or 42°C. This suggests that elongation has not been impaired in these mutants. Wildtype cell cultures showed typical phase distributions at both 30°C and 42°C. Mutants 58, 97, and 116 exhibited elevated levels of G_2/M cells at 30°C, but not at the restrictive temperature of 42°C. These distributions suggest elevated levels of damage at normal temperatures (producing G2 arrest), but that cell dying at 42°C occurs at all stages of the cycle. This suggests that elongation has not been affected. Mutant 67 shows a high number of cells in G_2/M phase (up to 59%) at the restrictive temperature suggesting that cells are being stalled at G_2/M. Mutant 99 maintains a significant percentage of cells in G_2/M at both 30°C and 42°C. This could be due to the cells accumulating chromosomal damage and being stalled at G_2/M at all temperatures.

Table 3. Distribution of G_1, S and G_2/M cells (%)

	30C (log)			42C (2hrs)			42C (4hrs)		
Strain	G1	S	G2/M	G1	S	G2/M	G1	S	G2/M
Wildtype	37	32	31	52	20	28	44	21	36
58	23	24	**53**	57	22	**20**	38	27	**35**
97	23	32	**46**	47	19	**34**	49	28	**24**
116	14	28	**61**	39	25	**37**	42	26	**34**
67	20	43	**37**	27	14	**59**	39	11	**51**
99	14	23	**63**	36	4	**61**	38	8	**54**

Sensitivity to 200 mM hydroxyurea

HU is a toxic compound that stops replication progression by depleting cellular deoxynucleotide triphosphate (dNTP) pools. As these pools decrease, DNA polymerase begins to randomly insert nucleotides or to leave nucleotides out leading to nicks and stalled replication forks. Inability to detect and correct such damage can lead to cell death (11). The first checkpoint-deficient allele of *POL2* was identified in a screen for mutants defective in a transcriptional response observed when HU was used as an inhibitor (21). Wildtype and mutant yeast cells were serially diluted 5-fold and pronged onto plates with and without 200 mM HU. Figure 13 shows the results of the pronging test. Wildtype cells showed no sensitivity to HU at this concentration. Mutant 116 showed sensitivity with strong growth inhibition at a greater than 125 fold killing compared to wildtype. Mutants 58 and 67 showed greatly decreased growth rates and killing with a 125 fold increase in

killing. Mutants 97 and 99 were resistant to HU, though 99 exhibited a slow growth phenotype.

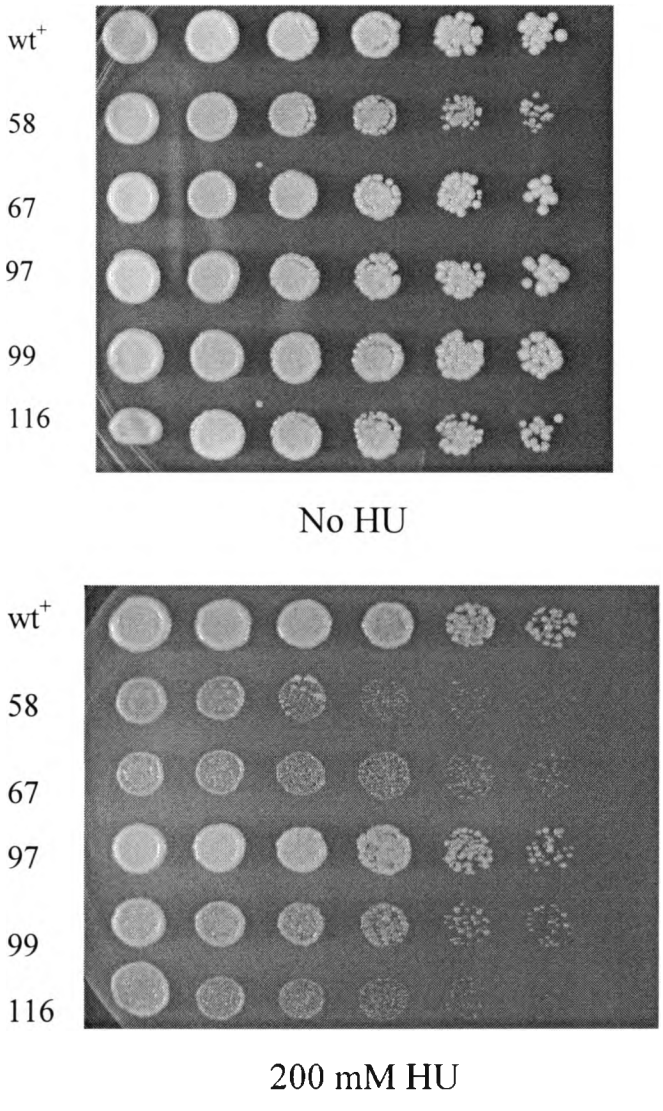
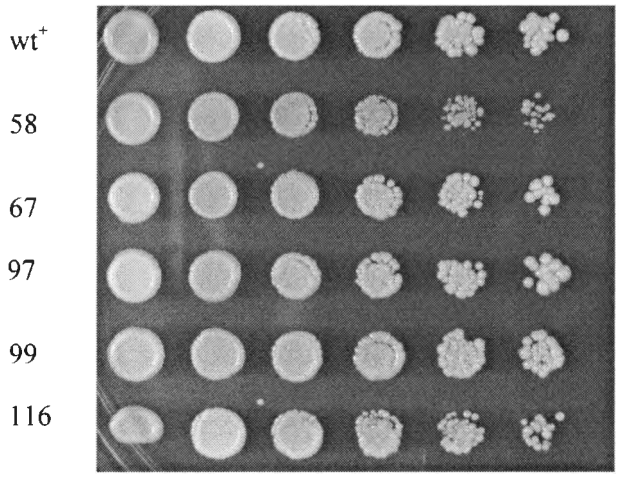


Figure 13 HU sensitivity assay

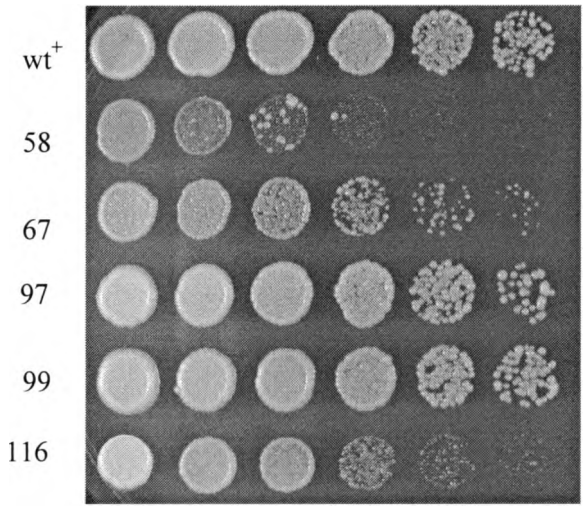
Sensitivity to 2 mM Methyl Methanesulfonate (MMS)

Methyl methanesulfonate is an SN2-type alkylating agent which predominately methylates nitrogen atoms in purines, primarily producing ^{7}meG and ^{3}meA, leading to the development of lesions in DNA (29). The compound is both highly mutagenic and toxic.

Excision of lesions leads to the formation of apurinic sites and A:T > T:A or G:C > T:A transversions as well as many single and double-strand breaks (29). Cells lacking proofreading and mismatch repair abilities are modestly sensitive to MMS and cells lacking DNA strand break repair gene functions are extremely sensitive to MMS (29). Previously characterized *pol2* mutations affecting the inter-zinc finger amino acids or the zinc fingers themselves are sensitive to MMS and have reduced ability to induce *RNR3* showing that the mutants are defective in the transcriptional response to DNA damage as well as the cell cycle response (21). Wildtype and mutant cells were serially diluted and pronged 5-fold onto plates containing 2 mM MMS. Figure 14 shows the results of the pronging test. Wildtype cells showed no sensitivity to 2 mM MMS. Mutant 58 showed strong sensitivity to MMS with a 3,125 fold increase in killing. Mutants 67, 97 and 99 were essentially as resistant as wildtype cells. Mutant 116 showed strong growth inhibition and moderate killing at 25-125 fold.



No MMS



2 mM MMS

Figure 14 MMS sensitivity assay

Pol2p Mutator Assay

Other than its proofreading ability, which is retained in the 5' exonuclease domain of the gene, little is known about the other regions of Pol2p that contribute to replication fidelity. The ability of each mutant to increase the mutation rate in the long A_{12} homonucleotide run of the *lys2::InsE-A12* allele was also investigated. This allele contains an insertion in *LYS2* and cannot grow on media not containing the amino acid lysine. The insertion results in a homonucleotide run of 12 A's in *LYS2*. A simple +1 frameshift mutation restores the normal *LYS2* open reading frame and cells become *LYS2*$^+$. Long homonucleotide runs are highly sensitive to minimal changes in replication fidelity and replication slippage that affect the appearence of mutations. The *lys2::InsE-A12* allele has been used previously in the study of various DNA metabolic mutants for their ability to influence +1 frameshift mutations. The strain used to study the *pol2*

mutants contains a mutation (*pol2-4*) within the Exo domain that causes a modest 10-20 fold increase in mutations in *LYS2*. This genetically sensitized background can increase the ability of detecting mutants as new mutations are combined with it.

Rectangular patches of cells were grown on normal YPAD plates and replica-plated to media without lysine. Mutants 58, 67, 97 and 99 produced Lys^+ paps at about the same frequency as wildtype cells and do not appear to be mutators. Only 116 appeared to be a *lys2* > $LYS2^+$ mutator. Note the large number of Lys^+ paps formed in Figure 15 (bottom image).

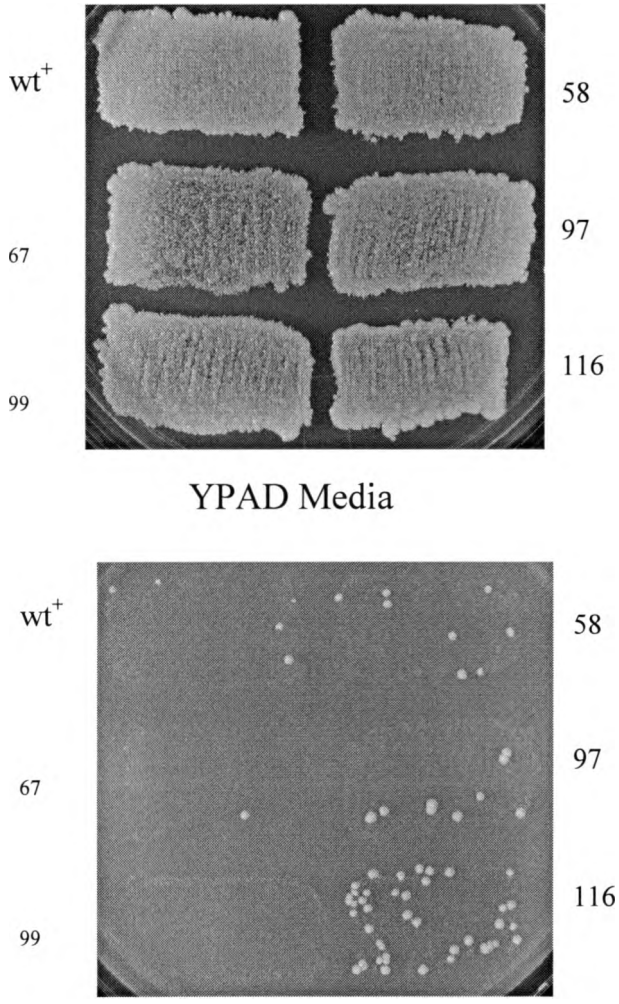
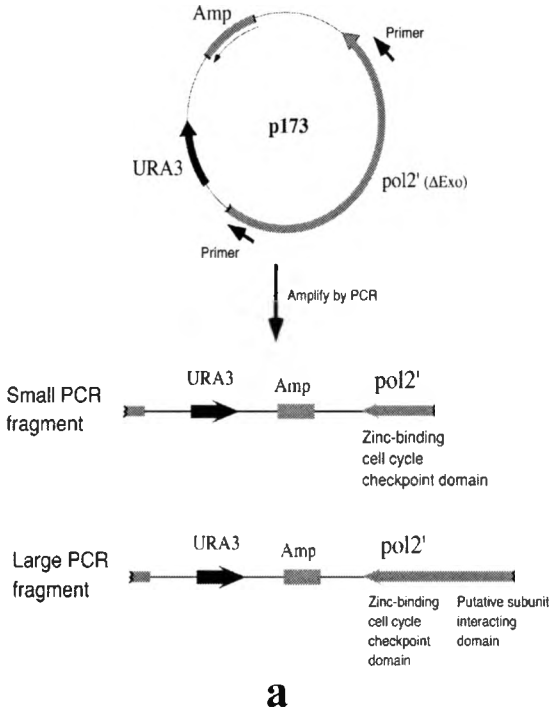


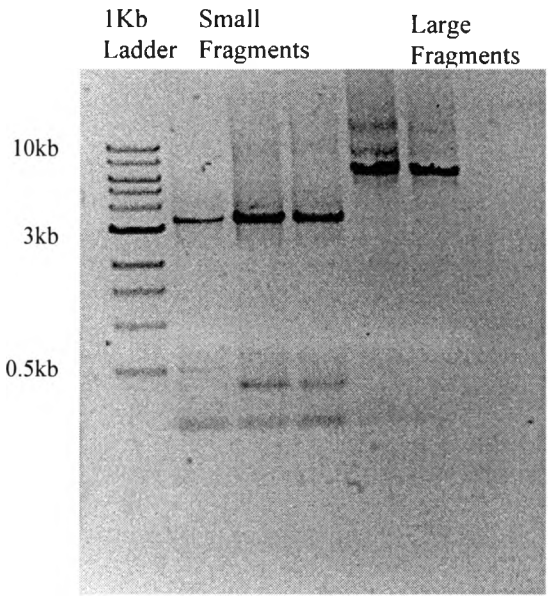
Figure 15 Mutator Assay

Determination of the locations of the mutations

To determine the approximate intragenic positions of the mutations, primers were designed to produce PCR fragments containing two different portions of *POL2*, each generated by amplification of sequences in p173. Figure 16a contains a diagram showing the extent of the gene in each fragment. Figure 16b shows agarose gel analysis of each fragment. The smaller fragment was approximately 3.3 kb long and contained the region encoding the zinc finger domain. The larger fragment was approximately 7 kb long and contained the region encoding the zinc finger as well as the putative subunit interacting domain of Pol2p.



a



b

Figure 16 **PCR Fragments**

After transformation of the two PCR fragments separately into each mutant strain, cells were spread onto -Ura plates. Patched colonies were replica-plated onto -Ura plates at 42°C. Isolates 58 and 116 reverted to ts^+ when transformed with the smaller fragment. Thus, when the zinc-binding coding region of these two ts^- mutants was replaced with normal sequences from the wildtype gene, the temperature-sensitivity disappeared. This indicates that the ts^- mutations for mutants 58 and 116 are in the 3' end region of the gene. Isolates 67, 97 and 99 could all grow at 42°C when transformed with the larger fragment, but not with the smaller fragment. Thus, when the putative subunit interacting domain was replaced with normal sequences temperature-sensitivity disappeared. Table 4 shows the results obtained from analysis of several patched transformants for each mutant.

Table 4. Location of Mutation

Mutant	Fragment Size	Number of patches tested	Number of patches able to grow at 42°C
97	Small	7	0
97	Large	14	14
99	Small	8	0
99	Large	15	15
67	Small	8	0
67	Large	58	54
116	Small	8	7
116	Large	11	9
58	Small	16	16
58	Large	21	21

Summary and Conclusions

Table 5 represents a summary of the results from several of the assays used to characterize all 5 mutants. Mutants 58 and 116 appear to have several common phenotypes, although mutant 58 was mutagenized with 5-azacytidine and 116 with 2-aminopurine. They both have sensitivity to MMS which is common with mutations in the zinc-finger binding domain. Their minimum restrictive temperatures and cell cycling phenotypes were also identical. Mutants 97 and 99 also share several phenotypes. They have sensitivity to neither MMS nor HU, their minimum restriction temperatures are similar and each mutation mapped to the subunit-interacting domain (Table 5). Mutant 67 is distinct due to its weak sensitivity to HU, minimum restrictive temperature of 42°C and resemblance to wildtype cells regarding lack of sensitivity to MMS.

Mutant 99 was the only mutant to exhibit the classical elongation-defective phenotype of an increased G_2/M phase (dumb-bell shaped) cell morphology at restrictive temperatures. This result might be due to the fact that none of the mutations were mapped to the polymerization domain of the enzyme, which is responsible for nucleotide addition during elongation.

Mutants 67, 97 and 99 are unusual in that they contain mutations in the Dpb-subunit interacting domain. Results obtained with these mutants suggest that at restrictive temperatures, disruptions in the interaction between Pol2p and one or more of its subunits (Dpb2p, Dpb3p and Dpb4p) is lethal to the cell. There are few mutations in this region that affect DNA replication and repair and cell cycling that have been isolated due to the little work done with *POL2*, which focused on catalytic regions and subunit domains.

In summary, we have isolated a collection of novel temperature-sensitive *pol2* mutants with unique sets of phenotypes. Three of the mutants have rarely seen alterations in the subunit-

interacting domain of Pol2p and therefore present a unique oppurtunity to investigate the importance of subunit-subunit interactions in DNA polymerase epsilon in future studies.

Table 5. Summary of mutant phenotypes[a]

pol2 mutant	Suggested Mutation Locus	Minimum Restrictive Temperature	Elongated G2/M phase	MMS^s	HU^s	$Lys^- \rightarrow Lys^+$ Mutator
58	Zinc-finger domain	35°C	30°C only	Yes	Yes	No
116	Zinc-finger domain	35°C	30°C only	Yes	Yes	Yes
97	Subunit-interacting domain	37°C	30°C only	No	No	No
99	Subunit-interacting domain	37°C	42°C only	No	No	No
67	Subunit-interacting domain	42°C	Both 30°C and 42°C	No	Yes (weak)	No

[a] Concentrations: MMS (2 mM) and HU (200 mM)

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VITA

Natashya Waring was born in Havre De Grace, Maryland, on July 25, 1969. After completing her work at Judson High School in Converse, Texas, in 1987 she entered the University of Texas at Austin and earned a bachelor's degree in social work. During the following years she was employed as a licensed social worker. In September, 2000, she entered the Graduate School of Southwest Texas State University, San Marcos, Texas.

Permanent address: 3201 Duval Road #1131

Austin, Texas 78759

This thesis was typed by Natashya Jean Waring.