In Vivo Consequences of Putative Active Site Mutations in Yeast DNA Polymerases α, ε, δ, and ζ

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ABSTRACT

Several amino acids in the active site of family A DNA polymerases contribute to accurate DNA synthesis. For two of these residues, family B DNA polymerases have conserved tyrosine residues in regions II and III that are suggested to have similar functions. Here we replaced each tyrosine with alanine in the catalytic subunits of yeast DNA polymerases α, δ, ε, and ζ and examined the consequences *in vivo*. Strains with the tyrosine substitution in the conserved SL/MYPS/N motif in region II in Polδ or Polε are inviable. Strains with same substitution in Rev3, the catalytic subunit of Polζ, are nearly UV immutable, suggesting severe loss of function. A strain with this substitution in Polα (*pol1-Y869A*) is viable, but it exhibits slow growth, sensitivity to hydroxyurea, and a spontaneous mutator phenotype for frameshifts and base substitutions. The *pol1-Y869A/pol1-Y869A* diploid exhibits aberrant growth. Thus, this tyrosine is critical for the function of all four eukaryotic family B DNA polymerases. Strains with a tyrosine substitution in the conserved NS/VxYG motif in region III in Polα, -δ, or -ε are viable and a strain with the homologous substitution in Rev3 is UV mutable. The Polα mutant has no obvious phenotype. The Polε (*pol2-Y831A*) mutant is slightly sensitive to hydroxyurea and is a semidominant mutator for spontaneous base substitutions and frameshifts. The Polδ mutant (*pol3-Y708A*) grows slowly, is sensitive to hydroxyurea and methyl methanesulfonate, and is a strong base substitution and frameshift mutator. The *pol3-Y708A/pol3-Y708A* diploid grows slowly and aberrantly. Mutation rates in the Polα, -δ, and -ε mutant strains are increased in a locus-specific manner by inactivation of *PMS1*-dependent DNA mismatch repair, suggesting that the mutator effects are due to reduced fidelity of chromosomal DNA replication. This could result directly from relaxed base selectivity of the mutant polymerases due to the amino acid changes in the polymerase active site. In addition, the alanine substitutions may impair catalytic function to allow a different polymerase to compete at the replication fork. This is supported by the observation that the *pol3-Y708A* mutation is recessive and its mutator effect is partially suppressed by disruption of the *REV3* gene.

THE fidelity of chromosomal DNA replication depends on the base selectivity of DNA polymerases, on exonucleolytic proofreading, and on DNA mismatch repair. Genetic studies of proofreading- and mismatch repair-deficient strains of yeast and *Escherichia coli* (*e.g.*, Morrison and Sugino 1992; Morrison *et al.* 1993; Schaaper 1993) indicate that, among these steps, the greatest contribution to a low spontaneous mutation rate is provided by the high base selectivity of replicative DNA polymerases. The major replicative DNA polymerases catalyze DNA synthesis with error rates ranging from 10^{-4} to 10^{-7} (reviewed in Roberts and Kunkel 1996; Kunkel and Bebenek 2000). At least three DNA polymerases are required for replication of the eukaryotic nuclear genome, DNA polymerase α (Polα), Polδ, and Polε (Sugino 1995; Burgers 1998; Waga and Stillman 1998). Recent studies (reviewed in Friedberg and Gerlach 1999; Friedberg *et al.* 2000; Goodman and Tippin 2000) suggest that additional, specialized DNA polymerases also operate under special circumstances. For example, Polζ and Polη are required when the replication machinery encounters lesions in DNA (Lawrence 1996; Johnson *et al.* 1999; Masutani *et al.* 1999) and Pol4 may be involved in nonhomologous end joining (Wilson and Lieber 1999).

Our understanding of the mechanisms responsible for the high base selectivity of polymerization has been greatly facilitated by structure-function studies of DNA polymerases. The crystal structures of family A (Pol I family) DNA polymerases (*E. coli* Pol I, Taq Pol, T7 Pol) suggest that the binding pocket for the newly forming base pair snugly accommodates correct Watson-Crick base pairs but excludes mispairs with abnormal geometry (reviewed in Kunkel and Bebenek 2000). The importance of this binding pocket to base selection is indicated by functional studies of mutant DNA polymerases whose fidelity is increased or decreased by amino acid

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substitutions in or near the DNA polymerase active site (Minnick *et al.* 1999). The present study was initiated on the basis of observations with two mutants of the large Klenow fragment of *E. coli* DNA polymerase I. These mutants contain alanine substituted for residues that are invariant in family A polymerases, Glu710 and Tyr766. Both mutant enzymes have reduced DNA synthesis fidelity *in vitro* (Carroll *et al.* 1991; Bell *et al.* 1997; Minnick *et al.* 1999). This is consistent with the structural location of these two amino acids at the polymerase active site and their importance in geometric selection of correct base pairs.

Just as for family A enzymes, the polymerase domains of three family B polymerases (Polα family), RB69 Pol (Wang *et al.* 1997) and two Archea polymerases (Hopfner *et al.* 1999; Zhao *et al.* 1999), are composed of fingers, thumb, and palm subdomains. The palm subdomains of these enzymes contain catalytic carboxylates in positions that are thought to be structurally equivalent to those in Pol I family polymerases. When these catalytic residues in Klentaq Pol (a family A homolog of Klenow fragment Pol) and RB69 Pol are superimposed, RB69 residues Tyr416 and Tyr567 are found in positions that are similar to those of the Glu710 and Tyr766 residues of Klenow fragment Pol mentioned above. This led to the suggestion (Wang *et al.* 1997) that Tyr416 and Tyr567 in RB69 Pol may have functions that are similar to those of Glu710 and Tyr766 in Klenow fragment Pol. Interestingly, these two tyrosine residues are conserved in family B polymerases from bacteriophage to man, including the yeast replicative Polα, -δ, and -ε and the specialized Polζ (Figure 1). This information led to this study, whose objective was to extend our previous structure-function analyses of DNA polymerase fidelity *in vitro* to an *in vivo* setting in eukaryotic cells. Here we examine the importance of two conserved tyrosine residues in the three yeast replicative DNA polymerases and in the Polζ involved in UV mutagenesis. These represent all the known yeast family B DNA polymerases. To obtain a more global view of mutator effects conferred by these mutant enzymes, we first constructed a novel yeast strain to concomitantly measure rates for various types of mutations at five different loci. We then introduced into this strain polymerase alleles encoding alanine instead of either of the two tyrosines in the catalytic subunits of Polα, -δ, -ε, and -ζ. We examined the effects of these mutations on growth, sensitivity to DNA damaging agents, and spontaneous (for replicative polymerases) or UV mutagenesis (for Polζ). These effects were examined in an otherwise wild-type background and in combination with defects in DNA mismatch repair or specialized DNA polymerases.

MATERIALS AND METHODS

Plasmids: Chromosomal DNA polymerase mutations were generated by an integration-excision method (see Morrison *et al.* 1991). Plasmid p173 (Figure 2; Kirchner *et al.* 2000) contains the *Bam*HI-*Bsp*EI fragment of *POL2* cloned in the *Bam*HI-*Ava*I site of pFL34* [similar to pFL34 but with *URA3* in the other orientation (Tran *et al.* 1995, 1997)]. Plasmid p174 (kindly provided by Dr. H. Tran) contains the *Bam*HI-*Sst*I fragment of *POL2* cloned in the *Bam*HI-*Sac*I site of pFL34*. These plasmid variants with desired DNA sequence changes were cut by *Age*I (p173), *Acc*65-I, or *Tth*111-I (p174) prior to transformation, to create the *POL2* (Polε) gene mutations. For creation of mutations in the *POL3* (Polδ) gene, plasmid p170 (Kokoska *et al.* 1998) variants with desired mutations made as described below were cut by *Hpa*I or *Bse*RI prior to transformation. For delivery of mutations into the *POL1* (Polα) gene, a new plasmid was constructed. First, the *Bgl*II fragment with the *URA*3 gene from plasmid p170 was made blunt ended using Klenow fragment of DNA polymerase I and then cloned into the *Bst*Z17-I site of the low copy number plasmid pMBL18 (also with blunt ends; Nakano *et al.* 1995), thus generating plasmid pYIYI18. In this construct, the α-complementing *lacZ* region with the polylinker remains intact. Transformants with a Ura^+ phenotype were selected by complementing the *pyrF* mutation in *E.coli* strain MC1066 (Casadaban *et al.* 1983). Active site mutations were created as described below in pPol1-8 (Budd and Campbell 1987). Then, 3.7-kb *Hin*dIII fragments with the N-terminal part of the *POL1* gene from pPol1-8 mutant variants were cloned into the *Hin*dIII site of pYIYI18 to generate pYIAL31-pol1-Y869A and pYIAL31-pol1-Y951A. These plasmids were cut with either *Eco*47-III or *Bsp*EI to target integration into the *POL1* locus. For delivery of mutations into the *REV3* (Polζ) gene, the plasmid pRevLCav2 was constructed. A 2.3-kb *Ava*II fragment containing the C-terminal part of the *REV3* from the plasmid pJA6 (Morrison *et al.* 1989) was cloned into *Sma*I site of pYIYI18. Site-directed mutagenesis was performed as described below and pRev3Cav2 variants with desired mutations were cut by *Sna*BI prior to transformation to target integration into the *REV3* gene. Plasmid pAM58 (Morrison *et al.* 1993) was used to disrupt the *PMS1* gene and plasmid pAM56 (A. Morrison, personal communication) was used to disrupt *REV3* with the *LEU2* gene.

Yeast strains: Yeast strains used in this study are listed in Table 1. To simultaneously determine forward mutation rates as well as rates of reversion of a frameshift mutation and rates of transitions and transversions, we constructed a new yeast strain, 8C-YUNI101. Standard methods of yeast genetics were used (Rose *et al.* 1990). We began with strain CG379-3-29RL, a derivative of the strain CG379Δ (Shcherbakova *et al.* 1996) with the *ura3-29* allele inserted into chromosome III in a RL orientation (Morrison and Sugino 1994) in the *BIK1* locus (see Morrison and Sugino 1994; Shcherbakova and Pavlov 1996 and Saccharomyces Genome Database). A *pol3-01* variant of this strain (exoD-CG379-3-29RL) was crossed to S111-2-11 (Budd and Campbell 1993) to generate the diploid strain SPJ1. A meiotic segregate of this diploid, 5B-SPJ1, was crossed to h1-27B-SPJ5, which is a His^+ revertant (generated by transformation with a wild-type *HIS3* PCR fragment) of 27B-SPJ5. 27B-SPJ5 resulted from a cross of CG379Δ to W303-1A (Thomas and Rothstein 1989). The cross between 5B-SPJ1 and 27B-SPJ5 generated the diploid strain YUNI101. Tetrad analysis of a YUNI101 diploid gave 8C-YUNI101 (*MAT***a** *his7-2 leu2-3,112 ura3*Δ *bik1::ura3-29RL trp1-1*$_{UAG}$ *ade2-1*$_{UAA}$). PCR analysis showed that this segregate does not have a mutation in *RAD5* gene that could be inherited from W303-1A (see McDonald *et al.* 1997).

Strain 8C-YUNI101 permits measurement of the forward mutation rate at the *CAN1* locus, the rate of reversion of the *ura3-29* missense mutation [originally induced by the base analogue HAP, reversion occurs mainly by intragenic events (Shcherbakova and Pavlov 1996 and our results below)],

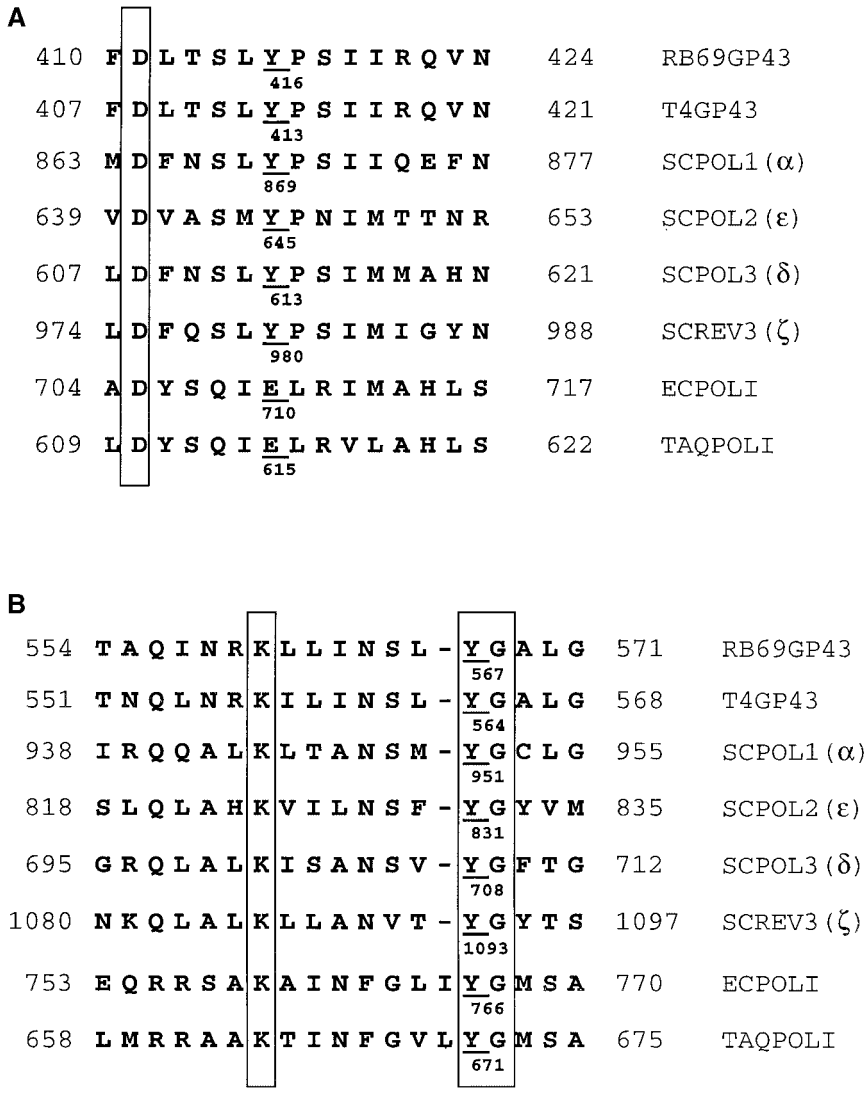

FIGURE 1.—Alignment of amino acid sequences of regions II (A) and III (B) of yeast DNA polymerases with RB69 and T4 polymerases, polymerase I, and Taq polymerase. Alignment is based on the work of DELARUE *et al.* (1990). Region II has a different aligning of E710 of Klenow and Taq polymerases as suggested by crystal structures of RB69 and Klenow DNA polymerase superimposition (WANG *et al.* 1997). Strictly conserved amino acids are boxed. Amino acids on which this study is focused are underlined. RB69GP43, DNA polymerase of bacteriophage RB69; T4GP43, DNA polymerase of bacteriophage T4; SCPOL1, SCPOL2, SCPOL3, and SCREV3, corresponding yeast *Saccharomyces cerevisiae* DNA polymerases Polα, Polε, Polδ, and Rev3; ECPOLI, *E. coli* DNA polymerase I; TAQPOLI, *Thermus aquaticus* DNA polymerase I.

reversion of nonsense mutations *trp1-1* and *ade2-1* that occurs via both intra- and intergenic events, and reversion of the *his7-2* mutant allele that occurs mainly via +1 or −2 frameshifts in a homopolymeric run (SHCHERBAKOVA and KUNKEL 1999 and our results below). Other strains used included YUNI190, a diploid strain resulting from crossing 8C-YUNI101 and CG379-3-29RL; YUNI192, a diploid resulting from crossing Del708-8C-YUNI101 and Del708-CG379-3-29RL (both containing the *pol3-Y708A* mutation); and YUNI200 diploid series resulting from crossing 8C-YUNI101 and E134 or their polymerase and mismatch repair defective derivatives.

Mutants of the basic strains were prepared using the integration-excision method and by targeted gene disruption via transformation with fragments of plasmids or PCR fragments carrying a selectable *kanMX* cassette or a hygromycin B-resistance marker flanked by short sequence homology to the target gene (WACH *et al.* 1994; GOLDSTEIN and MCCUSKER 1999). The *REV3* gene was disrupted either by *LEU2* using the pAM56 plasmid cut by *Xba*I or by PCR fragments with antibiotic resistance markers. The *RAD30* and *POL4* genes were disrupted by the *kanMX* cassette. The primers used are listed in Table 2A. Double and triple mutants were constructed by sequential transformations with corresponding plasmids. The last mutation generated was always in the DNA mismatch repair genes, since these mutations cause a strong mutator phenotype.

Generation of DNA polymerase point mutations: Mutations were introduced into relevant plasmids (Figure 2) by PCR using the primers shown in Table 2B. Plasmid DNA from four to six individual bacterial transformants after mutagenesis was purified and sequenced. At least two independent isolates containing the correct mutant sequence and no other changes in 0.5–1 kb in the vicinity of the alanine codon were used for constructing the yeast mutant strains. Consistent results were obtained with duplicate isolates in every case. The polymerase mutations were introduced into the chromosome by integration of appropriately cut plasmids with selectable *URA3* marker (described above) to target integration into polymerase genes (see MORRISON *et al.* 1991). Initial transformants possess duplication of part of a polymerase gene. Integrants were grown on 5′-fluoroorotic acid containing medium to select for excision of *URA3* and reconstitution of the original chromosomal structure. The presence of the mutation in the chromosomal DNA was detected by PCR using primers with 3′-ends complementary to either the mutant or wild-type sequence. We used a set of four primers for each DNA polymerase to detect both E710 and Y766 homolog mutations (Table 2, C–F, set 1). The strategy is explained here for Polα. Primer al 869Y WTL (Table 2C) is complementary to the wild-type sequence at the *pol1-Y869A* site. Primer al 869A L is complementary to the mutated sequence at the *pol1-Y869A* site. Primer al 951Y WTR is a reverse complement to the wild-type sequence

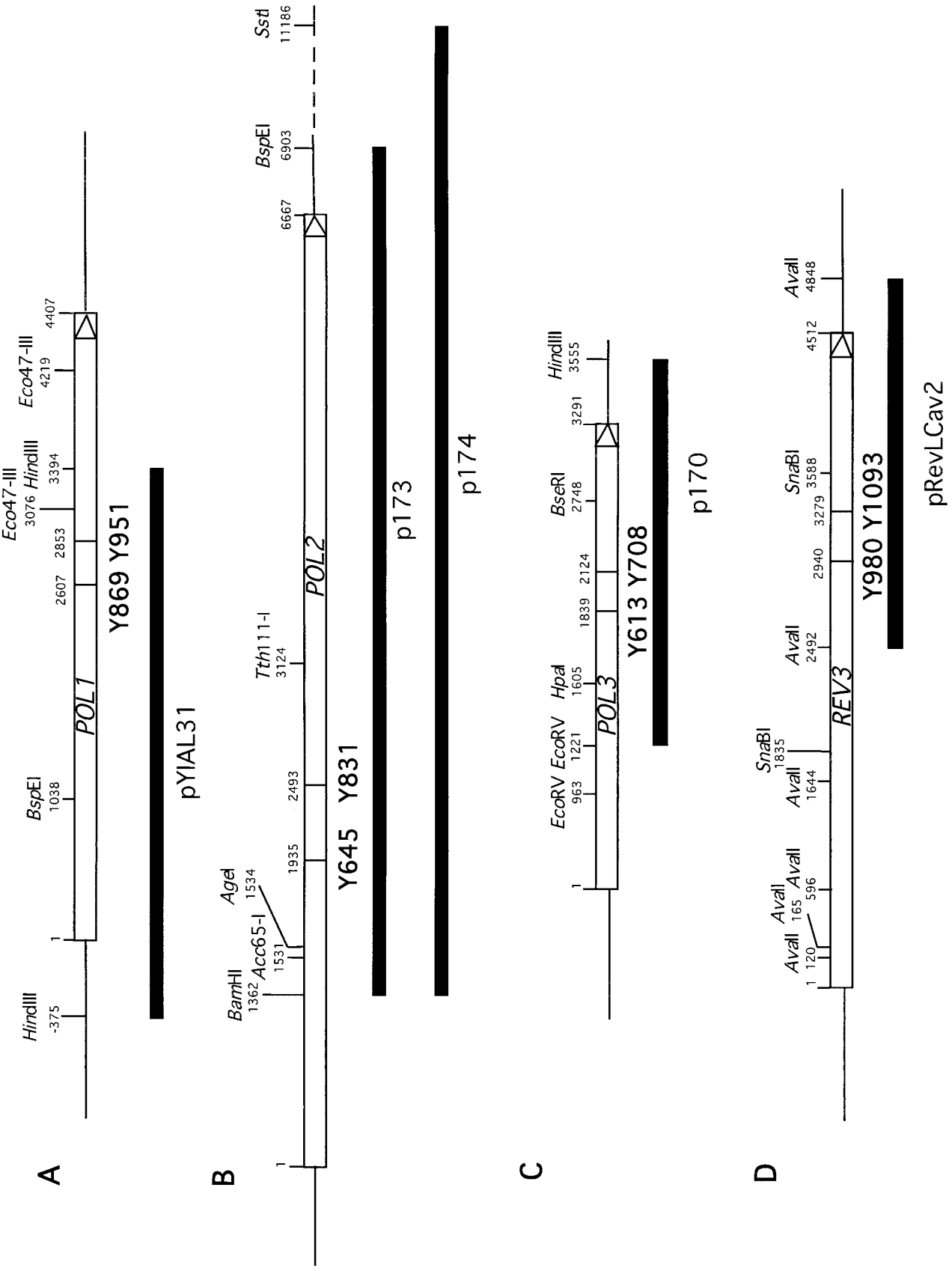
A
HindIII
-375
BspEI
1038
1
POL1
2607
Eco47-III
2853
HindIII
3076
3394
Eco47-III
4219
4407
Y869 Y951
pYIAL31
B
BamHI
1362
Acc65-I
1531
AgeI
1534
1935
Tth111-I
2493
3124
POL2
1
6667
BspEI
6903
SstI
11186
Y645 Y831
p173
p174
C
EcoRV
963
EcoRV
1221
HpaI
1605
POL3
1
1839
2124
BseRI
2748
3291
HindIII
3555
Y613 Y708
p170
D
AvaII
1
AvaII
120
AvaII
165
AvaII
596
SnaBI
1644
1835
AvaII
2492
REV3
2940
SnaBI
3279
3588
AvaII
4512
4848
Y980 Y1093
pRevLCav2

TABLE 1

Yeast strains used in this study

Strain	Genotype of haploids or names of haploid parents for diploids	Reference
CG379-3-29RL	*MAT*α *ura3*Δ *leu2-3,112 trp1-289 bik1::ura3-29RL his7-2 ade5-1 lys2-B15*	Shcherbakova and Pavlov (1996)
exoD-CG379-3-29RL	*MAT*α *ura3*Δ *leu2-3,112 trp1-289 bik1::ura3-29RL his7-2 ade5-1 lys2-B15 pol3-01*	Shcherbakova and Pavlov (1996)
S111-2-11	*MAT***a** *pol2-11 trp1-289 hisX leu2-3,112 ade2-101 his3 gal2 can1*	Budd and Campbell (1993)
CG379Δ	*MAT*α *ura3*Δ *leu2-3,112 trp1-289 his7-2 ade5-1 lys2-B15*	Shcherbakova *et al.* (1996)
SPJ1	Cross of exoD-CG379-3-29RL to S111-2-11	This work
5B-SPJ1	*MAT*α *ade2-201 trp1-289 his7-2 his3 leu2-3,112 ura3*-Δ *bik1::ura3-29RL*	This work
W303-1A	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 rad5-G535R*	Thomas and Rothstein (1989)
SPJ5	Cross of CG379Δ to W303-1A	This work
27B-SPJ5	*MAT***a** *ade2-1 his3-11,15 ura3*-Δ *leu2-3,112 trp1-1 lys2-B15*	This work
h1-27B-SPJ5	*MAT***a** *ade2-1 ura3*-Δ *leu2-3,112 trp1-1 lys2-B15*	This work
YUNI101	Cross of 5B-SPJ1 to h1-27B-SPJ5	This work
8C-YUNI101	*MAT***a** *his7-2 leu2-3,112 ura3*Δ *bik1::ura3-29RL trp1-1$_{UAG}$ ade2-1$_{UAA}$*	This work
E134	*MAT*α *ura3-52 leu2-3,112 trp1-289 his7-2 ade5-1 lys2::InsE$_{A14}$*	Shcherbakova and Kunkel (1999)
YUNI190	Cross of 8C-YUNI101 and CG379-3-29RL	This work
Del708-8C-YUNI101	*MAT***a** *his7-2 leu2-3,112 ura3*Δ *bik1::ura3-29 RL trp1-1$_{UAG}$ ade2-1$_{UAA}$ pol3-Y708A*	This work
Del708-CG379-3-29RL	*MAT*α *ura3*Δ *leu2-3,112 trp1-289 bik1::ura3-29RL his7-2 ade5-1 lys2-B15 pol3-Y708A*	This work
YUNI192	Cross of Del708-8C-YUNI101 and Del708A-CG379-3-29RL	This work
YUNI200	Cross of 8C-YUNI101 and E134	This work

at the *pol1-Y951A* mutation site and primer al 951A R is a reverse complement to mutated sequence at the *pol1-Y951A* mutation site (3′-end bases critical for discrimination are shown in boldface in Table 2). A combination al 869Y WTL/al951Y WTR amplifies a DNA fragment with the wild-type sequence of *POLI*; the al 869A L/al 951Y WTR combination amplifies a DNA fragment with the *pol1-Y869A* mutation, and the al 869Y WTL/al 951A R combination amplifies the *pol1-Y951A* mutation. The wild-type strain gives a positive signal only with the first pair of primers, the majority of initial integrants give signals with the wild-type and mutant primers, and the final mutants give a positive signal only with primers complementary to the mutant sequence. The same approach was taken with the other mutations, using the primers listed in Table 2, D, E, and F. Positive clones were finally analyzed by DNA sequencing of the relevant portion of the polymerase gene. Diploid transformants with the *pol3-Y613A* (Polδ) and *pol2-Y645A* (Polε) mutations in the heterozygous state were constructed in a similar manner. However, since selection for loss of the *URA3* gene due to plasmid excision is obscured by mitotic recombination, the initial Ura$^+$ transformants were examined by PCR with a different set of primers to confirm that they have a mutation in a full-length copy of the polymerase gene (Table 2, D and E, set 2). Primers ZABAM and ZAECOV are complementary to portions of polymerases ε and δ genes, respectively, that are beyond the fragment cloned in the plasmids used to create mutations (Figure 2). In combination with primers complementary to mutated or wild-type sequence these primers permit detection of the mutated sequence in the full-length portion of a polymerase gene. Such diploid transformants were plated onto sporulation medium and tetrads were dissected. Resulting colonies on YPD were examined after 4 days of incubation at 30°.

Qualitative tests for sensitivity to UV irradiation and chemical treatments: Serial 10-fold dilutions of each strain (starting from 5×10^7 cells/ml) were prepared in 96-well microtiter plates and plated with a 48-prong replicator (Sigma, St. Louis) onto YPD medium or YPD containing hydroxyurea (HU) or methyl methanesulfonate (MMS). For the UV irradiation sensitivity test, cells on plates were UV irradiated using two germicidal lamps at 1.5 J/m^2/sec at the dose indicated in the legend to Figure 3B.

Quantitative tests for mutability by UV irradiation: Appropriately diluted cells were plated onto YPD or selective medium and UV irradiated at doses indicated in the legend to Figure 6. Mutant frequencies were then calculated as the ratio of the number of mutants on selective plates to number of colonies on YPD medium multiplied by the dilution factor.

Figure 2.—Fragments of DNA polymerase genes used for polymerase mutant construction: (A) *POL1*, (B) *POL2*, (C) *POL3*, and (D) *REV3*. A schematic map of DNA polymerase genes is shown. Open reading frame sequences are represented by rectangles. The thick lines below the sequence depict DNA polymerase gene fragments cloned in integrative plasmids. Names of the corresponding plasmids are below these lines. Restriction sites (nucleotide numbering starts from the first ATG codon of the open reading frame) are shown above the polymerase regions. Sites of amino acids that were targeted for site-directed mutagenesis are shown as vertical lines crossing open reading frames (ORFs). Numbers above these bars refer to a third nucleotide in the codon coding for the corresponding amino acids. Numbers are shown in boldface below the ORF box.

TABLE 2

Primers used in this study

Primer name	DNA sequence (5′ → 3′)
	A. Primers for PCR disruptions
REV3: ZETADISL	GTCGAACGACACAATACAGAGCGATACGGTTAGATCATCCTCTAAATCACGTACGCTG CAGGTCGAC
ZETADISR	GACACGAGAGTAAAATACTGGACAGTCATATGAATTGCATTTACTAGCATCGATGAAT TCGAGCTCG
POL4: POL4DISL	AGTGGTAATAAGTAAAGGATAAACATGCGACCTGTTAGACAAATCGCCGTACGCTGCA GGTCGAC
POL4DISR	TAAGCTATAAAGATACAAGCCCAAGTCGCATAAAATTCAAATTATTGAGCATCGATGAA TTCGAGCTCG
RAD30: DISRAD30L	CAAAGCATGTCAAAATTTACTTGGAAGGAGTTGATTCAGCTTGGTTCCCGTACGCTGC AGGTCGAC
DISRAD30R	TTGGAAGATGTAACTTGTTTCTTCTGAGGTGTGGCAGTATGTTGTGAGATCGATGAAT TCGAGCTCG
	B. Primers for site-directed mutagenesis[a]
pol1-Y869A-L	G GAC TTT AAT TCT TTG **GCT** CCA TCT ATT ATC CAG G
pol1-Y951A-L	GCC AAT TCT ATG **GCT** GGT TGT TTG GGT TAT G
pol2-Y654A-L	GAT GTC GCC TCT ATG **GCC** CCA AAC ATC ATG AC
pol2-Y831A-L	GTT ATT TTG AAT TCG TTT **GCT** GGG TAT GTT ATG AGG
pol3-Y631A-L	G GAT TTC AAT TCT TTA **GCT** CCA AGT ATT ATG ATG G
pol3-Y708A-L	GCT AAC TCT GTC **GCT** GGT TTT ACA GGA GCG
rev3-Y980A	GAT TTC CAA TCA TTG **GCT** CCA TCC ATT ATG ATT GG
rev3-Y1093A	GCG AAT GTC ACC **GCC** GGT TAT ACA TCA GCT TC
	C. Primers for detecting Polα mutations[b]
Set 1: al 869Y WTL	A GTC ATG GAC TTT AAT TCT TTG **TA**
al 869A L	GTC ATG GAC TTT AAT TCT TTG **GC**
al 951Y WTR	ATC AAC ATA ACC CAA ACA ACC A**TA**
al 951A R	C AAC ATA ACC CAA ACA ACC A**GC**
	D. Primers for detecting Polε mutations[b]
Set 1: ep 645Y WTL	AT GTA GAT GTC GCC TCT ATG **TA**
ep 645A L	GTA GAT GTC GCC TCT ATG **GC**
ep 831Y WTR	CC TTT CCT CAT AAC ATA CCC A**TA**
ep 831A R	C TTT CCT CAT AAC ATA CCC A**GC**
Set 2: ZABAM	GCTGTTACTCAATCTAAGCTAGG
R645RealDetWT	ATTTGTAGTCATGATGTTTGGG**TA**
R645RealDetAla	GTAGTCATGATGTTTGGG**GC**
	E. Primers for detecting Polδ mutations[b]
Set 1: de 631Y WTL	CA ACT TTG GAT TTC AAT TCT TTA **TA**
de 631A L	A ACT TTG GAT TTC AAT TCT TTA **GC**
de 708Y WTR	C CGT CGC TCC TGT AAA ACC A**TA**
de 708A R	GT CGC TCC TGT AAA ACC A**GC**
Set 2: ZAECOV	TAACTTTATCATCAAAGTTGATCC
DEL631READET-WT	GCGCCATCATAATACTTGGA**TA**
DEL631READET-ALA	GCCATCATAATACTTGGA**GC**
	F. Primers for detecting Polζ mutations[b]
Set 1: ze 980Y WTL	GTGCTGGATTTCCAATCATTG**TA**
ze 980A L	GCTGGATTTCCAATCATTG**GC**
ze 1093Y WTR	AATGAAGCTGATGTATAACCG**TA**
ze 1093A R	TGAAGCTGATGTATAACCG**GC**

[a] The primers shown are for the coding strand, with the alanine codon in boldface.
[b] Terminal bases critical for discrimination between wild-type and mutant sequences in PCR reactions are in boldface and underlined.

Measurement of spontaneous mutation rates: The fluctuation tests to determine spontaneous mutation rates were performed using at least nine independent cultures. Single 2-day-old colonies from YPD plates were inoculated in 5 ml of liquid YPD medium and were grown with strong aeration for 2 days and processed as described (Shcherbakova and Kunkel 1999).

Revertant sequencing: Independent Ura$^+$ or His$^+$ revertants were grown as small patches on YPD plates, regions of corresponding genes were amplified by PCR on whole cells, ampli-

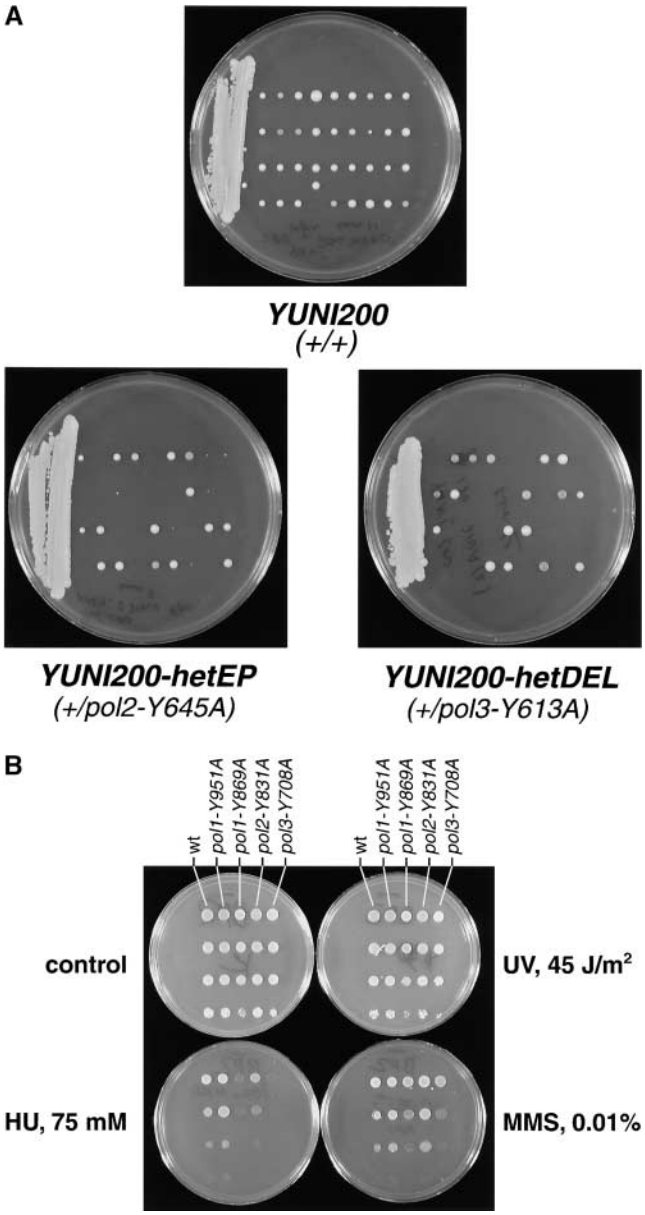

FIGURE 3.—Growth of yeast strains with polymerase mutations. (A) Tetrad analysis of control diploid, YUNI200 (top), and diploids heterozygous for *pol2-Y645A* (YUNI200-hetEP, bottom left) and for *pol3-Y613A* (YUNI200-hetDEL, bottom right). (B) Sensitivity of yeast polymerase mutants to HU, UV light, and MMS. Serial 10-fold dilutions of each strain starting from 5×10^7 cells/ml were prepared in a 96-well microtiter plate and plated onto corresponding medium with a 48-prong replicator (Sigma). Each column on the plate represents a dilution of one strain: wild type, *pol1-Y951A*, *pol1-Y831A*, *pol2-Y831A*, and *pol3-Y708A*.

fied DNAs were purified by QIAGEN (Valencia, CA) PCR purification kit and sequenced using an automated PE ABI377 DNA sequencer.

RESULTS

Tester strain to monitor mutator effects: For this study we constructed a new yeast strain, 8C-YUNI101, in which mutation rates can be measured at five different genetic loci. This includes forward mutations to canavanine resistance, reflecting a variety of substitution, frameshift, and more complex mutations (for example, see CHEN *et al.* 1998). 8C-YUNI101 harbors the *his7-2* allele containing a single base deletion in a run of 8 A·T base pairs, which can revert by addition of a base pair, loss of two base pairs or, more rarely, complex events (SHCHERBAKOVA and KUNKEL 1999 and this study). 8C-YUNI101 harbors the *ura3-29* allele, which reverts via three specific base pair substitutions at a single G·C base pair in a TCT codon encoding for serine (see SHCHERBAKOVA and PAVLOV 1996 and this study). Finally, 8C-YUNI101 also contains the *trp1-1* and *ade2-1* nonsense alleles, which revert presumably via a broad range of base pair substitution mutations. The *ade2-1* mutation imparts on this strain the growth-dependent accumulation of red pigment, which makes it possible to color-select slow-growing variants, such as respiratory-defective petite mutants. The concomitant analysis of five different mutational markers in one strain was informative regarding the mutator specificity of the mutant DNA polymerase alleles examined here.

Phenotypes conferred by replicative Pol mutations in haploid yeast strains: We used plasmids with truncated DNA polymerase α, δ, or ε genes to introduce coding sequence changes that replaced either of the two conserved tyrosine residues (Figure 1) with alanine. These six different mutant alleles were then independently integrated into the genome of strain 8C-YUNI101. To facilitate identification of strains with the desired allele, we controlled the construction process at the initial integration step and during the subsequent pop out of the wild-type allele, using PCR with allele-specific primers (see MATERIALS AND METHODS). This substantially reduced the number of clones to be analyzed and permitted identification of mutant clones without knowledge of phenotypes. It also proved to be an invaluable tool for constructing polymerase mutants in the heterozygous state. The data obtained with mutants are summarized in Table 3, which can also be used as a guide for mutant allele nomenclature.

Transformation of the haploid yeast strain 8C-YUNI101 with plasmids designed to generate the Y645A Polε mutant and the Y613A Polδ mutant did not yield the desired mutant strains, suggesting that these two changes are lethal in this haploid strain. To test this, we integrated the plasmids into the diploid strains YUNI190 or YUNI200 and verified the presence of the mutations in the heterozygous state in a full size chromosomal copy of the gene by PCR analysis (see MATERIALS AND METHODS). Tetrad analysis of the two mutant strains yielded 2:2 segregation for viability (Figure 3A). All viable spores were Ura^-. These results suggest that the Y645A mutation in Polε and the Y613A mutation in Polδ are incompatible with vegetative growth. Note that some spores with the Y645A mutation in Polε formed tiny residual colonies (Figure 3A), suggesting that the effect

TABLE 3

Phenotypes of yeast strains with mutant replicative DNA polymerase alleles

DNA Pol	Polymerase allele	Residue in Kf Pol	Growth on YPD	Sensitivity to HU	Sensitivity to MMS	Dominant or recessive	Mutator effect in		Chromosome stability
							PMS1$^+$	*pms1*	
				A. Pol region II substitutions in SL/MYPS/N motif[a]					
α	*pol1-Y869A*	E710	Slow	Sensitive	Sensitive	Recessive	Weak	Strong	Low
ε	*pol2-Y645A*	E710	Microcolonies	NA	NA	Recessive	NA	NA	NA
δ	*pol3-Y613A*	E710	None	NA	NA	Recessive	NA	NA	NA
				B. Pol region III substitutions in NS/VxYG motif[a]					
α	*pol1-Y951A*	Y766	Normal	Normal	Normal	NA	None	Weak (Ade$^+$ only)	Normal
ε	*pol2-Y831A*	Y766	Normal	Slightly sensitive	Normal	Semidominant	None	Strong	Normal
δ	*pol3-Y708A*	Y766	Slow	Sensitive	Sensitive	Recessive	Moderate	Strong and general[b]	Low

NA, not applicable.
[a] Tyrosine that was substituted is underlined.
[b] Mutator for all five markers tested.

of this mutation is less severe than for the Y613A Polδ mutation.

In contrast to the above effects, strains harboring the Y869A or Y951A mutations in Polα, the Y708A mutation in Polδ, or the Y831A mutation in Polε were all viable. The Y951A Polα strain and the Y831A Polε strain grew normally on YPD medium (Figure 3B, control plates and data not shown). In contrast, the Y869A Polα strain and the Y708A Polδ strains grew more slowly and had a reduced cell number in stationary phase cultures when compared to the wild-type strain (cell titers were 2–3 × 10^{-8} for wild-type and 0.9–1 × 10^{-8} for Y708A Polδ or Y869A Polα strains). None of the four mutant strains was highly sensitive to UV irradiation (Figure 3B). In contrast, three of the four mutant strains (*pol1-Y869A*, *pol2-Y831A*, and *pol3-Y708A*) were more sensitive than the wild-type strain to treatment with HU, a known inhibitor of DNA replication. The Y869A Polα mutant and Y708A Polδ mutant strains were also sensitive to 0.01% MMS (Figure 3B). These initial qualitative analyses indicate that the phenotypic consequences of these six polymerase mutations are in the following order of increasing severity (for summary, see Table 3): Y951A Polα, Y831A Polε, Y869A Polα, Y708A Polδ, Y645A Polε (microcolonies), and Y613A Polδ (inviable).

Diploid strains heterozygous or homozygous for replicative Pol mutations: Among the five mutant polymerase alleles that affected one or more phenotypes as haploids (the exception being *pol1-Y951A*, which had no phenotypic consequence), four did not yield detectable phenotypic effects on growth rate, MMS and HU sensitivity, or mutagenesis in the presence of the wild-type Pol allele in heterozygous diploid strains. Thus, these alleles are recessive (Table 3). However, as shown below, the *pol2-Y831A* mutation was semidominant as evidenced by elevated frameshift mutation rate in a mismatch repair-defective strain. Diploid strains homozygous for *pol1-Y951A* and *pol2-Y831A* grow normally, like the corresponding wild-type diploid strain. Homozygosity for *pol3-Y708A* produced very slow growth (Figure 4A, lower right diploid), and this was also seen with the *pol1-Y869A* mutation (data not shown). Cells in the colonies of these slow-growing diploid strains had abnormal morphology (not shown). These diploid strains are all *MAT***a**/*MAT*α and thus would not normally mate, as is the case for the wild-type diploid strain (left plate of Figure 4B). However, almost all single-colony isolates of the homozygous mutant diploid strains were able to mate with one or both of mating-type testers (Figure 4B). This indicates that they had either lost chromosome III or were losing it frequently while the colonies were growing (Mortimer *et al.* 1981). A possibility that these mating clones were generated by mitotic recombination is unlikely since mitotic recombination is not affected by the *pol3-Y708A* mutation to a level that could explain the observation (Y. I. Pavlov, unpublished data). The *pol1-Y869A* and *pol3-Y708A* mutants had a reduced ability to main-

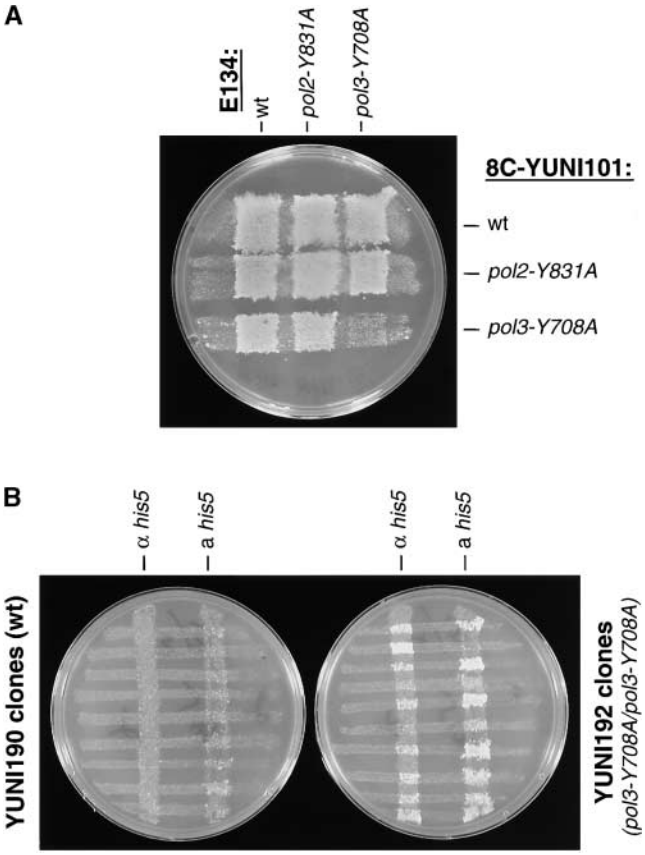

Figure 4.—Growth and mating phenotypes of diploids homozygous for DNA polymerase mutations. (A) Genetic crosses of strains with DNA polymerase mutations. Plate shown is replica plating of the cross from YPD to medium selective for hybrids. Diploids could be seen as rectangle areas of growth on the intersections of slightly visible traces of nongrowing haploid parent strains. (B) Mating of wild-type (left) and *pol3-Y708A* homozygous (right) diploids with mating-type testers. Individual clones of wild-type diploid (YUNI190) and clones of diploid arising from cross of two haploid *pol3-Y708A* mutants (YUNI192, bottom right diploid in Figure 4A) were crossed on YPD to standard mating-type tester strains and replica plated onto medium selective for hybrids.

tain centromeric plasmids (data not shown). Taken together, these two observations indicate that these polymerase mutations affect chromosome stability (Table 3).

Mutator effects of replicative Pol mutations in otherwise wild-type background: By analogy to the reduced DNA synthesis fidelity of mutant analogues of Klenow fragment Pol, a major objective of this study was to determine if putative active site mutants in eukaryotic replicative polymerases affected mutation rates *in vivo*. To test this, we measured spontaneous mutation rates in strain 8C-YUNI101 and its four polymerase mutants that were viable as haploids at the five loci that monitor base substitution and frameshift mutations.

For the Y951A Polα and Y831A Polε mutant strains, the mutation rates at all five loci were similar to those seen in the wild-type yeast strain (Table 4). The Y869A Polα mutant exhibited wild-type mutation rates at three loci, but had ~3-fold higher rates for canavanine resistance and for His^+ reversion. DNA sequence analysis of His^+ revertants of the Y869A Polα strain (Table 5) indicated that the rate of addition of one A·T base pair to the homonucleotide run at the *his7-2* mutation site is actually elevated by 22-fold compared to the wild-type strain. The Y708A Polδ mutant had a wild-type reversion rate of the *trp1-1* and *ade2-1* mutation, but mutation rates were elevated 3-fold, 4-fold, and 6-fold, respectively, at the *his7-2*, *ura3-29*, and *CAN1* loci. DNA sequence analysis of His^+ revertants of the Y708A Polδ strain (Table 5) indicated that the rate of addition of one A·T base pair to the homonucleotide run at the *his7-2* mutation site is elevated by 14-fold compared to the wild-type strain. DNA sequence analysis of Ura^+ revertants of the Y708A Polδ strain indicated that the rates of C → T and C → G substitutions at the *ura3-29* site are elevated by 4-fold and 11-fold, respectively, compared to the wild-type strain (Table 6). Thus, two of the polymerase mutants are modest spontaneous mutators for some point mutations in yeast strains that are proficient in DNA mismatch repair. The degree to which the four different polymerase gene mutations affect spontaneous mutation rates is in the same relative order as for the other phenotypes mentioned above (Table 3).

Mutator effects of replicative Pol mutations in mismatch repair-defective strains: Mutation rates were measured at the five loci in polymerase mutant strains that are also deficient in DNA mismatch repair due to disruption of the *PMS1* gene (Table 4B). At the *CAN1*, *trp1-1*, and *his7-2* loci, mutation rates in a *pms1::LEU2* strain that contains normal polymerase genes are substantially higher than are the rates in a mismatch repair-proficient strain (Table 4, compare A and B). This is expected due to lack of repair of mismatches generated by the wild-type replicative polymerases. However, the rate of reversion to Ura^+ is elevated only two-fold and reversion of Ade^+ is not elevated in a *pms1::LEU2* background. Replication errors in these latter sites may be efficiently corrected by exonucleolytic proofreading and/or they may be inefficiently corrected by *PMS1*-dependent mismatch repair.

In the *pms1::LEU2* strain background, strains harboring each of the four viable polymerase gene mutations had elevated mutation rates at one or more of the five loci examined (Table 4B). The Y951A Polα mutant had an 8-fold higher rate of Ade^+ reversion, with negligible effects observed at the other loci. The three other polymerase mutant strains all had mutation rates that were elevated by 2- to 180-fold in comparison to the rates in the *pms1::LEU2* strain with wild-type polymerases. The Y869A Polα mutant had an ~20-fold higher rate of Trp^+ and Ade^+ reversion and a 5- and 7-fold higher rate of forward Can^r and frameshift His^+ mutations, respectively. Note that variability of Trp^+ and Ade^+ mutation rates among independent cultures was much higher than that for Ura^+ and His^+ reversion, which makes

TABLE 4

Mutation rates in strains with mutant DNA polymerase alleles

Polymerase mutation in different genetic backgrounds	Mutation rate[a] (95% confidence limits)				
	Can^r ($\times 10^{-7}$)	Ura$^+$ ($\times 10^{-8}$)	His$^+$ ($\times 10^{-8}$)	Trp$^+$ ($\times 10^{-8}$)	Ade$^+$ ($\times 10^{-8}$)
A. Mismatch repair-proficient strains					
Wild-type Pols	4.4 (2.9–5.8)	1.3 (1.2–1.7)	1.9 (1.4–3.6)	4.1 (2.8–9.7)	1.2 (1.2–2.5)
pol1-Y869A	11.5 (6.2–28)	1.1 (0.8–2.2)	6.4 (4.1–16)	4.0 (2.1–7.4)	<0.9
pol1-Y951A	4.4 (3.0–6.8)	1.1 (0.8–1.5)	2.2 (0.9–2.3)	4.9 (1.3–6.3)	1.1 (0.8–3.2)
pol2-Y831A	7.4 (4.6–14)	1.2 (0.8–2.8)	2.8 (2.3–7.2)	6.1 (5.0–8.3)	<0.9
pol3-Y708A	28 (14–32)	6.1 (5.0–7.7)	6.0 (4.8–11)	2.5 (2.3–6.3)	2.5 (2.0–3.4)
pol3-Y708A rev3::LEU2	4.6 (3.4–9.5)	1.9 (1.7–2.6)	3.2 (1.9–6.1)	2.5 (1.4–2.8)	1.9 (1.3–2.9)
rev3::LEU2	1.4 (0.9–2.5)	1.2 (1.1–2.0)	2.7 (1.1–3.4)	<1.2	<1.2
B. Mismatch repair-deficient *pms1* strains					
Wild-type Pols	88 (73–150)	2.8 (2.4–4.1)	87 (54–150)	23 (16–26)	0.6 (0.5–1.5)
pol1-Y869A	420 (240–510)	6.6 (4.3–14)	640 (600–1900)	500 (130–1800)	16 (12–79)
pol1-Y951A	87 (56–120)	3.7 (2.5–4.4)	130 (88–170)	32 (15–47)	4.9 (2.8–9.0)
pol2-Y831A	340 (230–420)	12 (11–19)	470 (270–780)	140 (57–240)	48 (29–97)
pol3-Y708A	370 (150–540)	140 (100–260)	580 (340–800)	140 (110–150)	110 (59–130)
pol3-Y708A rev3::kanMX	330 (250–500)	16 (9–36)	450 (350–570)	71 (52–96)	18 (12–27)
rev3::kanMX	68 (45–150)	1.8 (1.5–3.3)	85 (58–175)	19 (4–46)	0.7 (0.5–1.5)

[a] Median for 9–27 independent cultures.

Trp$^+$ and Ade$^+$ reversions less reliable assays. The *pol1-Y869A* allele had only a small effect (~2-fold) on base substitutions at the *ura3-29* locus. The Y831A Polε mutant was an ~80-fold mutator for Ade$^+$ and an ~5-fold mutator for Ura$^+$, Trp$^+$, His$^+$, and canavanine resistance (Can^r). These relatively strong mutator effects contrast with the lack of a mutator effect for this polymerase allele in the wild-type background, suggesting that all errors arising in the *pol2-Y831A* strain are corrected by mismatch repair. The strain harboring the Y708A Polδ allele had the most broad effects on spontaneous mutation. Ade$^+$ reversion was increased by 180-fold, Ura$^+$ reversion was increased by 50-fold, and Can^r, His$^+$, and Trp$^+$ mutations were increased by 4- to 7-fold. Sequence analysis of Ura$^+$ revertants showed that the rates of three types of base substitutions at the *ura3-29* mutation site are elevated by 6- to 79-fold in the Y708A Polδ mutant strain, by up to 6-fold in the Y831A Polε mutant strain and by up to 3-fold in the Y869A Polα mutant strain (Table 6). Sequence analysis of His$^+$ revertants (Table 5) showed that the rate of addition of an A·T base pair to the homonucleotide run at the

TABLE 5

Rates of various frameshifts leading to His$^+$ reversion

Strain	Rates for specific types[a] of His$^+$ revertants $\times 10^{-8}$						Total rate $\times 10^{-8}$	Revertants sequenced
	+1 in run		−2 in run		Other[b]			
	Abs.[c]	Rel.[d]	Abs.	Rel.	Abs.	Rel.		
A. Mismatch repair proficient								
Wt	0.25	1	1.1	1	0.55	1	1.9	30
pol1-Y869A	5.7	22	0.35	0.3	0.35	0.6	6.4	18
pol2-Y831A	1.2	4.7	0.6	0.6	1	1.7	2.8	14
pol3-Y708A	3.6	14	2.1	2	0.3	0.6	6	17
B. Mismatch repair deficient								
Wt	87	1	<3.6	—	<3.6	—	87	24
pol1-Y869A	640	7.3	<43	—	<43	—	640	15
pol2-Y831A	470	5.4	<20	—	<20	—	470	23
pol3-Y708A	535	6.1	<22	—	45	>12	580	26

[a] Median mutation rate (Table 4) corrected for proportion of a particular mutational event among revertants sequenced.
[b] Other mutations detected were +1 and −2 frameshifts in a sequence outside of seven A·T base pair run.
[c] Absolute mutation rate for a particular reversion event.
[d] Mutation rate for a particular reversion event relative to the strain without DNA polymerase mutation.

TABLE 6

Rates of various base substitutions leading to Ura$^+$ reversion

Strain	Rates for specific types[a] of Ura$^+$ revertants $\times 10^{-8}$						Total rate $\times 10^{-8}$	Revertants sequenced
	C → T		C → A		C → G			
	Abs.[b]	Rel.[c]	Abs.	Rel.	Abs.	Rel.		
A. Mismatch repair proficient								
Wt	0.53	1	0.41	1	0.36	1	1.3	22
pol1-Y869A	0.92	1.7	<0.18	—	0.18	0.5	1.1	6
pol2-Y831A	0.34	0.6	0.52	1.2	0.34	1	1.2	7
pol3-Y708A	2.1	4	0.2	0.3	3.8	11	6.1	43
pol3-Y708A rev3::LEU2	0.3	0.6	1.6	3.8	<0.3	—	1.9	6
rev3::LEU2	0.5	0.9	0.7	1.8	<0.1	—	1.2	13
B. Mismatch repair deficient								
Wt	1	1	1.7	1	0.1	1	2.8	28
pol1-Y869A	1.2	1.2	5.4	3.1	<0.6	—	6.6	11
pol2-Y831A	2.3	2.3	9.7	5.7	<0.5	—	12	26
pol3-Y708A	79	79	55	32	6	6	140	23
pol3-Y708A rev3::kanMX	4.6	4.6	11.4	6.7	<1.1	—	16	14
rev3::kanMX	0.6	0.6	1.2	0.7	<0.1	—	1.8	12

[a] Median mutation rate (Table 4) corrected for proportion of a particular mutational event among revertants sequenced.
[b] Absolute mutation rate for a particular reversion event.
[c] Mutation rate for a particular reversion event relative to the strain without DNA polymerase mutation.

his7-2 locus was elevated by 7-fold in the Y869A Polα mutant strain. The rate was elevated by 5-fold in the Y831A Polε mutant strain and by 6-fold in the Y708A Polδ mutant strain.

Monitoring reversion at the *ura3-29* and *his7-2* loci coupled with DNA sequencing of revertants appeared to be a sensitive approach for characterizing the spontaneous mutator phenotypes of the DNA polymerase mutants. Therefore, further analysis of spontaneous mutagenesis was performed using these two markers.

Mutator effects of replicative Pol mutations in diploid strains: We examined whether the mutator phenotypes observed in haploid strains could be seen when mutant polymerase alleles were present with wild-type polymerase allele in heterozygous diploid strains. For this purpose, we measured the rate of *his7-2* reversion in the YUNI200 diploid strain series (Table 7). His$^+$ reversion rates in diploid strains homozygous for the polymerase mutations were similar to those observed in haploid strains, and the mutation rate ranking remained the same despite the fact that the *pol1-Y869A* and *pol3-Y708A* alleles severely impaired growth in the diploid state (see above). Reversion rates in the mismatch repair-proficient diploids heterozygous for any of three polymerase mutations were undistinguishable to the wild-type diploid (Table 7). The *pol1-Y869A* and *pol3-Y708A* were clearly recessive in the mismatch repair-deficient strain. In contrast, the *pol2-Y831A* allele was semidominant in the *pms1* background; the heterozygous diploid strain had a 3.6-fold increase in His$^+$ reversion rate over the wild-type diploid strain, as compared with a 7.5-fold increase in homozygous polymerase mutant diploid.

Partial suppression of mutator phenotype of *pol3-Y708A* mutation by disruption of *REV3*: To further investigate the genetic control of spontaneous mutagenesis in the polymerase mutant strains, we used semiquantitative tests to examine the effect of disruption of the *REV3*, *POL4*, and *RAD30* genes on Can^r mutation and Ura$^+$ and His$^+$ reversion rates in a set of mismatch repair-proficient and -deficient strains with or without polymerase mutations (data not shown). The only combination of polymerase mutations that showed genetic interaction was *REV3* disruption in a *pol3-Y708A* strain. *REV3* encodes the catalytic subunit of DNA polymerase ζ, a specialized polymerase involved in error-prone processing of both endogenous and induced DNA damage in yeast (Lawrence 1996). Mutation rates were measured at the five loci in the *rev3* and the *rev3 pol3-Y708A* polymerase mutant strains (Table 4). Disruption of *REV3* had a 3-fold antimutator effect on the rate of Can^r mutations in a wild-type background, but it had no detectable effect on spontaneous mutation rates at the *ura3-29* or *his7-2* loci (Table 4A). Disruption of *REV3* did not significantly affect the mutation rates in the mismatch repair-deficient *pms1* background (Table 4B). However, disruption of *REV3* decreased rates of Can^r mutations (6-fold) and Ura$^+$ reversions (3-fold) in the *pol3-Y708A* strain (Table 4A) and decreased rates of Ura$^+$ reversions (almost 9-fold) in the *pol3-Y708A pms1* strains (Table 4B; Figure 5A). No such reduction in rate of Ura$^+$ reversion was seen in strains with combinations of *pol3-Y708A* and disruptions of either *POL4* or *RAD30* (Figure 5A). No suppression of His$^+$ reversion was observed by any of three specialized polymerase gene dis-

TABLE 7

His$^+$ reversion rates in diploid strains homozygous or heterozygous for Pol mutations

Polymerase alleles	His$^+$ reversion rate $\times 10^{-8}$ (95% confidence limits)	Relative rate
Mismatch repair-proficient strains		
+/+	0.9 (0.2–1.4)	1
pol1-Y869A / pol1-Y869A	13 (5.1–20)	14
+/*pol1-Y869A*	1.1 (0.7–1.7)	1.2
pol2-Y831A / pol2-Y831A	1.7 (1.2–5.7)	1.9
+/*pol2-Y831A*	1.1 (0.7–1.6)	1.2
pol3-Y708A / pol3-Y708A	8.5 (5.8–9.4)	9.4
+/*pol3-Y708A*	0.7 (0.2–1.1)	0.8
Mismatch repair-deficient strains (*pms1/pms1*)		
+/+	110 (93–140)	1
pol1-Y869A / pol1-Y869A	950 (790–1300)	8.6
+/*pol1-Y869A*	170 (140–170)	1.5
pol2-Y831A / pol2-Y831A	820 (750–950)	7.5
+/*pol2-Y831A*	400 (270–600)	3.6
pol3-Y708A / pol3-Y708A	1000 (910–1700)	9.1
+/*pol3-Y708A*	83 (66–120)	0.8

Mutation rates are median for 9–18 cultures.

ruptions (Figure 5B). Sequence analysis of Ura$^+$ revertants in single *rev3* mutant showed that C → G transversions disappeared from the spectra. C → T transitions were approximately as frequent as in wild type and C → A transversions were more frequent in a mismatch repair-proficient strain and equal to wild type in a mismatch repair-deficient strain (Table 6). Sequence analysis showed that in a mismatch repair-proficient background, the antimutator effect of the *rev3* in the *pol3-Y708A* strain is mainly due to a decrease in the rate of C → G transversions (more than 12-fold comparing to the *pol3-Y708A* single mutant) and decrease in the rate of C → T transitions (7-fold), while C → A transversion rates were increased 8-fold (Table 6A). All types of base pair substitutions were decreased in the *pol3-Y708A pms1 rev3* strain (Table 6B). These data imply that Polζ may contribute to spontaneous mutagenesis at specific loci and for specific types of base pair substitutions in a strain with a mutation in the Polδ active site.

Notably, the mutation rates at the *ura3-29* locus in the triple *pol3-Y708A pms1 rev3* mutant strain remained substantially higher than those observed in the *pms1* strain and disruption of *REV3* did not suppress the frameshift mutator effect of the *pol3-Y708A*. Thus, disruption of *REV3* only partially suppresses the mutator effects of the *pol3-Y708A* mutation, implying that this tyrosine to alanine substitution in Polδ may directly reduce replication fidelity. Similarly, disruption of *REV3* did not suppress the mutator phenotypes of the *pol2-Y831A* (Polε) or *pol1-Y869A* (Polα) mutation at the *ura3-29* and *his7-2* loci, suggesting that these mutations either directly reduce DNA replication fidelity *in vivo* or their indirect effect is mediated by a Rev3-independent mechanism.

UV sensitivity and mutability of Polζ catalytic subunit active site mutants: The above experiments suggested that the active site mutations in replicative DNA polymerase genes might impair some function in addition to or other than polymerase fidelity. Therefore we investigated the effects of homologous tyrosine to alanine active site changes in the Rev3 Pol, which is not essential for growth. The *REV3* gene encodes the catalytic subunit of Polζ, which belongs to DNA polymerase family B (Figure 1), and it is necessary for UV mutagenesis. Unlike the situation with the mutant alleles of Polα, -δ, and -ε genes, the *rev3* mutations did not yield spontaneous

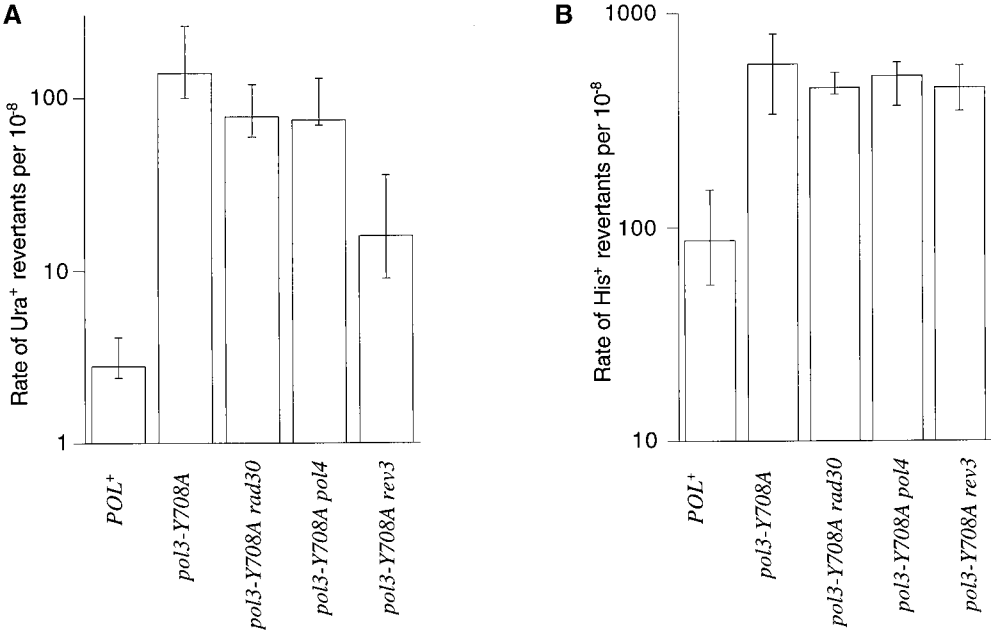

FIGURE 5.—Effects of mutations in specialized DNA polymerases on mutator effect of the *pol3-Y708A* in a mismatch repair-defective *pms1* strain. (A) Rates of Ura$^+$ reversion. (B) Rates of His$^+$ reversion. Rates are medians for 9–27 independent cultures. Error bars represent 95% confidence intervals.

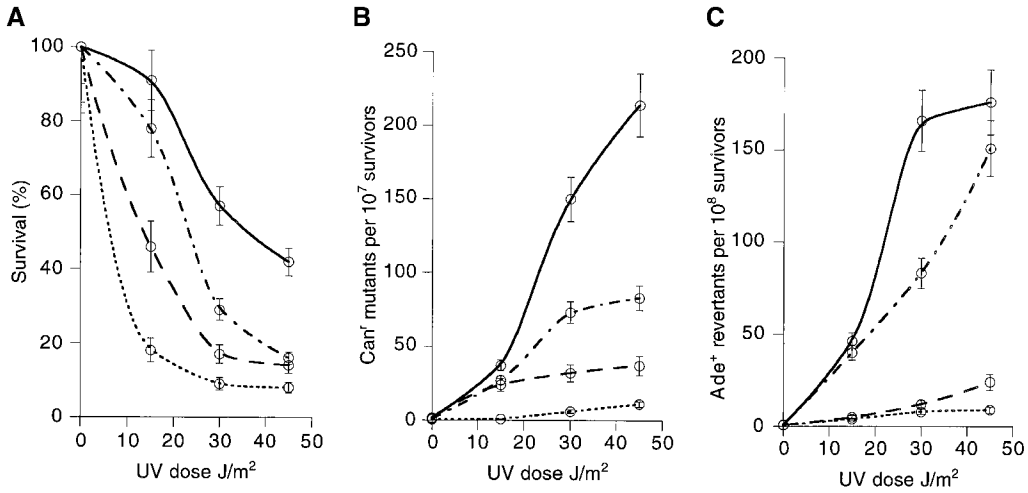

FIGURE 6.—UV irradiation effect on survival and mutagenesis in strains with mutations in the *REV3* gene. (A) Survival (percentage). (B) Frequencies of Can^r mutants. (C) Frequencies of Ade$^+$ mutants. Mean values from at least three experiments. Error bars are standard errors. —, *REV3*, - - -, *rev3-Y980A*; ···, Δ*rev3::LEU2*; -·-·-, *rev3-Y1093A*.

mutator effects (data not shown). We then examined UV radiation-induced mutagenesis in these strains. UV light was a potent inducer of Can^r and Ade$^+$ mutations in the wild-type strain (Figure 6, B and C). Disruption of *REV3* decreased cell survival after UV irradiation (Figure 6A) and severely reduced the frequency of UV radiation-induced Can^r (Figure 6B) or Ade$^+$ (Figure 6C) mutations. Active site mutations had intermediate effects. The region II tyrosine to alanine mutation *rev3-Y980A* (Figure 1A) had an effect that was closer to the effect of the *REV3* disruption—it lowered UV survival and strongly decreased UV radiation-induced mutagenesis, suggesting severe loss of Pol function. The effects of the region III mutation *rev3-Y1093A* (Figure 1B) were less pronounced, as this strain was partially UV mutable and its survival after UV irradiation was closer to that of the wild-type strain (Figure 6A). The degree of effects of the two mutations on Rev3 function is in the same relative order as for replicative polymerases.

DISCUSSION

Structure-function studies have identified conserved residues in family A DNA polymerase active sites that determine the efficiency and fidelity of DNA synthesis. This study uses this information to probe the functions of eukaryotic family B polymerases in the much more complex setting *in vivo*. The results indicate that two highly conserved tyrosine residues inferred from structural studies to be at the polymerase active sites of yeast DNA polymerases α, δ, ε, and ζ, the four known family B polymerases in yeast, have important roles in determining cellular growth potential, sensitivity to chemical treatments, and/or genomic stability. In a general sense, the severity of phenotypes conferred by the replicative polymerase mutations correlates with the amount of DNA synthesis these enzymes are thought to perform in cells. Thus, for replicative polymerases, the Polα mutants have the weakest phenotypes, the Polε mutants have intermediate phenotypes, and the Polδ mutants have the strongest phenotypes when the three corresponding Pol alleles are compared (Table 3). Exonuclease-deficient Polα is thought to incorporate only a small number of nucleotides to initiate Okazaki fragments and is then replaced by other polymerases that may proofread errors made by Polα, an idea suggested by PERRINO and LOEB (1990). In addition, some DNA synthesized by Polα could be removed during Okazaki fragment maturation. On the opposite end of this continuum, the Polδ mutants are severely defective in several ways, consistent with the idea that Polδ performs the bulk of chromosomal DNA replication in eukaryotes (BURGERS 1998; WAGA and STILLMAN 1998). Each of the mutant replicative polymerase alleles studied here differs from the others in one or more phenotypes (Table 3), indicating that the functions of the two conserved tyrosines are polymerase and residue specific. In addition, homologous mutations in *REV3* (Polζ) conferred defects in UV mutability, suggesting partial loss of function.

Strains with Tyr to Ala substitutions in conserved region II: Among the many amino acids that form DNA polymerase active sites, we focused here on two that were anticipated from previous studies to be important for DNA synthesis fidelity. One is a tyrosine in conserved region II of family B enzymes (Figure 1A). The X-ray crystal structures of three family B polymerases reveal that this tyrosine is at the polymerase active site (WANG *et al.* 1997; HOPFNER *et al.* 1999; ZHAO *et al.* 1999). It is in a location suggested by WANG *et al.* (1997) to be functionally equivalent to a critical glutamate residue in family A enzymes, specifically Glu710 in the Klenow fragment Pol and Glu615 in Klentaq Pol. In the structure of Klentaq Pol complexed with DNA and a dNTP that is poised for catalysis, the side chain of Glu615 closely approaches the 2′ position of the deoxyribose of the incoming dNTP in a manner that excludes incorporation of rNTPs during DNA synthesis. Replacement of this side chain with alanine in Klenow fragment Pol reduces catalytic efficiency by 20-fold (POLESKY *et al.*

1992) and strongly reduces discrimination against rNTP incorporation (Astatke *et al.* 1998). Similar effects are seen in studies of a homologous mutant of another Pol A family member, T7 DNA Pol (Donlin and Johnson 1994). By functional analogy, reduced replication efficiency and/or enhanced rNTP incorporation could explain why the yeast *pol3-Y613A* (Polδ) and *pol2-Y645A* (Polε) alleles are incompatible with vegetative growth in haploids and are recessive in diploids (Table 3; Figure 3A). Reduced polymerase activity could also explain why the *rev3-Y980A* strain is almost as UV immutable as the strain with a complete deletion of *REV3* (Figure 6). Spores containing the *pol2-Y645A* (Polε) mutation do produce microcolonies (Figure 3A), indicating minimal retention of some *POL2* gene function. However, these microcolonies apparently stop growing and never reach the size of normal colonies. The inability of the *pol2-Y645A* allele to support vegetative growth appropriately may not simply reflect loss of Polε catalytic function, as was reported for mutations of catalytic residues (Dua *et al.* 1999) or an in frame deletion of the Polε catalytic domain that still permitted slow growth (Kesti *et al.* 1999). Instead, this may be due to some form of aberrant catalysis by Polε, such as incorporation of rNTPs or incorrect dNTPs. Efforts are currently underway to purify the mutant polymerases in order to characterize their biochemical properties.

The haploid strain with the *pol1-Y869A* substitution is viable. However, it grows slowly, is sensitive to hydroxyurea, and is recessive. This suggests that, although sufficient to carry out the essential function of this polymerase in replication, the catalytic efficiency of this Polα mutant is reduced in comparison to wild-type Polα. Indeed, purified human Polα with a Y865S mutation (where human Tyr865 is homologous to yeast Tyr869) has lower catalytic efficiency than does wild-type Polα (Dong *et al.* 1993). The yeast Polα Y869A mutation also destabilizes the genome, as indicated by reduced chromosome stability and the enhanced rate of single-base additions (22-fold, Tables 3 and 5) and base substitutions (>20-fold for Trp^+ and Ade^+ nonsense mutations reversion, Table 4B). The mutator effects were strongest in a *pms1* strain lacking mismatch repair, suggesting that the point mutations may directly reflect replication errors made by the Y869A Polα, at least at specific regions of DNA encoding for tRNA genes, known suppressors of nonsense mutations. This suggestion is in agreement with the 15-fold reduced *in vitro* fidelity of human Polα with a Y865S mutation (Dong *et al.* 1993) and with the reduced fidelity of Klenow fragment Pol containing the putative functionally analogous E710A substitution (Minnick *et al.* 1999). However, the effect of the *pol1-Y869A* on base pair substitutions leading to the *ura3-29* reversions was small (3-fold for C → A transversions, Table 6B). Klenow fragment Pol with a E710A mutation extends from a T·G mismatch almost 40-fold less efficiently than wild-type enzyme (Minnick *et al.* 1999) suggesting a possibility that Y869A Polα may pause after making an error, possibly allowing the mismatch to be proofread or extended by another polymerase. It is also possible that the Y869A yeast Polα mutation may alter replication so as to enhance the rate of double strand breaks formation (as implied by the low chromosome stability), thereby indirectly increasing mutagenesis. It was reported earlier that double strand break repair at the *TRP1* locus is mutagenic (Holbeck and Strathern 1997). Collectively, the observations with the Y869A Polα mutation imply that it may enhance mutagenesis by directly reducing replication fidelity or by contributing to genome instability indirectly through unknown pathway.

Strains with Tyr to Ala substitutions in conserved region III: The second amino acid residue anticipated to be important for DNA synthesis fidelity is the tyrosine in conserved region III of family B enzymes (Figure 1B). Structural studies (Wang *et al.* 1997; Hopfner *et al.* 1999; Zhao *et al.* 1999) indicate that this tyrosine is at the polymerase active site and may be functionally equivalent to a conserved tyrosine in family A enzymes, specifically Tyr766 in Klenow fragment Pol. This amino acid is suggested to contribute to the proper base pairing geometry in the binding pocket for the nascent base pair and/or to act as a chaperone in delivering the dNTP to the binding pocket (reviewed in Kunkel and Bebenek 2000). Replacement of this side chain in Klenow fragment Pol with alanine reduces its DNA binding affinity, catalytic efficiency, base selectivity, and mismatch extension efficiency (Bell *et al.* 1997; Minnick *et al.* 1999). The putative functionally analogous mutant of RB69 Pol (a family B member) also has slightly reduced catalytic efficiency (Yang *et al.* 1999) and reduced misincorporation fidelity *in vitro* and it is a strong spontaneous base substitution mutator *in vivo* (Bebenek *et al.* 2000).

Haploid yeast strains with analogous tyrosine to alanine mutations in conserved region III (Figure 1B) of Polα, -δ, and -ε are all viable and the homologous mutant of Polζ is UV mutable (Figure 6). The *pol1-Y951A* (Polα) allele had almost no effect on spontaneous mutagenesis, increasing only Ade^+ reversion in the *pms1* background. It may not synthesize DNA at the loci examined, it may not have reduced fidelity, or errors made by this polymerase may be efficiently corrected by proofreading or during Okazaki fragment maturation. The latter possibility is less likely because no genetic interaction of the *pol1-Y951A* with the proofreading exonuclease mutations *pol2-4* and *pol3-01* or the flap endonuclease *rad27* mutation has yet been found (Y. Pavlov, unpublished data). In contrast, the *pol2-Y831A* Polε mutant strain exhibited normal growth and the wild-type sensitivity to MMS treatment and chromosomal stability, but was slightly sensitive to HU and exhibited clear spontaneous base substitution and frameshift mutator phenotypes when the *PMS1* was disrupted (Tables 4–6). These mutator effects were not suppressed by disruption of special-

ized polymerase genes and the mutator effect at the *his7-2* locus is semidominant (Table 7). Given the effects of Tyr to Ala substitutions in Klenow fragment Pol and RB69 Pol mentioned above, these data suggest that the *pol2-Y831A* allele encodes a mutant Polε whose catalytic efficiency is not severely reduced, but whose base selectivity may be reduced, leading to replication errors that can be corrected by mismatch repair.

Among replicative polymerase mutants that were viable in the haploid state, the most severe phenotypes were exhibited by Y708A Polδ mutant (Table 3). A strain with the *pol3-Y708A* allele grew slowly and the mutation was recessive, indicating that the mutant polymerase does not compete effectively with wild-type Polδ. The haploid *pol3-Y708A* strain was sensitive to hydroxyurea and MMS, suggesting that both DNA replication and DNA repair are defective. Chromosomal stability is low in this strain, as judged by an elevated rate of loss of centromeric plasmids in haploid (our unpublished observations) and by the bipolar mating of homozygous diploid (Figure 4). The *pol3-Y708A* strain also has strongly elevated rates of spontaneous base substitution and frameshift mutations (Tables 4–7). Larger deletions and more complex mutations in the *CAN1* gene were abundant in the *pol3-Y708A* strain (our unpublished observations). The mutator effects were strongest in the mismatch repair-defective background (Table 4), especially for base substitutions at the *ura3-29* and *ade2-1* loci (Table 4). The base substitution specificity of the *pol3-Y708A* Polδ mutant is quite different from that of the *pol2-Y831A* Polε mutant (Table 6). Thus the homologous region III Tyr to Ala changes in DNA polymerases δ and ε both enhance spontaneous errors that are subject to mismatch repair, but with quite different specificity. This could reflect a different misinsertion specificity of these mutant enzymes, or a differential ability to proofread errors. The latter could result from altered communication between the polymerase and exonuclease active sites due to the mutation in the polymerase active site as has been suggested for other mutant family B enzymes (see, for example, Reha-Krantz and Nonay 1994; Bebenek *et al.* 2000). It is worth noting that the *pol3-Y708A* and *pol2-Y831A* alleles encode wild-type active sites for their intrinsic exonucleases. Thus, the mutagenic potential of these mutant polymerases could be underestimated if misinsertions and misalignments are proofread by these exonucleases.

Klenow fragment Pol with a Y766A mutation analogous to the *pol3-Y708A* Polδ allele has reduced capacity to extend a template-primer with a terminal mismatch (Carroll *et al.* 1991). The Y567A mutation in RB69 Pol, a family B homolog of the *pol3-Y708A* Polδ mutant, also extends terminal mismatches less efficiently than does its wild-type parent (Bebenek *et al.* 2000). If the *pol3-Y708A* Polδ mutant also extends mismatched termini poorly, then the observed strong base substitution mutator phenotype may reflect low base selectivity by this mutant Polδ followed by fast dissociation (and thus escape from proofreading by intrinsic exonuclease) and then by mismatch extension catalyzed by a second polymerase that extends mismatched termini more efficiently. This hypothesis is supported by the observation that disruption of *REV3*, encoding DNA polymerase ζ that is devoid of proofreading exonuclease, reduces the rate of the *pol3-Y708A*-dependent spontaneous base substitutions that revert the *ura3-29* mutation (Tables 4 and 6; Figure 5). A major component of this antimutator effect in a mismatch repair-proficient strain is suppression of C → G transversions that may arise due to mismatches poorly correctable by proofreading and mismatch repair (see Morrison and Sugino 1994; Luhr *et al.* 1998). Indeed, the ratios of different base substitutions in a mismatch repair-deficient to a mismatch repair-proficient *pol3-Y708* strain are 38 for C → T, 275 for C → A, and 1.6 for C → G (Table 6). Thus, mismatches leading to C → A transversions in the *pol3-Y708A* background at *ura3-29* locus are most vulnerable to mismatch repair, while those leading to C → G transversions are resistant. From the data of Table 6A we can estimate that rate of C → G in *pol3-Y708A* is decreased by the *rev3* deletion at least 12-fold. In a mismatch repair-deficient *pol3-Y708A* strain two other types of base substitutions are also suppressed by the *rev3* (C → T 17-fold, C → A 5-fold; Table 6B). Yeast Polζ is known to efficiently extend mismatched primer termini (Lawrence 1996; Johnson *et al.* 2000), such that loss of this function could explain the observed antimutator effect. Another possibility is that mutant Polδ is unable to bypass spontaneous DNA damage and dissociates giving opportunity for Polζ-dependent error-prone bypass (see Harfe and Jinks-Robertson 2000). The moderate mutator effect of the *rev3* mutation for C → A transversions in a mismatch repair-proficient *pol3-Y08A* strain raises a possibility that there is a relatively small proportion (seen only when most errors are corrected by a mismatch repair) of mispairs leading to C → A transversions that are processed by Rev ζ in an error-free way.

Note that the suppression of spontaneous mutagenesis in replicative Pol mutants by *REV3* disruption is specific in three ways. It is observed for disruption of *REV3* but not for disruption of *RAD30* (Polη) or *POL4* (Figure 5). This suggests that the latter two polymerases may not compete for extending aberrant termini generated by Polδ. Suppression by disruption of *REV3* is observed specifically for *pol3-Y708A*-dependent mutagenesis but not for other replicative DNA polymerase mutations. This suggests that either Polζ does not compete with mutant Polε or Polα for aberrant termini or that the latter polymerases may not need assistance in extending mismatches. Finally, disruption of *REV3* strongly reduces rates of some substitution mutations (for example, those arising in the *pol3-Y708 pms1* strain), but not all (for example, arising in other polymerase mutants), and disruption of *REV3* does not reduce rates of frameshift

mutations. The frameshifts may result from misaligned DNA intermediates with termini that are more easily extended and therefore do not require a specialized polymerase for continued synthesis. The high rate of frameshift mutations and the still higher than normal rate of base substitutions even when the Polζ gene is disrupted suggest that the *pol3-Y708A* mutation at the polymerase active site reduces the base selectivity of the major eukaryotic replicative DNA polymerase.

In the *pms1* background, *pol3-Y708A* stimulated frameshift reversion at *his7-2* substantially more than it stimulated base pair substitutions that revert *ura3-29* (Tables 5B and 6B). This implies a strong effect of the *pol3-Y708A* allele on frameshift mutagenesis in homopolymeric tracts. This may explain why *rev3* deletion did not decrease the rate of Can^r forward mutations in the *pol3-Y708A pms1* strain (Table 4B). For example, it is well known that most mutations at *CAN1* in mismatch repair-defective strains are frameshifts in long homopolymeric tracts (Marsischky *et al.* 1996). If the *pol3-Y708A*-dependent mutations at *can1* are also primarily frameshift mutations in long homopolymeric tracts, they would likely involve misaligned substrates that do not contain a terminal mismatch (as mentioned above). These intermediates may not require extension by pol ζ, and the mutations would therefore not be suppressed by deletion of *rev3*. Thus when the proportion of frameshifts is high, the *CAN1* forward mutation system is rather insensitive to changes in rates of base substitutions.

It was shown earlier that the *REV3* disruption does not suppress spontaneous mutagenesis for Can^r in the *pol3-01* strain, which is defective in Polδ proofreading exonuclease and does not exhibit growth defects (Shcherbakova *et al.* 1996; Datta *et al.* 2000). Thus, unedited replication errors arising when the Polδ active site is normal result in a Polζ-independent increase in mutation rate. On the other hand, the mutator effect of the *pol2-1* mutation (the *URA3* insertion into the center of *POL2* gene), which results in extremely slow growth, is *REV3* dependent (Shcherbakova *et al.* 1996). It is possible that the magnitude of the replication defect conferred by a polymerase mutation determines the extent of Polζ participation in replication of undamaged DNA.

Effect of active site mutations in Polζ on UV survival and mutagenesis: Mutation of the region II tyrosine in the *REV3* gene yields a strong defect in survival and mutagenesis after UV irradiation, while mutation of the region III tyrosine gives milder effects. This implies that these *rev3* missense mutations, which are inferred to be at the active site and reduce the fidelity of other polymerases, actually impair rather than improve the ability of Polζ to participate in mutagenic translesion synthesis with UV photoproducts. It may be informative to study homologous missense mutations with a partial defect in Polζ function in transgenic mice, since complete deletion of mouse Polζ results in early embryonic lethality (Bemark *et al.* 2000; Esposito *et al.* 2000; Wittschieben *et al.* 2000).

Dual mechanism of mutagenesis in replicative DNA Pol mutants: The magnitude of increase and specificity of base pair substitutions in the *ura3-29* locus are different in polymerase α, ε, and δ mutants, which is consistent with different involvement of each of these Pols in replication. Another notable mutator effect of polymerase mutations in a *pms1* background is a strong increase in frameshift mutation rate. The magnitude of the frameshift mutator effect was the same in strains with any of three mutations. We detected almost exclusively +1 frameshifts but we know that Pol mutations lead to elevated levels of −1 frameshifts, too (Y. Pavlov, unpublished observations). Frameshift mutator effects were also observed for other alleles of *POL2* and *POL3* in combination with defective mismatch repair, with one allele of *POL2* exhibiting a specific preference for +1 frameshift mutagenesis (Tran *et al.* 1999; Kirchner *et al.* 2000). The frameshift mutator effect we report here may reflect reduced frameshift fidelity of these mutant polymerases. At least two models could be considered in this respect, misinsertion slippage (Bebenek and Kunkel 1990) and melting misalignment (Fujii *et al.* 1999).

Alternatively or in addition, the mutator effects seen here could be indirectly due to loss of coordination at the replication fork, perhaps leading to substitution of one DNA polymerase for another. Such a switch might be more likely to occur under conditions of lowered catalytic efficiency and/or a high level of misinsertions by a mutant replicative DNA polymerase as well documented in *E. coli* (Rangarajan *et al.* 1997, Strauss *et al.* 2000). This model can explain the base substitution mutator effect of the *pol3-Y708A* mutation that is dependent on Polζ (Figure 5) and may also be relevant to the mutator effects of mutant alleles of replication accessory proteins like RPA, RFC, and PCNA (McAlear *et al.* 1996; Chen *et al.* 1998, 1999). The observation that *rev3* suppression of a mutator effect is locus and mismatch specific (Tables 5 and 6; Figure 5) could be explained by the different nature of the mismatched intermediates and their presence in different local sequence environments. Thus, the extent of involvement of auxiliary polymerase could differ. Finally, it is also possible that altered replication efficiency or fidelity in the strains harboring mutant polymerase alleles may lead to activation of a checkpoint response that results in the accumulation of mutations (Datta *et al.* 2000).

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