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CONSTRUCTION AND *IN VIVO* CHARACTERIZATION OF A *SACCHAROMYCES CEREVISIAE* DNA POLYMERASE DELTA EXONUCLEASE MUTANT

by

MICHALIS IOANNOU HADJIMARCOU ©

A THESIS

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MASTER OF SCIENCES

in

MOLECULAR BIOLOGY AND GENETICS
DEPARTMENT OF BIOLOGICAL SCIENCES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CONSTRUCTION AND *IN VIVO* CHARACTERIZATION OF A *SACCHAROMYCES CEREVISIAE* DNA POLYMERASE DELTA EXONUCLEASE MUTANT submitted by Michalis Ioannou Hadjimarcou in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in MOLECULAR BIOLOGY AND GENETICS.

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ABSTRACT

DNA polymerase exonucleolytic proofreading is a major determinant of the fidelity of DNA replication. Mutational analysis of the bacteriophage T4 DNA polymerase has been used to identify amino acid residues and structures required for the proofreading pathway. These studies revealed a protein loop structure in the exonuclease domain of the enzyme that functions in strand separation. The protein loop is proposed to be conserved in yeast and human replicative DNA polymerases. This hypothesis was tested by constructing a *Saccharomyces cerevisiae* DNA polymerase δ mutant with a G447S substitution in the proposed analogous protein loop structure. A decrease in replication fidelity (mutator phenotype) is expected if proofreading is reduced by the substitution. A mutator phenotype was detected, an observation that supports the hypothesis that a protein loop structure functions in the bacteriophage T4 and yeast DNA polymerase δ proofreading pathways.

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LIST OF ABBREVIATIONS

A	alanine
Ala	alanine
AMV	avian myeloplastosis virus
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
C	cysteine
$CaCl_2$	calcium chloride
cpm	counts per minute
D	aspartate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUMP	deoxyuracil monophosphate
dUTP	deoxyuracil triphosphate
E	glutamate
EDTA	ethylenediaminetetraacetic acid
F	phenylalanine
G	glycine
Glu	glutamate
Gly	glycine
H	histidine
I	isoleucine
IPTG	isopropyl-1-thio-β-D-galactoside
K	lysine
KCl	potassium chloride
L	leucine
LB	Luria Broth
Leu	leucine
Lys	lysine
M	methionine
$MgCl_2$	magnesium chloride
N	asparagine
NaCl	sodium chloride

NaOH	sodium hydroxide
NEN	New England Nuclear
$(NH_4)_2SO_4$	ammonium sulphate
OD	optical density
P	proline
PCR	polymerase chain reaction
Q	glutamine
R	arginine
RNase	ribonuclease
rpm	revolutions per minute
S	serine
SDS	sodium dodecyl sulphate
Ser	serine
T	threonine
Taq	*Thermus aquaticus*
Thr	threonine
Tris-HCl	Tris Hydroxymethyl Aminomethane Hydrochloride
Tyr	tyrosine
V	valine
W	tryptophan
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
Y	tyrosine
YPD	Yeast extract, Peptone, Dextrose

LIST OF SYMBOLS

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
φ	phi
Δ	deletion

INTRODUCTION

Throughout the history of life, propagation of all species depends on the faithful transmission of genetic information from one generation to the next. In both prokaryotic and eukaryotic organisms, DNA polymerases contribute significantly in maintaining genome integrity by replicating DNA with high fidelity. DNA polymerases, as part of elaborate multiprotein replication complexes, incorporate nucleotides with an insertion error frequency of about 10^{-5}, as measured for replication of the *Escherichia coli* chromosome (Loeb and Kunkel, 1982). Proofreading, a 3'→5' exonuclease activity associated with many DNA polymerases, further reduces replication errors by 100-fold or more. The accuracy of DNA replication is also enhanced by post-replication mismatch repair, which corrects errors that escape proofreading, and increases the accuracy of DNA replication by another several hundred-fold. The net result is an overall nucleotide misincorporation frequency of about 10^{-10} (Schaaper, 1993).

The two DNA polymerase functions, template-directed nucleotide incorporation and 3'→5' exonuclease activity, are illustrated in the simple diagram shown below, where n represents the length of the primer strand in nucleotides, and PPi is pyrophosphate.

$$\begin{tikzcd}
\begin{array}{l} \mathrm{DNA}_n + \mathrm{dNTP} \\ \;+ \\ \mathrm{H_2O} \end{array} \arrow[r, shift left, "\text{extension}"] \arrow[d, "\text{proofreading}"] & \mathrm{DNA}_{n+1} + \mathrm{PPi} \arrow[l, shift left, "\text{pyrophosphorolysis}"] \\
\mathrm{DNA}_{n-1} + \mathrm{dNMP} &
\end{tikzcd}$$

Primer extension occurs by incorporation of a deoxyribonucleoside monophosphate (dNMP) with simultaneous release of PPi. Accurate nucleotide incorporation is achieved by the ability of DNA polymerases to recognize the proper specific geometry of the terminal base pair (Moran *et al.*, 1997). The extension reaction can be reversed by pyrophosphorolysis, but cellular pyrophosphotases prevent DNA degradation by degrading PPi.

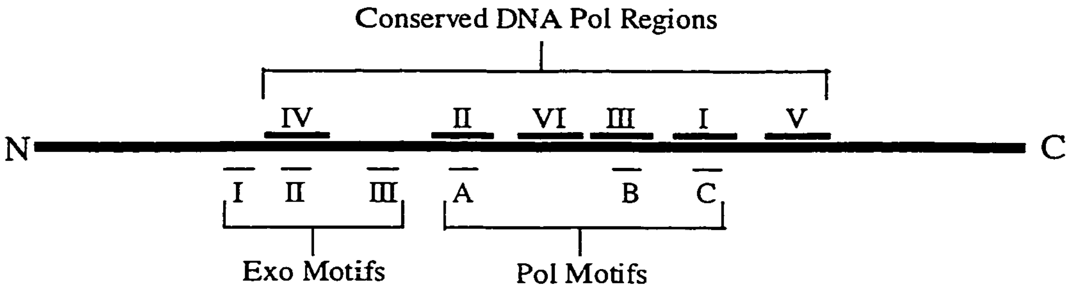
Nucleotide incorporation is in competition with the exonucleolytic proofreading pathway. Proofreading is triggered by incorporation of a non-complementary nucleotide, which produces a mispaired primer-terminus. Extension of a mispaired primer-terminus is slow, which makes initiation of the proofreading pathway the kinetically favored reaction. Once the incorrect nucleotide has been removed, the primer is returned to the polymerase active center and DNA replication resumes. Since the 3'→5' exonucleolytic function of the DNA polymerase works preferentially to correct replication misincorporation errors, it is called a proofreading activity.

Considering the essential role that DNA polymerases have in all organisms, it comes as no surprise that many DNA polymerases share functional and sequence similarities. Based on amino acid sequence similarities, three DNA polymerase families have been identified (Wong *et al.*, 1988; Braithwaite and Ito, 1993). Families A, B and C include DNA polymerases with homology to the proteins encoded by the *E. coli polA*, *polB*, and *polC* genes, respectively. An additional group of enzymes, Family X, includes the mammalian DNA pol β and terminal transferases (Ito and Braithwaite, 1991).

Family B members, also called α-like, include the DNA polymerases of bacteriophage T4, φ29 and RB69; DNA polymerases of herpes, vaccinia, and adeno viruses; and the eukaryotic alpha (α), delta (δ) and epsilon (ε) DNA polymerases (Wang *et al.*, 1989; Braithwaite and Ito, 1993). These enzymes contain in their primary sequence three or more of six conserved regions that form the catalytic active centers. These regions are designated I-VI according to their degree of conservation, with region I being the most conserved and region VI the least (Fig. 1). All six regions show similar spatial arrangements for each DNA polymerase polypeptide in the order IV-II-VI-III-I-V. Regions II, III and I contain the highly conserved amino acid motifs A, B and C, respectively, which contain aspartate residues that are essential for the polymerase activity (Wong *et al.*, 1988; Delarue *et al.*, 1990; Braithwaite and Ito, 1993; Fig. 1). Region IV is located in the exonuclease domain of DNA polymerases and is required for proofreading activity. The 3'→5' exonuclease active center is composed of three amino acid motifs, Exo I, II, and III, which are conserved in all proofreading DNA polymerases (Blanco *et al.*, 1992; Fig. 1). The three Exo motifs contain highly conserved acidic residues that are essential for 3'→5' exonuclease activity (Reha-Krantz *et al.*, 1991; Morrison *et al.*, 1991; Blanco *et al.*, 1992).

One of the most characterized Family B members is the bacteriophage T4 DNA polymerase (T4 DNA pol). Genetic (Reha-Krantz, 1994; Reha-Krantz, 1995), biochemical (Nossal and Alberts, 1983; Young *et al.*, 1992; Capson *et al.*, 1992), and structural (Wang *et al.*, 1996; Wang *et al.*, 1997) studies have realized a level of characterization that makes the T4 DNA polymerase a good research model for other

Figure 1. **Locations of Family B DNA polymerase signature sequences**. Conserved regions I-VI were identified by Wang *et al.* (1989). Residues essential for the 3'→5' exonuclease activity are found in the Exo I, II and III motifs (Blanco *et al.*, 1992). Essential residues in the polymerase activity are located in motifs A, B and C (Delarue *et al.*, 1990).


Conserved DNA Pol Regions
IV
II
VI
III
I
V
N
C
I
II
III
A
B
C
Exo Motifs
Pol Motifs

Family B DNA polymerases. Since the T4 DNA polymerase shows functional similarity to human DNA polymerase δ (Tsurimoto and Stillman, 1990) and yeast DNA polymerase δ (Bauer and Burgers, 1988), as well as homology with both of these enzymes at the protein sequence level (Braithwaite and Ito, 1993), information from T4 DNA polymerase studies can be used to guide studies of these eukaryotic DNA polymerases. The subject of this investigation is to use information learned about the T4 DNA pol proofreading pathway to study proofreading by the yeast DNA pol δ.

The T4 DNA pol proofreading pathway begins with identification of a wrong nucleotide at the primer terminus in the polymerase active center. The next step is strand separation and transfer of the primer terminus from the polymerase active center to form a pre-exonuclease complex (active-site-switching). Formation of the pre-exonuclease complex serves as a kinetic barrier to indiscriminate DNA degradation by preventing proofreading unless the rate of primer elongation is significantly reduced by the presence of an incorrect base pair at the primer-terminus. Further strand separation allows binding of the primer-terminus in the exonuclease active center, where the hydrolysis reaction takes place (Capson *et al.*, 1992; Marquez and Reha-Krantz, 1996; Reha-Krantz *et al.*, 1998; Baker and Reha-Krantz, 1998).

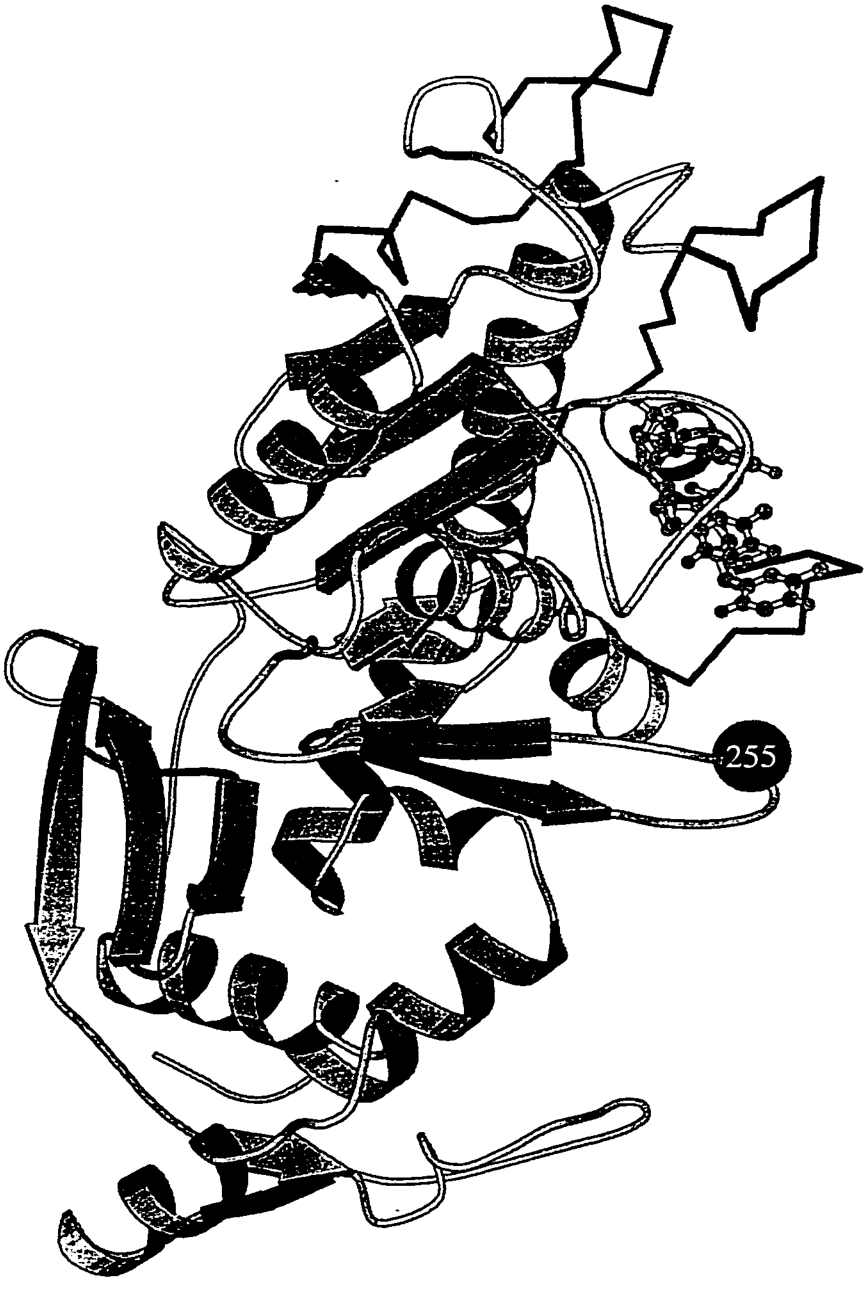
Selection of mutant T4 DNA polymerases on the basis of a strong mutator phenotype (Reha-Krantz, 1988) and as active-site-switching mutants (Stocki *et al.*, 1995) has allowed identification of amino acids that are essential for exonucleolytic proofreading. One T4 mutant DNA pol that was isolated repeatedly, the G255S-

DNA pol, has a serine substitution for glycine at position 255 and produces a strong mutator phenotype *in vivo* (Reha-Krantz, 1988; Stocki *et al.*, 1995). Residue G255 resides near the tip of a novel protein loop structure in the exonuclease domain of the enzyme, between the Exo II and Exo III regions (Wang *et al.*, 1996; Figs. 1 and 2). The exonuclease activity of the G255S-DNA pol is near the wild-type level for single-stranded DNA substrates, which indicates that the ability to excise the 3' terminal nucleotide from the primer strand is not affected (Stocki *et al.*, 1995). The mutant enzyme, however, has very low exonuclease activity on fully double-stranded DNA. Together, these observations suggest that residue G255 and the associated protein loop structure are required for some aspect of strand-separation and/or in moving the primer terminus from the polymerase to the exonuclease active center (Marquez and Reha-Krantz, 1996).

A similar involvement for a protein-loop structure for transfer of DNA from the polymerase to the exonuclease active center is proposed for other Family B DNA polymerases (Stocki *et al.*, 1995; Marquez and Reha-Krantz, 1996). The T4 DNA pol residue G255 is proposed to be analogous to residue G447 in the *S. cerevisiae* DNA pol δ and G442 in the human DNA pol δ (Stocki *et al.*, 1995; Marquez and Reha-Krantz, 1996). To test the hypothesis that other Family B DNA polymerases use a protein-loop structure in the proofreading pathway as observed for the T4 DNA pol, the yeast G447S-DNA pol δ (serine substitution for glycine at position 447) was constructed.

Three DNA polymerases are essential in *S. cerevisiae* for DNA replication: DNA pol δ (Sitney *et al.*, 1989; Boulet *et al.*, 1989), DNA pol α (Campbell, 1986;

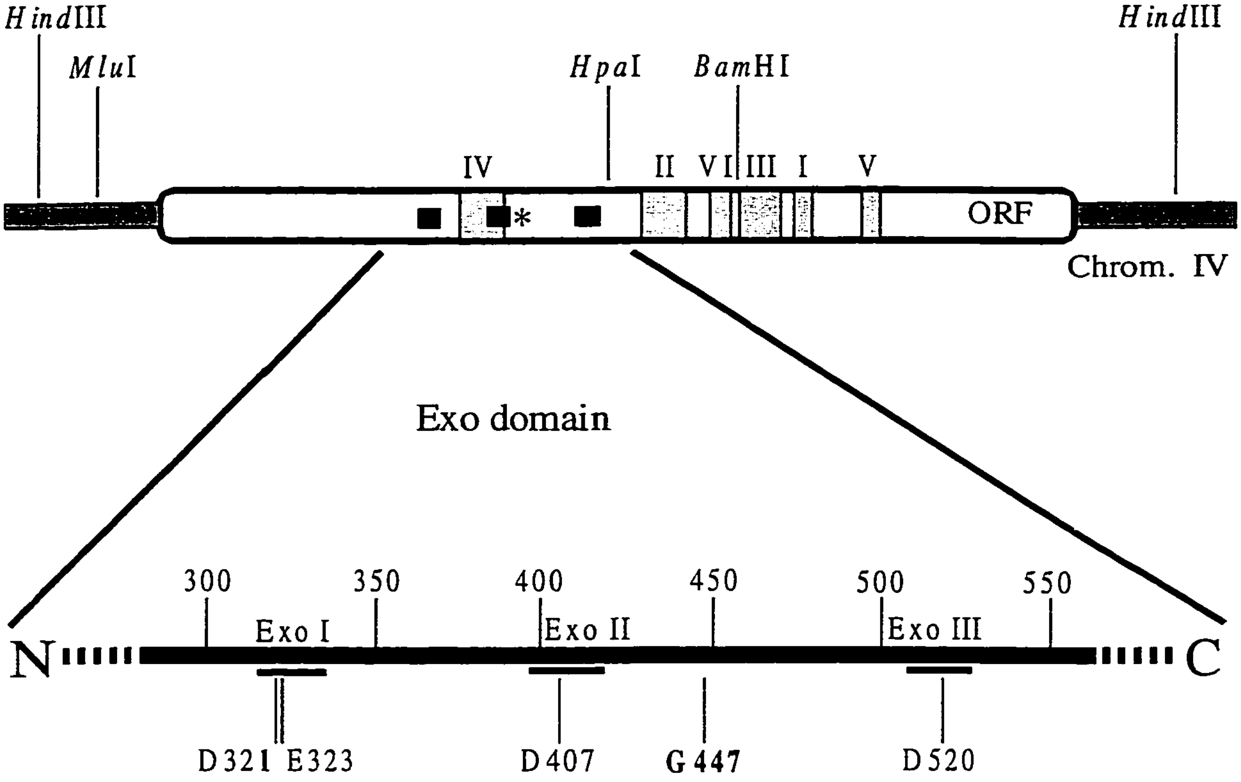
Figure 2. ***Ribbon* and *arrow* drawing of the overall backbone structure of the amino-terminal and exonuclease domains of T4 DNA polymerase**. The position of glycine 255 is shown in a loop structure formed by beta strands 11 and 12. An oligonucleotide [p(dT_4)] is bound in the exonuclease active center of the enzyme. The drawing is by Wang *et al.* (1996).


255

Pizzagalli *et al.*, 1988), and DNA pol ε (Morrison *et al.*, 1990). The processivity of DNA pol δ is enhanced by association with the proliferating cell nuclear antigen, PCNA (Bauer and Burgers, 1988). DNA pol δ, purified from yeast cells, consists predominantly of two subunits of 125 kDa and 55 kDa (Bauer and Burgers, 1988). The large subunit is encoded by the *POL3* gene (formerly *CDC2*; Boulet *et al.*, 1989; Fig. 3), and exhibits both 5'→3' polymerase and 3'→5' exonuclease activities (Simon *et al.*, 1991; Brown *et al.*, 1993). The function of the 55 kDa subunit remains unknown but studies suggest that it is required for stimulation of DNA pol δ by PCNA (Sun *et al.*, 1997).

The G447S substitution in the yeast DNA pol δ was constructed by site-directed mutagenesis of a cloned portion of the *POL3* gene, which encodes the amino-terminal and exonuclease domains. The mutations encoding the G447S substitution were then introduced into the chromosomal *POL3* gene by homologous recombination. Mutant yeast cells were examined for temperature sensitivity, but none was found, which indicates that the polymerase function of the mutant enzyme was not affected adversely. DNA replication fidelity of the G447S-DNA pol δ *in vivo* was also assessed. The frequency of frameshift mutations was found to be similar to that of the wild-type enzyme, but base substitution mutations were elevated. The same pattern of mutational specificity was detected for the T4 G255S-DNA pol. These results indicate that the yeast G447S-DNA pol δ resembles the T4 G255S-DNA pol, which supports the hypothesis that a protein loop structure functions in the proofreading pathway of the T4 and other Family B DNA polymerases.

Figure 3. **Schematic diagrams of the *S. cerevisiae POL3* gene**. The *POL3* open reading frame (ORF) is shown as a thick white box, flanked by non-coding yeast DNA (shaded, medium thickness boxes). The six Family B DNA polymerase conserved regions (I-VI) and restriction sites important for this study are also indicated. The location of glycine 447 is marked with an asterisk. The three conserved Exo I, II, and III motifs are depicted as small black boxes near conserved region IV. The exonuclease domain is expanded in the lower diagram to show the relative positions of the Exo motifs, the conserved acidic active site residues, and glycine 447. Numbers correspond to amino acid positions.


*Hind*III
*Mlu*I
*Hpa*I
*Bam*HI
*Hind*III
IV
II
V I
III
I
V
*
ORF
Chrom. IV
Exo domain
300
350
400
450
500
550
Exo I
Exo II
Exo III
N
C
D321
E323
D407
G447
D520

MATERIALS AND METHODS

Microbiological media and methods

Bacterial strains are described in Table 1. Bacterial cultures were grown with aeration at 37 °C in Luria Broth (LB) or on solid LB medium prepared as described by Sambrook *et al.* (1989; p. A1). Antibiotics were supplemented as indicated. Solid media used for blue/white colony selection contained 0.1 mM IPTG and 20 µg/ml X-gal. Bacterial cells were made competent by the $CaCl_2$ method and transformations were performed as described by Cohen *et al.* (1972).

Yeast cultures (strains described in Table 2) were grown with aeration at 30 °C in YPD medium (Sherman *et al.*, 1983) or on solid YPD medium. Complete minimal media and complete minimal omission media were prepared according to Sherman *et al.* (1983). Medium containing 5-fluoroorotic acid (5-FOA) was prepared as described by Boeke *et al.* (1984). Yeast cells were made competent by the lithium acetate method and transformations were carried out as described by Ito *et al.* (1983).

DNA replication fidelity was measured by forward mutation and reversion assays. Yeast cultures were grown from single colonies to saturation at 30 °C in 5 ml YPD. The cells were pelleted at 1,200 rpm for 5 min in a tabletop centrifuge, washed in 5 ml water, pelleted again as above, and resuspended in 1 ml water. A final centrifugation was performed at 14,000 rpm for 30 sec and the cells were resuspended in 0.2 ml water at a concentration of approximately $2x10^9$ cells/ml. To measure the frequency of *ade5-1* reversion, 0.1 ml cells (~$2x10^8$ cells) were plated onto complete minimal plates lacking adenine and the plates were incubated at 30 °C

Table 1. ***Escherichia coli* strains**

Strain	Genotype	Relevant Characteristics and Reference
CJ236	*dut1 ung1 thi1 relA1* [F′: *pCJ105 (Cm*^{r}*)*]	Lacks dUTPase and uracil-N-glycosylase activities; permissive for M13; resistant to chloramphenicol (Joyce and Grindley, 1984)
DH5α	*endA1 hsdR17* (rK$^-$mK$^+$) supE44 *thi1 recA1 gyrA* (Nal^r) *relA1* Δ (*lacZYA-argF*) *U169 deoR* (φ80 Δ (*lacZ*) M15)	Active dUTPase and uracil-N-glycosylase activities; degrades uracil-containing DNA (Woodcock *et al.*, 1989)

Table 2. ***Saccharomyces cerevisiae* strains**

Strain	Genotype	Relevant Characteristics and Reference
MS71	*Matα ade5-1 trp1-289 ura3-52 his7-2*	*ade5-1* (UAC^{tyr}→UAA^{ochre}); *ura3-52* (non-reverting Ty insertion); *his7-2* (-1 frameshift) (Morrison *et al.*, 1991; Strand *et al.*, 1995)
MIH1	MS71 *pol3-447*	*pol3-447* (G447S-DNA pol δ)

for 5 days. The total cell titer was determined by plating a 5x10^6 dilution of the concentrated cells onto complete minimal plates. The plates were incubated at 30 °C for 3 days. The reversion frequency for each culture was calculated by dividing the number of revertants by the number of viable cells. Reversion assays at room temperature, 20 °C, and at 37 °C, were performed in an identical fashion except that cultures were grown at the indicated temperatures. The same procedure was followed to measure reversion of the *his7-2* allele except that complete minimal medium lacking histidine was used.

DNA replication accuracy was also measured by a forward mutation assay to canavanine resistance. Cultures, grown from single colonies, were concentrated to about 4x10^8 cells/ml. A 0.1 ml sample of the concentrated cells was plated onto complete minimal medium lacking arginine but containing 60 µg/ml canavanine, to select for canavanine resistance mutants. The total cell count was determined by plating on complete minimal medium. The plates were incubated at 30 °C for 3 days. The mutation frequency for each culture was calculated by dividing the number of canavanine-resistant cells by the total cell number.

Synthetic oligonucleotides

Synthetic oligonucleotides used in this study are described in Table 3. Oligonucleotides designated "LRK" were prepared by the DNA Synthesis Lab, Department of Biological Sciences, University of Alberta. Oligonucleotides with names beginning with "ADE5" or "HIS7" were prepared by the DNA Synthesis Lab, Department of Pathology, University of North Carolina at Chapel Hill.

Table 3. **Synthetic oligonucleotides used in site-directed mutagenesis, PCR and DNA sequence analysis**

Oligo[a]	DNA Sequence (5'→3')	Location and function
LRK114	CTTAGTTTATACTCACGCTG	*POL3*-reverse primer
LRK125[b]	P-TCGAAGGCTTATCTTACAAGAGAA ACC	*POL3*-site-directed mutagenesis
LRK126	CGCTAAAGGTGAATGATTTCCC	*POL3*-forward primer
LRK127	GGTTGAAGGCTCTCAAGGGC	YIpMH1S-forward primer
LRK128	ATCATGGTGTTGGAAGTGAACC	*POL3*-forward primer
LRK129	AGATGAAAATACATCTACCGTGG	*POL3*-forward primer
LRK130	GTCAACAAACTGCGTACTGCG	*POL3*-forward primer
LRK131	CTTTGATATCGAGTGTGCTGG	*POL3*-forward primer
LRK140	GCGACCACACCCGTCCTGTG	YIpMH1S-reverse primer
LRK145	GAGACATATTTGGCAGCTTCGG	*POL3*-reverse primer
*ADE5*F1	TAAGAGAACCATTCTCCCTCCC	*ADE5*-forward primer
*ADE5*R1	AAAACACTTGCGCCAAATCGGT	*ADE5*-reverse primer
*ADE5*F2	AATGTCAGATTCGGTGACCCAG	*ADE5*-forward primer
*ADE5*R2	CGGTACCATTAGTATCGTAGTGG	*ADE5*-reverse primer
*ADE5*F3	AAAGTGGTTGTGCTCTTGTGGG	*ADE5*-forward primer
*ADE5*R3	TTATGCGGAAAAGTAAAAGCGAAC	*ADE5*-reverse primer
*HIS7*F1	GAAGTAGCAGTATCCAGTTTAGG	*HIS7*-forward primer
*HIS7*R1	ATGTTACTTCATCCGCACCCTG	*HIS7*-reverse primer
*HIS7*F2	TTGGTAAGCCTGTTCAGTTGGC	*HIS7*-forward primer
*HIS7*R2	CCTCTTGTGTTGTGAAATGTCTG	*HIS7*-reverse primer

[a] Synthetic oligonucleotides were purchased as described in Materials and Methods.

[b] Oligonucleotide LRK125 is shown with a phosphate group attached to the 5'-end. The TCT codon, encoding the G447S substitution in yeast DNA pol δ, is underlined.

DNA extraction and analysis

Extraction of plasmid DNA from bacterial cells was performed by the alkaline lysis method (Birnboim and Doly, 1979). Plasmid DNA extracted from large culture volumes (1.5 l) was purified further by equilibrium centrifugation in a CsCl-ethidium bromide density gradient (Radloff *et al.*, 1967). Genomic DNA from yeast cells was extracted by a variation of the "smash and grab" method (Hoffman and Winston, 1987), as described by Ausubel *et al.* (1995; pp. 13.11.2-13.11.3). Single-stranded phagemid DNA was isolated from polyethylene glycol-precipitated phage particles and purified using phenol/chloroform extractions as described by Sambrook *et al.* (1989, p. 4.48)

Agarose gel electrophoresis was used to characterize the purified DNAs. DNA samples (single-stranded and double-stranded DNAs and restriction endonuclease reaction products) were diluted in loading buffer (4.8% sucrose, 0.025% bromophenol blue, 1.2 mM EDTA) and analyzed by electrophoresis on 0.6% agarose gels containing 0.5 µg/ml ethidium bromide, and TBE buffer (100 mM Tris base, 100 mM boric acid, 2.5 mM EDTA). Restriction endonuclease digests were performed according to the conditions suggested by the suppliers. Restriction products were heated at 65 °C for 5 min and quick-chilled on ice before electrophoresis on 0.6% agarose gels as described above. A 1 kb DNA ladder (1 µg/µl; GIBCO BRL) was electrophoresed next to the samples on the gel to provide size markers.

PCR amplifications

DNA sequences (plasmid and genomic) were amplified by the polymerase chain reaction method (Saiki *et al.*, 1985; Mullis *et al.*, 1986). Amplifications were performed in 0.6 ml PCR tubes containing about 100 ng genomic DNA or 0.2 ng plasmid DNA, 0.2 mM each dNTP, 0.5 µM each of forward and reverse primers, 1.5 mM $MgCl_2$, PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl; GIBCO BRL), and 2 units Taq DNA polymerase (GIBCO BRL) in a total reaction volume of 50 µl. The reactions were overlaid by 2 drops (about 60 µl) of paraffin oil. The amplification reactions were initiated with a denaturation step at 95 °C for 5 min, and were followed by 30 cycles of annealing at 56 °C for 1 min, extension at 72 °C for 1 min/kb sequence amplified, and denaturation at 92 °C for 1 min. A final polymerization step was carried out at 72 °C for 6 min to ensure complete extension of the amplification products.

Whole-cell PCR was performed by a modification of the standard protocol. Single colonies of about 1 mm in diameter were transferred into 0.6 ml PCR tubes, mixed with 1 µl water and microwaved at high power for 1 min. The cell mixture was then resuspended in 50 µl PCR mix containing forward and reverse primers, dNTPs, $MgCl_2$ and PCR buffer, at the concentrations specified above. The cell debris was pelleted by centrifugation (12,000 rpm for 30 sec) and the supernatant was transferred to fresh tubes. Two units Taq DNA polymerase were added and amplification reactions were carried out as described above.

DNA sequencing

DNA sequencing was performed by the chain termination sequencing method (Sanger *et al.*, 1977). Three procedures were used: sequencing with AMV reverse transcriptase, cycle sequencing and automated sequencing.

In reactions with AMV reverse transcriptase, single-stranded template DNA was prepared from double-stranded plasmid DNA by standard alkaline denaturation techniques using NaOH. Single-stranded DNA was also produced by asymmetric PCR (Gyllensten and Erlich, 1988) using the components and conditions described above for the basic PCR protocol except that forward primers were used in limiting concentrations (8 pM). The asymmetric PCR products were precipitated with ammonium acetate and ethanol to remove amplification buffer components. Sequencing primers were 5'-end-labeled in 10 µl reactions containing 10 pmol primer, 3.5 µl [γ-^{32}P]ATP (NEN, 3000 Ci/mmol), 1 µl 10X kinase buffer (0.5 M Tris-HCl [pH 7.6], 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) and 2 units T4 polynucleotide kinase (10 units/µl, Pharmacia). The reactions were incubated at 37 °C for 30 min and the labeled primers were stored frozen at -20 °C, until needed. Annealing reactions were performed with 3-5 pmol labeled primer mixed with about 3-5 pmol DNA, and 2.4 µl 5X annealing buffer (250 mM Tris-HCl [pH 8.3], 300 mM NaCl, 50 mM DTT) in a total volume of 12 µl. After incubation at 60°C for 3 min, the annealing mixture was quick-frozen on a dry ice/ethanol bath. Sequencing reactions contained 2.5 µl of the primer-template mix, 1 µl of 0.4 mM dNTPs, 1 µl of 250 µM ddNTPs, and 1 µl AMV reverse transcriptase mix (2.5 units/µl AMV reverse transcriptase [25 units/µl; Boehringer Mannheim] in reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 10 mM DTT, 30 mM $MgCl_2$]). Primer extension was carried out at 48 °C for 30 min before termination by addition of 5 μl loading dye (94% deionized formamide, 0.4X TBE buffer, 0.04% bromophenol blue/xylene cyanol blue). The sequencing reactions were heated at 100 °C for 5 min and quick-chilled on ice. DNA sequencing products were separated by electrophoresis on 7% polyacrylamide, 8 M urea sequencing gels. The gels were then dried at 80 °C for 1 h and exposed to phosphor imager screens.

DNA sequencing was performed by the cycle-sequencing method (Murray, 1989), using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham).

The sequence of the *S. cerevisiae ade5-1* and *his7-2* alleles was determined from double-stranded PCR products of genomic DNA from the wild-type strain MS71 (Table 2). The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN). Sequencing was performed with approximately 100 ng purified DNA and 10 pmol primer by the University of North Carolina at Chapel Hill Automated DNA Sequencing Facility.

Southern hybridization analysis

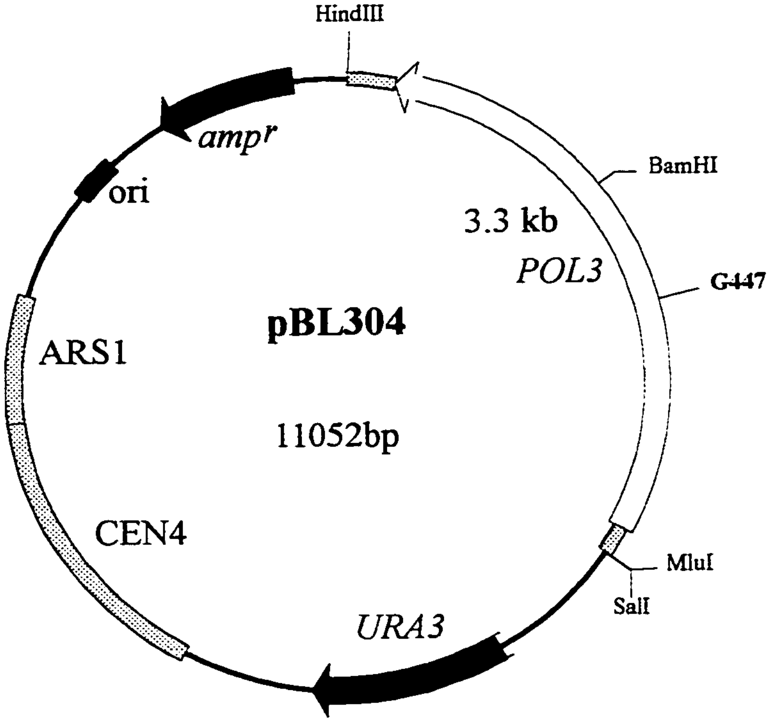
Southern hybridization analysis (Southern, 1975) was performed with about 5 μg yeast genomic DNA digested with 30 units *Hind*III (GIBCO BRL) in 40 μl reactions at 37 °C, overnight. The restriction fragments were separated on a 0.8% agarose gel at low voltage (40 V) for about 22 h. The DNA was transferred onto a Hybond-N^+ membrane (Amersham) using 0.4 M NaOH as transfer buffer according to standard procedures (Southern, 1975; Reed and Mann, 1985). Prehybridization was performed with 25 ml prehybridization solution (5X SSC [750 mM NaCl, 75 mM sodium citrate (pH 7.0)], 5X Denhardt solution [0.1% ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin fraction V], 1% SDS) containing 100 µg/ml denatured salmon sperm DNA, in a rotating hybridization oven at 68 °C for 3 h. The ^{32}P-labeled hybridization probe was generated by a variation of the basic PCR protocol described above. Amplification of about 0.5 kb of *POL3* sequence was performed in a 20 µl reaction volume with double-stranded YIpMH1S plasmid DNA, oligonucleotides LRK131 and LRK114 (Table 3), and [α^{32}P] dCTP. Reaction components and conditions were standard except that only 0.01 mM dCTP was used, supplemented by 8 µl [α^{32}P]dCTP (NEN, 3000 Ci/mmol). The probe was purified through a G-50 Sephadex column as described by Ausubel *et al.* (1995; pp.3.4.10-11). Approximately 100 ng of double-stranded PCR products were labeled to a specific activity of 9×10^8 cpm/µg DNA. The probe was denatured at 100 °C for 10 min, quick-chilled on ice, and added to 25 ml fresh prehybridization solution. Hybridization was allowed to proceed at 68 °C overnight. The membrane was washed to high stringency with a solution of 0.1% SSC and 0.1% SDS at 68 °C for 15 min. The membrane was covered with plastic wrap and exposed overnight to a phosphor imager screen.

Site-directed mutagenesis

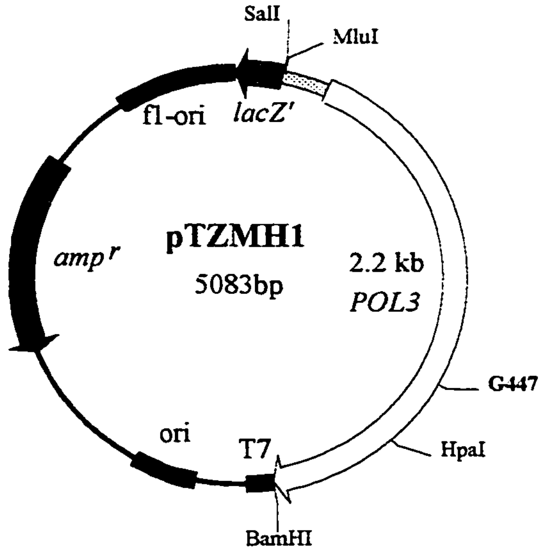
Site-directed mutagenesis, developed by M. Smith (Hutchison *et al.*, 1978), was performed as modified by Kunkel (1985). Briefly, the 5'-2.2 kb *POL3* gene fragment, containing the amino-terminal and exonuclease domains of yeast DNA pol δ (Fig. 3), was subcloned from plasmid pBL304 (Fig. 4a) into phagemid vector pTZ18U (Mead *et al.*, 1986) to give the 5.1 kb pTZMH1 (Fig. 4b). Propagation of pTZMH1 in *dut*$^-$ *ung*$^-$ *E. coli* CJ236 cells (Table 1) allowed incorporation of uracil into DNA during replication. Superinfection of CJ236 with helper phage M13KO7 (Vieira and Messing, 1987) resulted in production of phage-like particles (phagemids) containing single-stranded uracil pTZMH1 DNA. The phagemid DNA was isolated and purified as described above. The mutagenic oligonucleotide, LRK125 (5' P-TCGAAGGCTTATCTTACAAGAGAAACC 3'; Table 3), which contains the TCT codon for serine (underlined) in place of the GGT codon for glycine, was annealed to the single-stranded uracil pTZMH1 DNA. The phosphate group (P) shown attached at the 5'-end was incorporated to allow ligation of the nascent strand to the LRK125 primer at the completion of the *in vitro* synthesis reaction. In a 20 μl annealing reaction, 2 pmol single-stranded uracil-containing template pTZMH1 DNA was mixed with 46 pmol LRK125 and 0.1 M NaCl, incubated at 95 °C for 3 min and quick-chilled on ice. T4 DNA polymerase was then used to extend the annealed oligonucleotide. Full replication of the circular template produces double-stranded DNA that can be joined to the 5'-P at the end of the mutagenic oligonucleotide by DNA ligase to form covalently-closed duplex DNA. The 20 μl synthesis reaction contained 0.2 pmol of the annealed primer-template, T4 DNA polymerase buffer (67 mM Tris-HCl [pH 8.8], 0.5 mM DTT, 15 mM (NH$_4$)$_2$SO$_4$), 0.2 mM each dNTP, 0.1 μg/ml bovine serum albumin, 2 pmol T4 DNA polymerase and 8 mM MgCl$_2$. The initial primer extension reaction was at

Figure 4. ***POL3* plasmids**. (A) Plasmid vector pBL304 carrying the entire *POL3* gene. pBL304 contains the yeast *URA3* gene selection marker, a yeast centromere (CEN4) and replicon (ARS1), the *E. coli* β-lactamase gene selection marker (*amp*^{r}) and the pMB1 *E. coli* replicon (ori). The *POL3* ORF is flanked by short segments of yeast DNA. (B) Phagemid pTZMH1 carrying the 5'-2.2 kb *POL3* fragment. Phagemid pTZMH1 contains: the *E. coli* β-lactamase gene selection marker (*amp*^{r}); the bacteriophage f1 and *E. coli* pBR322 origins of replication for production of single-stranded and double-stranded DNA molecules; the *lacZ'* gene (α-complementation fragment of the β-galactosidase gene); the multiple cloning site cassette from phagemid pUC18 (Norrander *et al.*, 1983); and the bacteriophage T7 promoter. A short yeast DNA fragment is present directly upstream from the beginning of the *POL3* ORF.

(A)


HindIII
BamHI
G447
MluI
SalI
ampr
ori
3.3 kb
POL3
pBL304
ARS1
11052bp
CEN4
URA3

(B)


SalI
MluI
fl-ori
lacZ'
ampr
pTZMH1
5083bp
2.2 kb
POL3
G447
HpaI
ori
T7
BamHI

room temperature for 5 min. T4 DNA ligase in 1 µl (1 unit/µl; GIBCO BRL) and 1 µl of 100 mM ATP were added to the reaction and synthesis continued for another 10 min at 42 °C. The reaction was terminated by addition of 2 µl of 0.2 M EDTA.

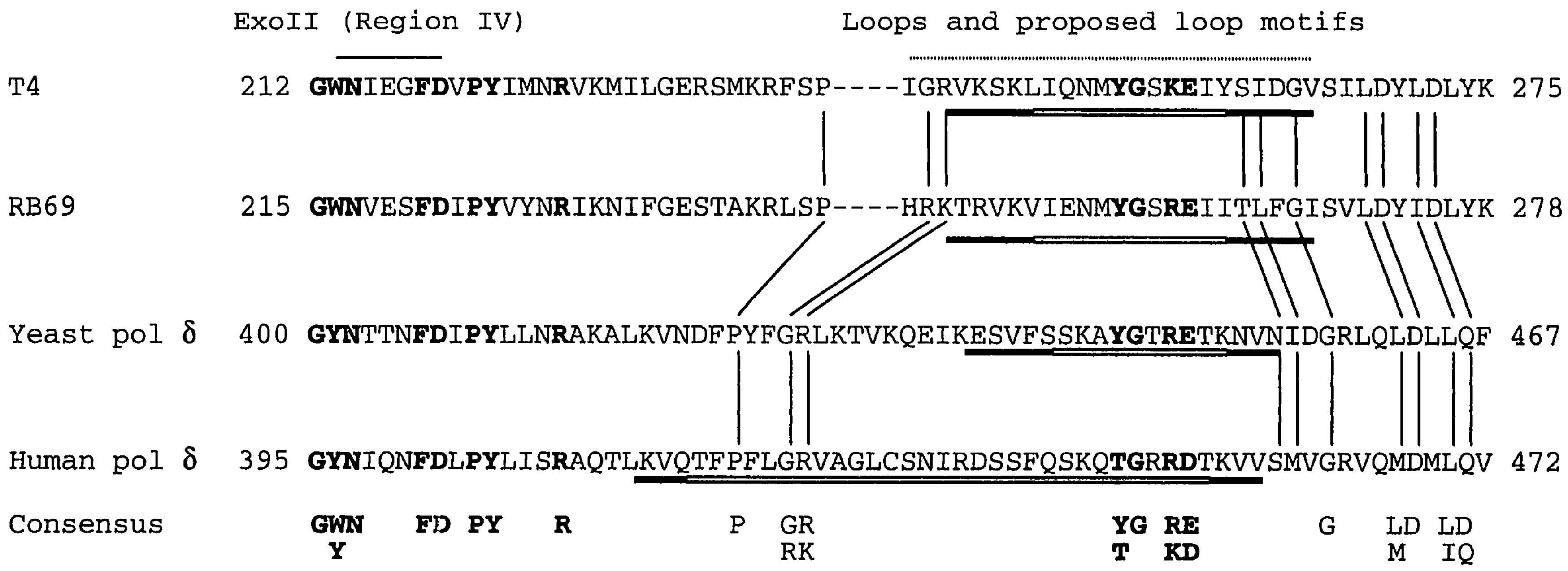
A sample of the reaction mixture (5 µl) was used directly to transform the *dut*$^+$ *ung*$^+$ *E. coli* strain DH5α (Table 1), which selects preferentially for the non-uracil strand (Kunkel, 1985). Transformants were selected on LB plates containing 50 µg/ml ampicillin. Six transformant colonies, from a total of about 3000, were selected for further characterization.

RESULTS

Protein sequence alignment and secondary structure analysis

Glycine 255 in the T4 DNA pol is located 36 amino acid residues downstream from residue D219 in the Exo II motif (Fig. 5). Protein sequence homologies for the T4 DNA pol, the T4-like bacteriophage RB69 DNA pol, the *S. cerevisiae* DNA pol δ, and the human DNA pol δ are evident in the Exo II region (Wang *et al.*, 1989). Additional protein sequence similarities downstream of the Exo II motif are proposed in the region that includes the G255 loop in the T4 DNA pol. The alignment in figure 5 maximizes sequence similarities among the enzymes compared and identifies a proposed new conserved exonuclease motif. Secondary structure analysis suggests the probability of a loop structure in this region for the yeast and human enzymes (Fig. 5), as observed for the T4 and RB69 DNA polymerases (Fig. 2; Wang *et al.*, 1996; Wang *et al.*, 1997). One way to test the hypothesis that the G255 protein loop structure in the T4 DNA pol is found in other Family B DNA polymerases is to use mutational analysis. Mutations encoding the G255S amino acid substitution were isolated repeatedly in mutational studies of the T4 DNA pol proofreading function (Reha-Krantz, 1988; Stocki *et al.*, 1995). Protein sequence alignments suggest that residue G447 in the yeast DNA pol δ may be analogous to residue G255 in the T4 DNA pol (Fig. 5). Thus, the G447S substitution in the yeast DNA pol δ may reduce the proofreading activity as observed for the G255S substitution in the T4 DNA pol. The G447S yeast DNA pol δ was constructed and DNA replication fidelity of the G447S-mutant strain was assessed to test the hypothesis.

Figure 5. **Amino acid sequence alignment and secondary structure analysis of an exonuclease region from four Family B DNA polymerases.** The alignment shows the highly conserved Exo II region and the proposed new conserved exonuclease motif. Alignments between the T4 DNA pol (T4), the *S. cerevisiae* DNA pol δ (Yeast pol δ), and the human DNA pol δ (human pol δ) are as proposed by Stocki *et al.* (1995). The sequence of the RB69 DNA pol (RB69) is aligned with the T4 DNA pol sequence as proposed by Wang *et al.* (1995). Amino acid residues conserved in all four DNA polymerases and conserved positions of basic (arginine [R] and lysine [K]), acidic (glutamate [E] and aspartate [D]) and hydroxyl (tyrosine [Y] and threonine [T]) residues in the proposed conserved loop structure are shown in bold and are indicated in the consensus sequence. Additional potential conserved residues are indicated by lines. Dashes are added in order to illustrate optimum alignment. Numbers preceding and following the sequences indicate amino acid positions. The secondary structure analysis is shown for the proposed new conserved exonuclease region and is indicated below the primary sequence for each DNA polymerase. Thick, horizontal black lines and double-lines correspond to amino acid residues that are part of protein beta-strands and turns/loops, respectively. The secondary structure of the T4 and RB69 DNA polymerases is derived from the crystal structure of the enzymes (Fig. 2; Wang *et al.*, 1996; Wang *et al.*, 1997). The secondary structures for the yeast and human DNA polymerases have been predicted by the method of King and Sternberg (1996).


ExoII (Region IV)
Loops and proposed loop motifs
T4 212 GWNIEGFDVPYIMNRVKMILGERSMKRFSP----IGRVKSKLIQNMYGSKEIYSIDGVSILDYLDLYK 275
RB69 215 GWNVESFDIPYVYNRIKNIFGESTAKRLSP----HRKTRVKVIENMYGSREIITLFGISVLDYIDLYK 278
Yeast pol δ 400 GYNTTNFDIPYLLNRAKALKVNDFPYFGRLKTVKQEIKESVFSSKAYGTRETKNVNIDGRLQLDLLQF 467
Human pol δ 395 GYNIQNFDLPYLISRAQTLKVQTFPFLGRVAGLCSNIRDSSFQSKQTGRRDTKVVSMVGRVQMDMLQV 472
Consensus GWN FD PY R P GR YG RE G LD LD
Y RK T KD M IQ

Site-directed mutagenesis of the yeast DNA pol δ gene

The entire *POL3* gene (3294 bp; Fig. 3) was cloned in the lab of Peter Burgers as a 3.7 kb *MluI/Hind*III fragment into *SalI/Hind*III digested YCp50, a yeast centromere-containing shuttle vector (Ma *et al.*, 1987), to create the 11 kb plasmid pBL304 (Fig. 4a). The yeast DNA pol δ is toxic to *E. coli* (Brown and Campbell, 1993). Uncontrolled expression, even at low levels, results in loss of the expression vector or in rearrangements of the cloned *POL3* gene. In order to avoid the toxic effects that can result from even low, leaky expression of the *POL3* gene, the 2.2 kb *SalI/Bam*HI fragment of pBL304, which encodes the amino-terminal and exonuclease domains of *POL3*, was subcloned into the phagemid vector pTZ18U (Mead *et al.*, 1986), to give the 5.1 kb pTZMH1 (Fig. 4b). In pTZMH1, the POL3 sequence is in the opposite orientation from the T7 promoter, thereby, minimizing the possibility of expression. Construction of the pTZMH1 phagemid was performed by ligating together the 2.2 kb *SalI/Bam*HI *POL3* insert from pBL304 and the 2.9 kb *SalI/Bam*HI-linearized pTZ18U vector DNA. The pTZMH1 construct was then transformed into *E. coli* DH5α cells (Table 1). Phagemid DNA was extracted from transformants and examined for the presence of the *POL3* sequence by restriction endonuclease mapping. A triple-digest with *Sal*I and *Bam*HI, which flank the cloned *POL3* fragment, and *Hpa*I, which cuts once within the *POL3* sequence, produced three DNA fragments with sizes of approximately 0.5 kb, 1.7 kb and 2.9 kb, as expected (Fig. 4b).

The mutation encoding the G447S substitution was introduced on the cloned *POL3* gene fragment by site-directed mutagenesis (Kunkel, 1985) using single-stranded uracil-containing pTZMH1 phagemid DNA and the mutagenic oligonucleotide LRK125 (Table 3). Double-stranded pTZMH1 DNA was first transformed into *dut*$^-$ *ung*$^-$ *E. coli* strain CJ236 (Table 1), which allows for uracil incorporation into DNA (Kunkel, 1985). Thus, replication of pTZMH1 in CJ236 resulted in substitution of uracil for thymine at several positions. Infection of the pTZMH1-transformed CJ236 strain with helper phage M13KO7 (Vieira and Messing, 1987) produced single-stranded uracil-containing pTZMH1 DNA, packaged in phagemids. The phagemids were isolated and the single-stranded uracil-containing DNA was extracted.

Oligonucleotide LRK125 contains the *POL3* sequence from nucleotide positions 1327-1353 except for a two-nucleotide substitution at positions 1339 and 1340. These alterations change the GGT codon for glycine at position 447, to the TCT codon for serine. The wild-type *POL3* and LRK125 sequences are shown below. A phosphate group (P) is indicated at the 5'-end of LRK125.

```
wild-type  5'[1327] TCGAAGGCTTAGGTTACAAGAGAAACC [1353]3'
LRK125             5'P-TCGAAGGCTTATCTTACAAGAGAAACC3'
```

With LRK125 as a primer and the single-stranded uracil-containing pTZMH1 DNA as template, the complementary strand was synthesized *in vitro* to produce double-stranded, circular DNA (Materials and Methods). Covalently-closed circular DNA was produced by the addition of DNA ligase. The synthesis reaction products were then transformed into *dut*$^+$ *ung*$^+$ *E. coli* DH5α cells (Table 1) to select against the uracil-containing strand and to enhance recovery of the mutagenized strand. The success of the site-directed mutagenesis step was assessed by sequencing DNA from two transformants. The GG→TC mutations, encoding the G447S substitution, were detected in both transformants. A control sequencing reaction with pTZMH1 DNA gave the wild-type sequence. The plasmid carrying the mutant 2.2 kb *pol3* gene fragment was called pTZMH1S.

The next step was to subclone the mutant *SalI/Bam*HI 2.2 kb *pol3* fragment into the yeast integrating plasmid vector, YIp5 (Struhl *et al.*, 1979) to form the 7.5 kb YIpMH1S construct (Fig. 6). The mutant *pol3* fragment was sequenced to verify presence of the mutations and absence of any other undesirable sequence alterations that may have arisen during the site-directed mutagenesis and/or the various cloning procedures. No deviations from the published *POL3* sequence (Boulet *et al.*, 1989; Morrison and Sugino, 1992a) were found except for the engineered mutations.

Introduction of the mutations encoding the G447S substitution into the chromosomal POL3 gene

YIpMH1S DNA was introduced into the yeast chromosome by integrative transformation (Hinnen *et al.*, 1978). The YIpMH1S plasmid was first linearized with *Hpa*I, which cuts within the *pol3* sequence, downstream from the mutation site (Fig. 7). Linearization increases the efficiency of integration of DNA into the yeast chromosome by homologous recombination (Orr-Weaver *et al.*, 1981). The linear DNA was transformed into Ura$^-$ *S. cerevisiae* MS71 cells (Table 2). The *ura3-52* mutation in strain MS71 is a non-reverting Ty insertion mutation in the *URA3* gene (Rose and Winston, 1984), which can be complemented by the *URA3* gene on the YIpMH1S plasmid. Ura$^+$ transformants were selected on complete minimal media

Figure 6. **Yeast integrating plasmid YIpMH1S**. YIpMH1S contains the 2.2 kb mutant *pol3* gene fragment, the yeast *URA3* gene selection marker, the *E. coli* β-lactamase gene selection marker (*amp*^{r}) and the pMB1 *E. coli* replicon (ori). The *POL3* ORF is preceded by a short segment of yeast DNA. Small numbered arrows represent annealing positions for the synthetic oligonucleotides LRK127, -128, -129, -130, -131, -126, -114, and -140 (Table 3) that were used for DNA sequencing and PCR reactions.

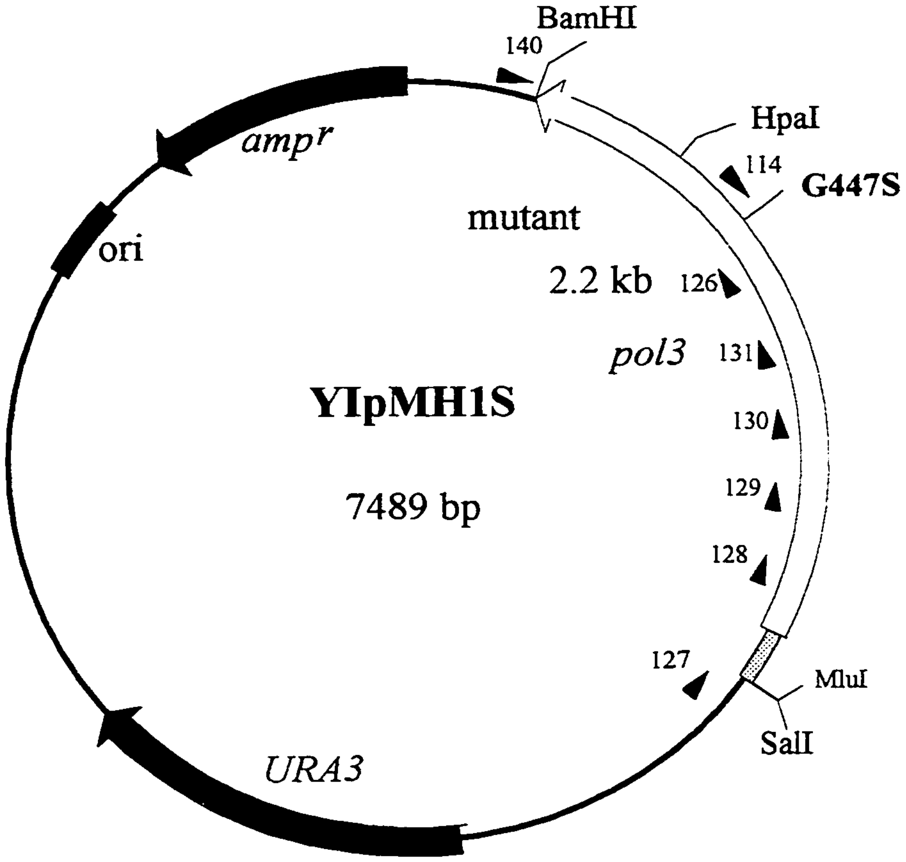
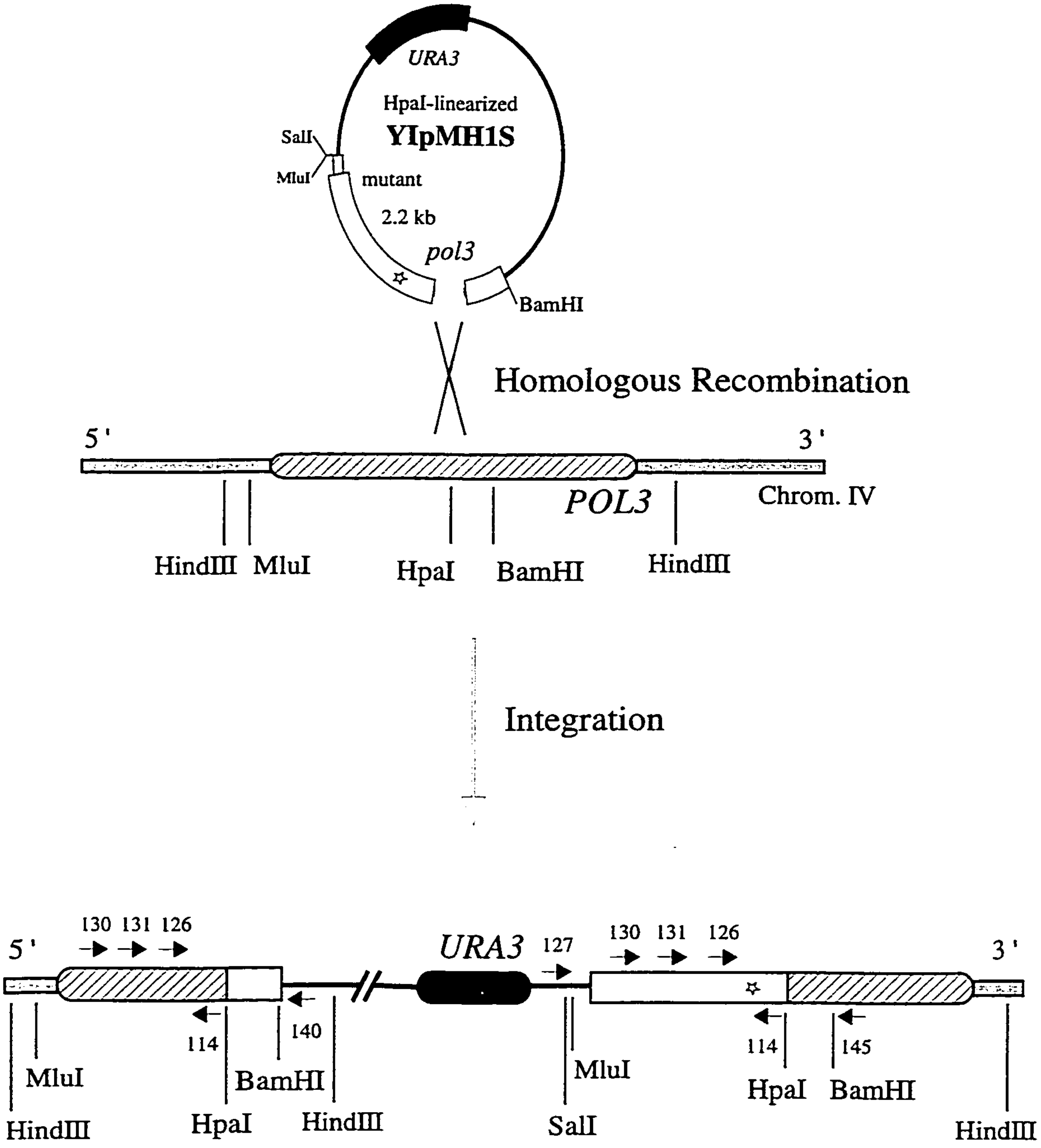

BamHI
140
HpaI
114
G447S
mutant
2.2 kb
pol3
126
131
130
129
128
127
MluI
SalI
ampr
ori
YIpMH1S
7489 bp
URA3

Figure 7. **Integration of YIpMH1S into the chromosomal *POL3* locus**. Homologous recombination between wild-type *POL3* sequences (striped) located on yeast chromosome IV (shaded) and mutant *pol3* sequences (clear) from plasmid YIpMH1S. Plasmid YIpMH1S is portrayed with a double-stranded break at the *Hpa*I site. Plasmid sequences are represented by solid black lines; the *E. coli* β-lactamase gene selection marker and the pMB1 *E. coli* replicon are not shown. Non-coding yeast sequences on YIpMH1S and on chromosome IV are shaded. The *URA3* gene is shown in black. Only part of the plasmid sequence is shown in the *POL3*-YIpMH1S integration construct. Small numbered arrows represent annealing positions for the synthetic oligonucleotides LRK114, -126, -127, -130, -131, -140, and -145 (Table 3).

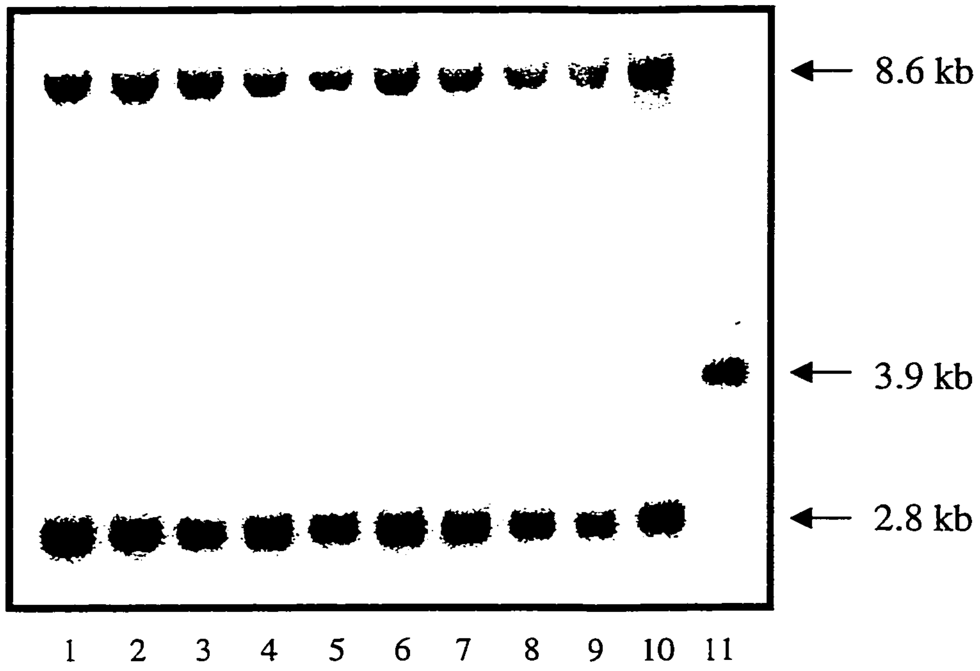

URA3
HpaI-linearized
YIpMH1S
SalI
MluI
mutant
2.2 kb
pol3
BamHI
Homologous Recombination
5'
3'
POL3
Chrom. IV
HindIII
MluI
HpaI
BamHI
HindIII
Integration
130 131 126
5'
URA3
127
130 131 126
3'
114
140
114
145
MluI
BamHI
HpaI
BamHI
HindIII
HpaI
HindIII
SalI
MluI
HindIII

POL3-YIpMH1S integration construct

lacking uracil. Complementation requires integration of the *URA3* gene into a yeast chromosome since the YIpMH1S plasmid lacks ARS and CEN elements required for stable extrachromosomal maintenance. Homologous recombination between the chromosomal *POL3* gene and the mutant 2.2 kb *pol3* fragment disrupts the chromosomal *POL3* gene (Fig. 7). Integration of plasmid YIpMH1S was verified by PCR using primers LRK131 and LRK140 (Fig. 7 and Table 3). A 1.1 kb PCR product was expected from strains with YIpMH1S DNA inserted into the *POL3* locus. The 1.1 kb PCR product was detected in twenty six out of twenty six transformants tested.

Integration was also verified by Southern hybridization analysis (Fig. 8). Genomic DNA was extracted from ten of the strains examined by PCR. The entire *POL3* gene is located within a 3.9 kb *Hind*III fragment on chromosome IV (Figure 3). Integration of YIpMH1S into *POL3* separated the two *Hind*III sites by an additional 7.5 kb, and introduced a third *Hind*III site between the original two. A genomic DNA digest with *Hind*III was expected to produce a single 3.9 kb band for the undisrupted *POL3* copy while 2.8 kb and 8.6 kb bands were expected from strains transformed to Ura$^+$ with YIpMH1S. *POL3* fragments were detected with a radiolabeled probe prepared by amplification of a 0.5 kb *POL3* sequence from YIpMH1S DNA, using primers LRK131 and LRK114 (Fig. 7 and Table 3; Materials and Methods). All ten strains examined produced the expected 2.8 kb and 8.6 kb bands. A control sample from a digest of genomic DNA from wild-type cells gave a single 3.9 kb band.

Figure 8. **Southern hybridization analysis of YIpMH1S-integrant strains**. Lanes 1-10 show samples from MS71 strains transformed to Ura^+ with plasmid YIpMH1S. The control sample in lane 11 is from a wild-type MS71 strain.


8.6 kb
3.9 kb
2.8 kb
1
2
3
4
5
6
7
8
9
10
11

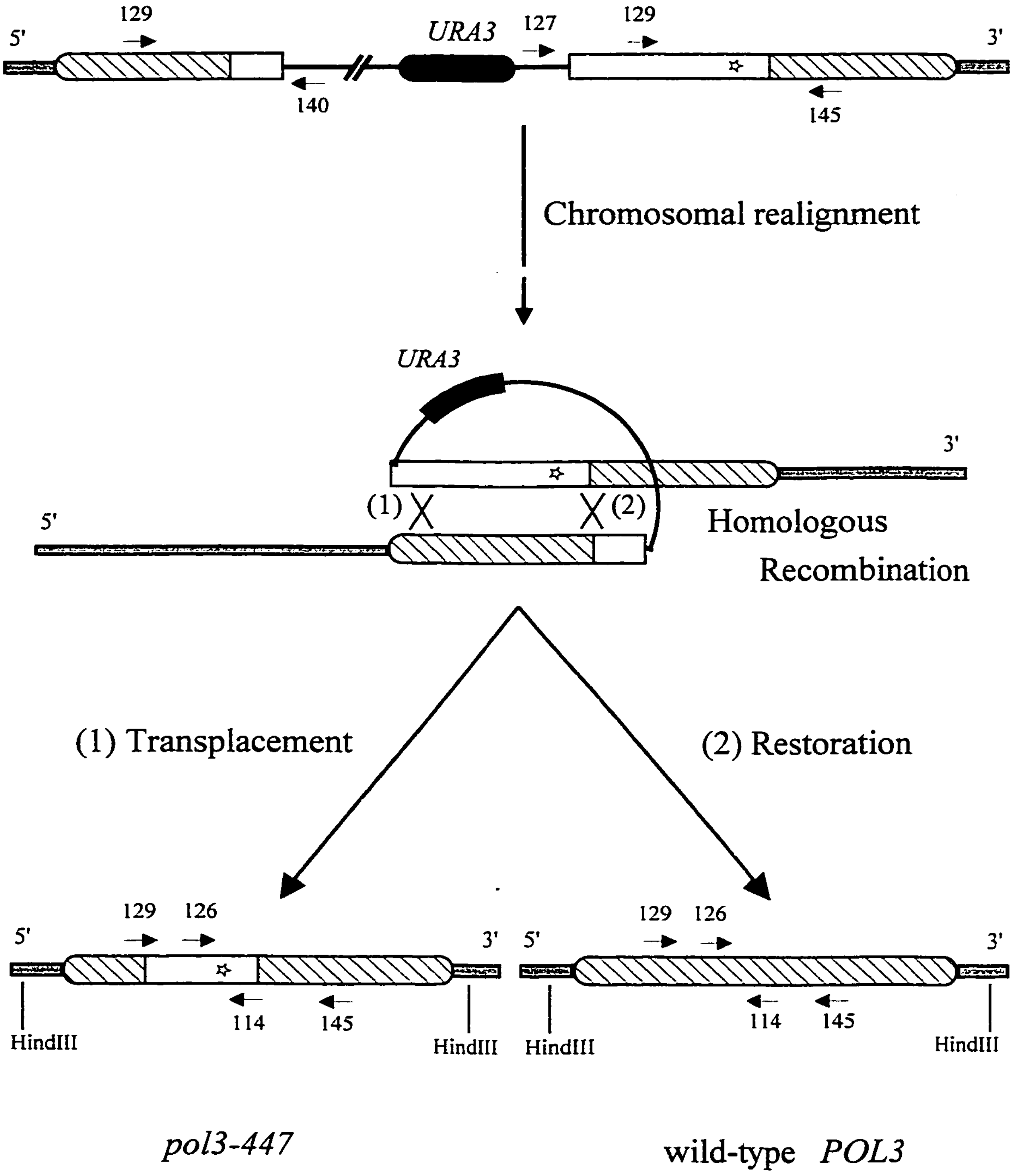
The results of the Southern hybridization analysis confirmed the presence of two copies of the 5'-2.2 kb region of the *POL3* gene in the yeast chromosome, separated by plasmid sequences and the *URA3* gene (Fig. 7). The *POL3* fragment located upstream from the *URA3* gene was expected to carry the wild-type sequence while the downstream complete gene should contain the engineered mutation. To confirm the presence of the mutation, DNA from three integrant strains was examined by sequencing. Only one of the three samples examined had the engineered mutation. Gene conversion, during integration of YIpMH1S into the chromosomal *POL3* locus, appeared to result in loss of the engineered mutations in the other two strains (Orr-Weaver *et al.*, 1988).

Restoration of the DNA configuration at the chromosomal POL3 locus

The strain carrying the mutant *pol3* gene was propagated in the presence of uracil to allow loss of the *URA3* gene. This sequence loss relies on the inherent genetic instability produced by the duplicated *POL3* sequences (Scherer and Davis, 1979). First, a chromosomal realignment brings the duplicated *POL3* and *pol3* sequences side-by-side (Fig. 9). Subsequently, recombination between the repeated sequences results in loss of one repeat, the *URA3* gene, and the plasmid DNA. Recombination could result either in transplacement, where the DNA segment containing the desired mutation remains in the chromosome, or in restoration of the wild-type DNA sequence (Fig. 9). The outcome depends upon the position of the crossover with respect to the mutation. Ura$^-$ cells were selected on medium containing 5-FOA. The drug 5-FOA prevents growth of yeast cells that carry the

Figure 9. **Restoration of the DNA configuration at the chromosomal *POL3* locus.** Homologous recombination between original chromosomal *POL3* sequences (striped) and mutant *pol3* sequences (clear) results in loss of the *URA3* gene, the YIpMH1S plasmid sequences and a fragment from either the original chromosomal *POL3* (1) or the mutant *pol3* (2) gene sequence that contains codon 447. The position of the mutation which encodes the G447S substitution is indicated by an asterisk. DNA sequences flanking the chromosomal *POL3* gene are shaded. Small numbered arrows represent annealing positions for the synthetic oligonucleotides LRK114, -126, -127, -129, -140, and -145 (Table 3).

POL3 -YIpMH1S integration construct


5'
129
URA3
127
129
3'
140
145
Chromosomal realignment
URA3
3'
(1)
(2)
5'
Homologous Recombination
(1) Transplacement
(2) Restoration
129
126
5'
3'
114
145
HindIII
HindIII
129
126
5'
3'
114
145
HindIII
HindIII
pol3-447
wild-type POL3

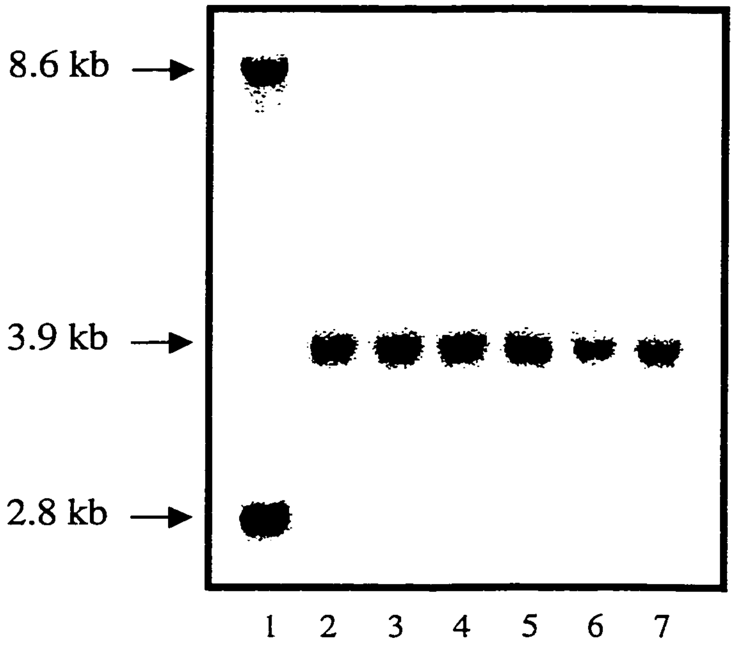
wild-type *URA3* gene but permits growth of cells (5-FOA^r) that cannot produce a functional *URA3* product (Boeke *et al.*, 1984).

To show that the selected 5-FOA^r strains had restored the DNA configuration at the chromosomal *POL3* locus, genomic DNA samples from ten 5-FOA^r strains were examined by PCR using primer pairs LRK129/140 and LRK127/145 (Table 3). PCR products of about 1.8 kb would be produced for strains with YIpMH1S integrated into the *POL3* locus, but no product was expected from 5-FOA^r strains in which the plasmid DNA was deleted. No PCR amplification product was observed. A control reaction with genomic DNA from a Ura$^+$ integrant strain showed the expected 1.8 kb band (data not shown).

DNA sequencing was used to determine whether restoration or transplacement had occurred. Six of ten strains sequenced had the wild-type sequence and four had the mutations encoding the G447S amino acid substitution. Mutant identification was confirmed by sequencing the complementary strand. The strains containing the G447S-DNA pol δ were named MIH1 (Table 2).

The DNA configuration at the chromosomal *pol3-447* locus in the four mutant strains was examined by Southern hybridization analysis (Fig. 10). A radiolabeled probe was prepared as described in Materials and Methods and used for detection of *pol3-447* sequences in *Hind*III-digested genomic DNA. All samples showed a single 3.9 kb band, which indicates that all plasmid DNA, the *URA3* gene and the wild-type copy of the duplicated *POL3* region had been removed by homologous recombination (Fig. 10).

Figure 10. **Southern hybridization analysis of *pol3-447* strains**. The samples in lanes 2-5 are from *pol3-447* strains. Control samples include a wild-type MS71 strain (lane 7) and an MS71 strain transformed to Ura$^+$ with plasmid YIpMH1S (lane 1). The sample in lane 6 is from a 5-FOA^r strain that kept the wild-type *POL3* sequence.


8.6 kb
3.9 kb
2.8 kb
1
2
3
4
5
6
7

Growth of strain pol3-447

To determine whether the G447S substitution in the yeast DNA pol δ affected DNA replication or cell division, the growth of wild-type and mutant cells was compared (Fig. 11). Similar growth curves were obtained for the wild-type and mutant strains at 30 °C (Fig. 11), and at 20 °C and 37 °C (data not shown).

DNA replication fidelity assays

The DNA replication accuracy of the G447S-DNA pol δ was assessed *in vivo* by one forward mutation assay and two reversion assays. For the forward mutation assay, mutations that produced canavanine resistance were counted. Canavanine is a lethal arginine analog. Both canavanine and arginine enter yeast cells through a permease encoded by the *CAN1* gene. Wild-type cells are sensitive to canavanine but mutation of the *CAN1* gene to prevent uptake of canavanine produces resistance (Grenson *et al.*, 1966). A total of thirty independent *pol3-447* cultures were grown in YPD to stationary phase at 30 °C. The mutation frequency was determined by first measuring the number of canavanine resistant cells in each culture and then finding the median value (Materials and Methods). The median mutation frequencies for the wild-type and mutant strains were 2.0×10^{-6} and 1.8×10^{-6}, respectively (Table 4). Mutation rates were also calculated by the method of Lea and Coulson (1949). Values of 3.4×10^{-7} and 3.1×10^{-7} for the mutant and wild-type strains were obtained, respectively. The results indicate that there is no significant difference between the mutant and wild-type strains in the frequency and rate of mutational events that confer resistance to canavanine.

Figure 11. **Growth curves for the wild-type and *pol3-447* strains, MS71 and MIH1.** The curves were constructed by titering a wild-type (◆) and a *pol3-447* (■) cultures at the indicated time-points during growth at 30 °C.

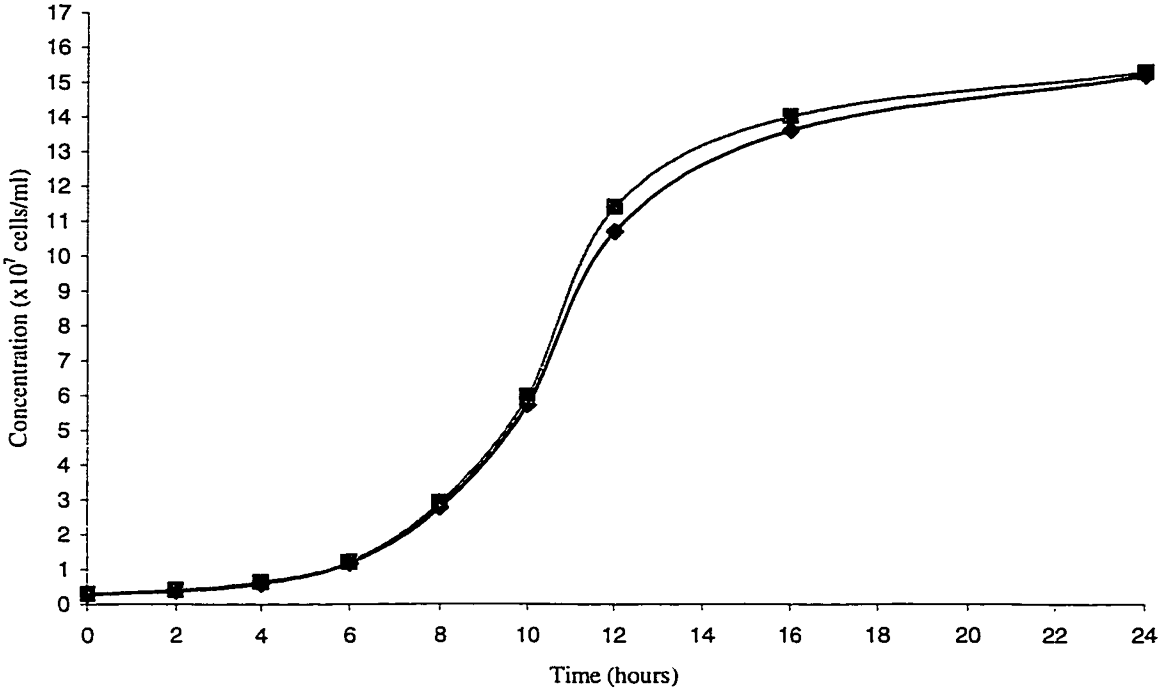

Concentration (x10^7 cells/ml)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Time (hours)
0
2
4
6
8
10
12
14
16
18
20
22
24

Table 4. **DNA replication fidelity in wild-type and G447S-DNA pol δ strains**

Marker gene	No. of cultures	Spontaneous mutation frequencies[a]		Relative reversion frequency
		POL3	*pol3-447*	
ade5-1	20	5.5×10^{-9}	43.0×10^{-9}	7.8
his7-2	10	11.5×10^{-9}	17.5×10^{-9}	1.5
CAN1	30	[b] 2.0×10^{-6}	1.8×10^{-6}	0.9

[a] Spontaneous mutation frequencies for the wild-type and *pol3-447* strains were determined as described in Materials and Methods. Data from individual cultures are shown for the *ade5-1* reversion assay in Figure 12 and for *his7-2* in Figure 14.

[b] Thomas Petes, personal communication.

The canavanine resistance assay detects mutational events that inactivate the *CAN1* gene function, e.g. base substitutions, frameshifts, additions and deletions. To assess the effect of the G447S substitution in the yeast DNA pol δ specifically on base substitutions, the frequency of reversion from adenine auxotrophy to adenine prototrophy for the wild-type and *pol3-447* strains was measured. Both strains carry the *ade5-1* allele, which contains a C to A base substitution (Fig. 12). This mutation changes the wild-type TAC codon for tyrosine to the TAA ochre codon. Reversion events at the *ade5-1* site are most likely to occur by base substitution mutations that replace the ochre chain termination codon with an amino acid codon. The amino acid codon may not necessarily encode tyrosine, if other amino acids can support the function of the ADE5 protein. Twenty cultures of wild-type and mutant cells were grown to stationary phase and dilutions were plated onto complete minimal medium lacking adenine to select for revertants, or on complete minimal medium to determine the total cell count (Materials and Methods). The median reversion frequency was $4.3\text{x}10^{-8}$ for strain *pol3-447* and $5.5\text{x}10^{-9}$ for the wild-type strain (Fig. 13 and Table 4). These results indicate an 8-fold mutator phenotype for strain *pol3-447*.

The DNA replication accuracy with the adenine reversion assay was also tested at 20 °C and 37 °C. A mutator phenotype was again observed for the *pol3-447* strain. The median reversion frequency for strain *pol3-447* was $4.6\text{x}10^{-8}$ and $4.5\text{x}10^{-8}$ at 20 °C and 37 °C, respectively. The median reversion frequency for wild-type cells was $9.6\text{x}10^{-9}$ at 20 °C and $7.3\text{x}10^{-9}$ at 37 °C (Fig. 14).

Figure 12. **Sequence of the *S. cerevisiae ade5-1* and *his7-2* alleles.** The nucleotide sequence of the *ade5-1* (a) and *his7-2* (b) alleles is shown for the region containing the mutations. The encoded amino acid residues are presented below each nucleotide codon. The numbers preceding and following the DNA and protein sequences represent nucleotide and amino acid positions, respectively. The numbers in brackets indicate the number of nucleotides/amino acids present downstream from the sequence shown. The nucleotide sequence affected by the mutation in each of the two alleles is underlined. In the *his7-2* allele, deletion of any of the eight adenines (underlined) produces the same effect.

(a)

1147 GGT ACC AAA TAC GAT TCC GCC 1167 [1242]
383 Gly Thr Lys Tyr Asp Ser Ala 389 [413]

1147 GGT ACC AAA TAA GAT TCC GCC 1167 [1241]
383 Gly Thr Lys **Ochre**

(b)

466 GAA AAG AAA AAA AAC CTA GAA 486 [1173]
156 Glu Lys Lys Lys Asn Leu Glu 162 [390]

466 GAA AAG AAA AAA ACC TAG AA 485 [1172]
156 Glu Lys Lys Lys Thr **Amber**

Figure 13. **Adenine reversion frequencies for the wild-type and *pol3-447* strains at 30 °C.** Reversion of *ade5-1* was determined for 20 cultures of wild-type (◆) and *pol3-447* (■) strains. The median value for the wild-type and *pol3-447* reversion frequencies is indicated by the solid and dotted lines, respectively.

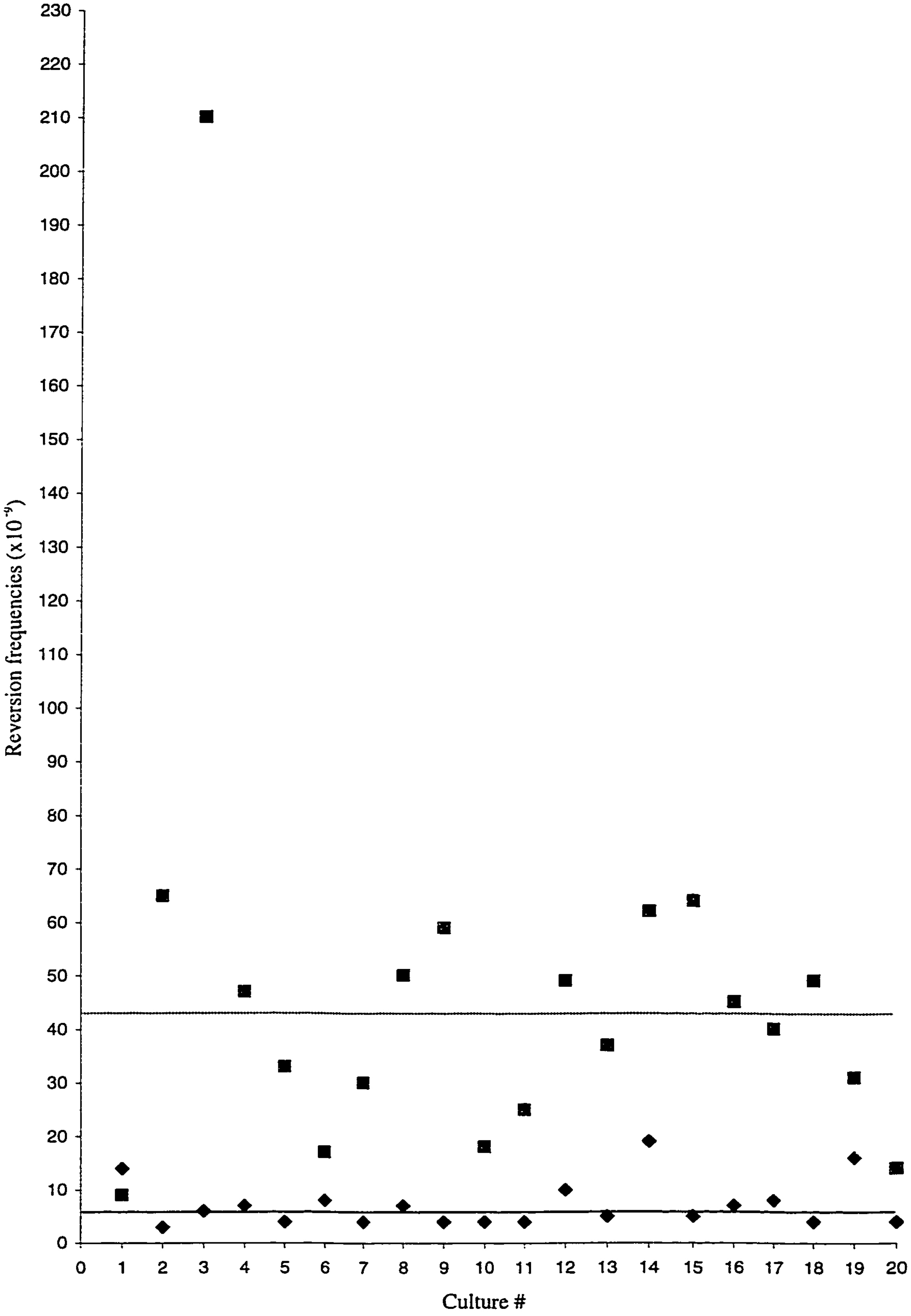
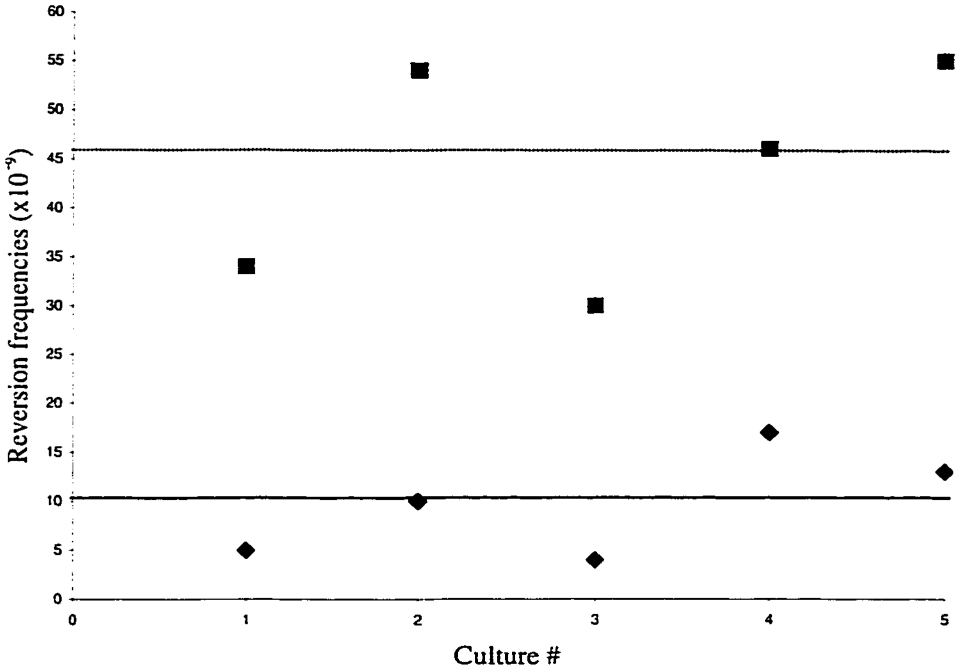
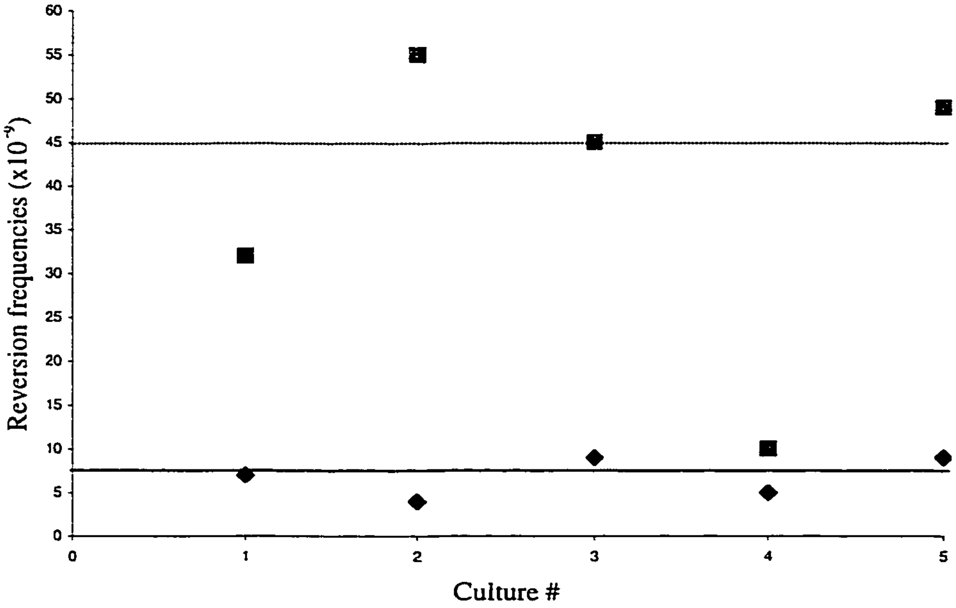

Reversion frequencies ($x10^{-9}$)
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
Culture #

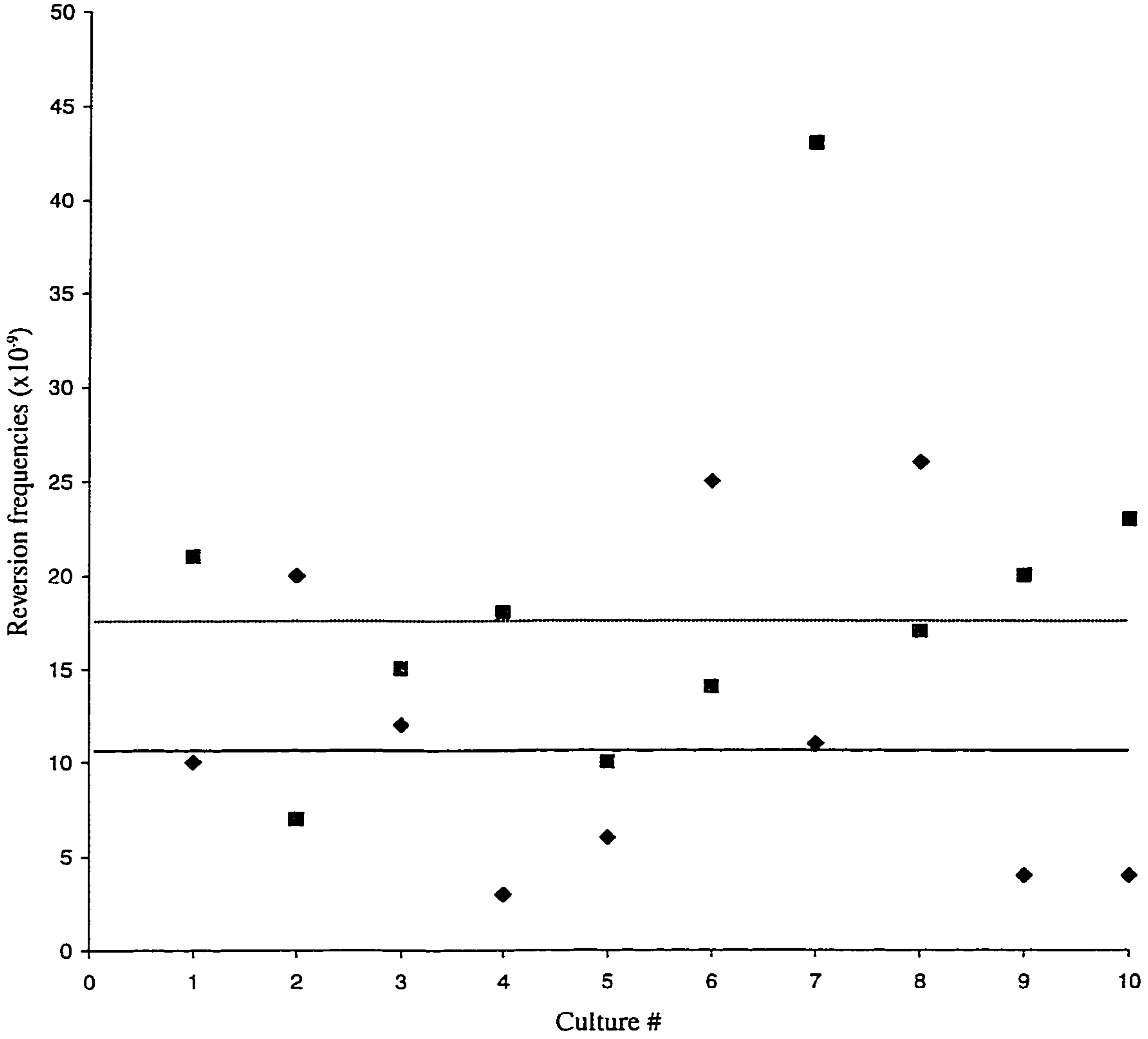
Figure 14. **Adenine reversion frequencies for the wild-type and *pol3-447* strains at 20 °C and 37 °C.** Reversion of *ade5-1* was determined for 5 cultures of wild-type (◆) and *pol3-447* (■) strains at 20 °C (a) and 37 °C (b). The median value for the wild-type and *pol3-447* reversion frequencies is indicated by the solid and dotted lines, respectively.


Reversion frequencies ($\times 10^{-9}$)
0
5
10
15
20
25
30
35
40
45
50
55
60
0
1
2
3
4
5
Culture #


Reversion frequencies ($\times 10^{-9}$)
0
5
10
15
20
25
30
35
40
45
50
55
60
0
1
2
3
4
5
Culture #

The effect of the G447S-DNA pol δ on frameshift mutations was examined by measuring the reversion frequency at the *his7-2* locus. The *his7-2* allele contains a single adenine deletion from a monotonous run of eight adenines (Fig. 12). The resulting –1 frameshift creates an amber codon immediately adjacent to the A tract. The most direct pathway to revert the original mutation is by a second frameshift that would restore the proper reading frame of the *HIS7* gene. Ten mutant and ten wild-type cultures were grown and titered on complete minimal medium and complete minimal medium lacking histidine. The median reversion frequencies with the *pol3-447* and the wild-type strains were similar, 17.5×10^{-9} and 11.5×10^{-9}, respectively (Fig. 15 and Table 4). The fluctuation in reversion frequencies for the wild-type and mutant strains is more than the difference between the median values (Fig. 15). Thus, no significant differences are detected for the mutant and wild-type strains in mutational events at the *his7-2* locus.

Figure 15. **Histidine reversion frequencies for the wild-type and *pol3-447* strains at 30 °C**. Reversion of *his7-2* was determined for 10 cultures of wild-type (◆) and *pol3-447* (■) strains. The median value for the wild-type and *pol3-447* reversion frequencies is indicated by the solid and dotted lines, respectively.


Reversion frequencies (x10^{-9})
50
45
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
10
Culture #

DISCUSSION

The proofreading pathway of the T4 DNA polymerase has been the subject of extensive investigation for many years. Mutant T4 DNA polymerases with decreased 3'→5' exonuclease activity produce more errors during DNA replication (Muzyczka *et al.*, 1972). Kinetic and mutational studies (Capson *et al.*, 1992; Reha-Krantz *et al.*, 1991; Reha-Krantz and Nonay, 1993) have revealed various aspects of the hydrolysis reaction, but until recently, relatively little has been known about how the primer-terminus is prepared for the hydrolysis reaction. Baker and Reha-Krantz (1998) and Reha-Krantz *et al.* (1998) proposed a DNA polymerase proofreading pathway with the following steps: (a) detection of a mismatched base pair in the polymerase active center, (b) DNA strand separation of at least two nucleotides, (c) transfer of the primer-terminus from the polymerase active center to form a pre-exonuclease complex, (d) positioning of the 3'-end of the primer strand in the exonuclease active center, and (e) excision of the terminal nucleotide. Formation of the transient, pre-exonuclease complex (Marquez and Reha-Krantz, 1996; Baker and Reha-Krantz, 1998) serves as a kinetic barrier to the proofreading pathway. Proofreading is prevented unless the rate of primer elongation is reduced significantly by the presence of an incorrect base pair at the primer-terminus. Further strand separation allows binding of the primer-terminus in the exonuclease active center, where the nucleotide excision reaction is catalyzed.

Is the DNA polymerase proofreading pathway discovered for the T4 DNA pol used by other DNA polymerases? This question was addressed by studying proofreading by the yeast DNA pol δ. One feature of T4 DNA pol proofreading is the requirement of a protein loop structure in forming the pre-exonuclease complex (Fig. 2; Marquez and Reha-Krantz, 1996; Baker and Reha-Krantz, 1998; Reha-Krantz *et al.*, 1998). A serine substitution for glycine 255 in the protein loop decreases the rate of formation of the pre-exonuclease complex and, thus, elevates the kinetic barrier to proofreading. As a consequence, there is an increase in the DNA replication errors. A series of studies are presented here to determine if a similar loop structure is also present in the yeast DNA pol δ, and if there, whether it functions in the proofreading pathway.

A first step was to compare protein sequences for the T4 DNA pol, the RB69 DNA pol, the yeast DNA pol δ, and the human DNA pol δ. Regions of potential sequence similarity are proposed to be located downstream from the highly conserved Exo II motif, and at about the same distance for all four DNA polymerases (Fig. 5). One of the proposed consensus sequences includes the T4 DNA pol residue glycine 255, the RB69 DNA pol glycine 258, the yeast DNA pol δ glycine 447, and the human DNA pol δ glycine 442. Structural studies show that a protein loop structure held by β strands is formed between residues 249 and 259 in the T4 DNA pol (Figs. 2 and 5) and between residues 252 and 262 in the RB69 DNA pol (Wang *et al.*, 1997). Residues G447 and G442 in the yeast and human DNA polymerases, respectively, may also reside in a loop structure, as indicated by secondary structure predictions performed by the method of King and Sternberg (1996). The same secondary structure prediction method also placed residues G255 in the T4 DNA pol and residue G258 in the RB69 DNA pol in protein turns (data not shown). One aspect of the alignment in Figure 5 is that a larger protein loop structure appears to be present in the yeast and human DNA polymerases, compared to that in the T4 and RB69 DNA polymerases (Fig. 5).

In order to examine whether the proposed loop structure in the yeast and human DNA polymerases has a function analogous to that of the G255-protein loop in the T4 DNA pol, the yeast *pol3-447* strain was constructed (Figs. 3, 4, and 7-10). Cell division in strain *pol3-447* was identical to the wild-type isogenic strain (Fig. 11), which indicates that DNA replication was not affected significantly by the G447S-DNA pol δ. This result is consistent with observations that the T4 G255S-DNA pol also does not affect DNA replication (Reha-Krantz, 1988; Stocki *et al.*, 1995).

Exonucleolytic proofreading by the G447S-DNA pol δ was examined by assessing the DNA replication fidelity of strain *pol3-447 in vivo*. A mutator phenotype was expected for strain *pol3-447* if proofreading is reduced by the G447S substitution. This prediction was tested by two reversion assays and one forward mutation assay. Strain *pol3-447* was found to elevate the reversion frequency from adenine auxotrophy to adenine prototrophy by about 8-fold, compared to the isogenic wild-type strain (Figs. 13-14 and Table 4). Since the *ade5-1* allele contains a C to A nonsense mutation ($UAC^{tyr} \rightarrow UAA^{ochre}$), it is assumed that reversion to adenine prototrophy is due to base substitution mutations. Thus, the G447S-DNA pol δ appears to be more error-prone than the wild-type enzyme for base substitution mutations.

Two possibilities were considered that could account for the elevated mutation frequency seen for base substitution mutations by the G447S-DNA pol δ.

The G447S substitution could distort the 3-dimensional conformation of the exonuclease domain. This possibility would imply that reduction in proofreading by the G447S substitution is not specific. Alternatively, residue G447 or the structure in which it resides could be an integral part of the DNA pol δ proofreading pathway. In this case, the G447S substitution affects primarily the protein loop structure. The location of glycine 447 in a proposed protein loop structure (Fig. 5), as found for G255 in the T4 DNA pol (Fig. 2), is consistent with the second possibility.

If the yeast residue G447 performs a role in proofreading as detected for residue G255 in the T4 DNA pol, a large reduction in DNA polymerase replication fidelity is expected since the T4 G255S-DNA pol confers about a 60-fold mutator phenotype (Stocki *et al.*, 1996). Yet, only an 8-fold mutator phenotype was observed in *pol3-447* cells (Figs. 13-14 and Table 4). A number of reasons can account for the difference in the magnitude of the mutator phenotypes. First, the T4 DNA pol 3'→5' exonuclease activity is the only DNA polymerase error-correcting mechanism in bacteriophage T4. Yeast, however, has a highly effective post-replication mismatch repair activity that is absent in T4. Another difference is that T4 has a single DNA polymerase, while several are present in yeast. The essential yeast DNA pol ε also has a 3'→5' exonuclease proofreading activity (Morrison *et al.*, 1990).

In a model for DNA replication error correction in yeast, DNA is replicated by DNA polymerases α, δ and ε. Incorrect nucleotides are removed by the 3'→5' exonuclease activities of DNA pol δ and DNA pol ε, and any remaining errors are corrected by the mismatch repair system (Morrison and Sugino, 1992b). Studies with proofreading-defective D321A+E323A DNA pol δ (*pol3-01*) and D290A+E292A DNA pol ε (*pol2-4*) mutants, indicate a synergistic relationship between the proofreading functions of the two polymerases (Morrison and Sugino, 1994). Synergy, i.e. the effect of the double mutation is greater than the sum of the single mutations, can result from action in series or from competition for the same pool of replication errors. The synergy observed for the two proofreading DNA polymerases resulted from competition for the same pool of replication errors (Morrison and Sugino, 1994). Replication errors in yeast can also be corrected by mismatch repair, an activity that requires the *PMS1* gene, which is analogous to the bacterial *mutL* gene (B. Kramer *et al.*, 1989; W. Kramer *et al.*, 1989). The mismatch repair pathway acts in series with the 3'→5' exonuclease activities of both DNA pol δ and DNA pol ε (Morrison *et al.*, 1993, Morrison and Sugino, 1994). A *pol3-01 pms1* haploid was inviable, presumably due to a catastrophically high mutation rate. A double homoallelic *pol3-01/pol3-01 pms1/pms1* diploid was viable and displayed a relative mutation rate that is a multiple of the relative mutation rates of the respective *pol3-01/pol3-01* and *pms1/pms1* mutants, indicating action in series on the same potential mutations. A similar effect was also observed with a *pol2-4 pms1* double mutant, although in this case the haploid strain was viable.

Additional evidence for action in series between the proofreading and mismatch repair pathways in yeast is the apparent similarity in the specificity for single base mutations for both systems *in vivo* (B. Cramer *et al.*, 1989; Morrison and Sugino, 1994). Strains *pol3-01* and *pol2-4* produced all classes of single-base mutations (substitutions, insertions, and deletions) except G:C→C:G transversions.

Interestingly, the mismatch repair system acts with high or intermediate efficiency on single nucleotide loops and all single base mismatches, except C:C. Thus, mutations that escape proofreading could potentially be recognized and corrected by the mismatch repair system, except for C:C.

The conclusion from the above discussion is that yeast cells, as opposed to bacteriophage T4, have the ability to compensate for a deficiency in the exonucleolytic proofreading pathway of DNA pol δ (or DNA pol ε). Therefore, it is possible that the mutator phenotype observed for strain *pol3-447* with the adenine reversion assay is an underestimate of the decreased efficiency of the G447S-DNA pol δ to proofread. This reasoning can explain the smaller mutator effect observed with the G447S-DNA pol δ, compared to that of the G255S-T4 DNA pol. The same arguments can also help explain the fact that a mutator phenotype was not observed with the forward mutation assay to canavanine resistance. As discussed, elevation of mutation frequencies caused by the G447S-DNA pol δ may be difficult to detect in cells equipped with additional proofreading mechanisms.

Studies were also performed to assess replication by the G447S-DNA pol δ on sequences with simple repeats. Frameshifts can result from additions or deletions of any number of nucleotides that lead to a shift in the proper reading frame of a gene. Streisinger *et al.* (1966) proposed that during DNA replication or repair synthesis of DNA templated with simple repeats, the primer and template strands separate and occasionally reanneal in a misaligned configuration. The result is a loop of unpaired bases in one of the DNA strands, which if not repaired, will lead to either an addition or a deletion in the number of repeat units. It is not known if the

DNA strands misalign spontaneously or if misalignment is created by the DNA polymerase. If the DNA polymerase plays a role in strand misalignment, proofreading provides an opportunity since the primer strand is separated from the template. The T4 G255S-DNA pol is defective in strand separation during proofreading and thus, may *reduce* frameshift mutagenesis, even though this defect *increases* base substitution mutagenesis. If the G447S substitution in the DNA pol δ is analogous to the T4 G255S substitution, then the G447S-DNA pol δ is predicted to reduce frameshift mutations. This hypothesis was tested with the histidine reversion assay. Strain *pol3-447* carries the *his7-2* allele, which contains a –1 frameshift, caused by deletion of a single adenine from a run of eight consecutive adenines (Fig. 12). Reversion events can most likely occur by either a +1 addition or a –2 deletion that would restore the proper reading frame in the *HIS7* gene. The reversion frequency measured for strain *pol3-447* was about the same as that observed for the wild-type strain (Fig. 15 and Table 4). The predicted reduction in frameshift mutations could also have been masked by the presence of the mismatch repair system. It would be difficult to observe a decrease in frameshifts when mismatch repair reduces these types of mutations to near background levels.

Ongoing experiments and future directions

To better characterize the G447S-DNA pol δ it would be informative to know the nature of the DNA replication errors made by this mutant enzyme. This can be accomplished by DNA sequence analysis of the *ade5-1* and *his7-2* sites in Ade$^+$ and His$^+$ revertant strains, respectively. By comparing the mutational spectra obtained from the *pol3-447* and wild-type strains that lead to reversion events of the *ade5-1* and *his7-2* sites, it will be possible to determine if the G447S-DNA pol δ makes the same type of errors as the wild-type enzyme.

The DNA replication fidelity of strain *pol3-447* should also be assessed in the absence of mismatch repair, to avoid the masking effects caused by this powerful error correction pathway. The mismatch repair deficient *msh2* strain elevates reversion rates of frameshift mutations by about 1000-fold (Reenan and Kolodner, 1992). If DNA polymerase-assisted strand separation is important for misalignment mutagenesis, and the yeast G447S-DNA pol δ has a function that is analogous to that of the T4 G255S-DNA pol, then, a *pol3-447 msh2* double-mutant strain is predicted to have reduced rates of frameshift mutations. Frameshift mutagenesis can be studied with the *his7-2* allele (Fig. 12). A series of plasmids with different repeats have also been constructed by Thomas Petes to study misalignment mutagenesis in yeast (Strand *et al.*, 1993). Using these plasmids, simple repeat instability can be examined in the *pol3-447 msh2* double mutant *in vivo*.

The yeast DNA pol δ amino acid sequence that contains the predicted G447-protein loop structure is proposed to be functionally analogous to the G255-protein loop in the T4 DNA pol. A direct way of testing this hypothesis is by constructing a chimeric T4 DNA pol in which the phage sequence from the Exo II motif to the proposed new conserved exonuclease region has been replaced by the corresponding yeast sequence (Fig. 5). If the function of the chimeric T4 DNA polymerase is similar to that of the wild-type T4 DNA pol, it would be strong evidence in support of the hypothesis. Alternatively, a chimeric yeast DNA pol δ, containing T4 DNA pol sequences, could be constructed and examined for DNA replication accuracy.

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