The fidelity of DNA synthesis by yeast DNA polymerase zeta alone and with accessory proteins

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ABSTRACT

DNA polymerase zeta (pol ζ) participates in several DNA transactions in eukaryotic cells that increase spontaneous and damage-induced mutagenesis. To better understand this central role in mutagenesis *in vivo*, here we report the fidelity of DNA synthesis *in vitro* by yeast pol ζ alone and with RFC, PCNA and RPA. Overall, the accessory proteins have little effect on the fidelity of pol ζ. Pol ζ is relatively accurate for single base insertion/deletion errors. However, the average base substitution fidelity of pol ζ is substantially lower than that of homologous B family pols α, δ and ε. Pol ζ is particularly error prone for substitutions in specific sequence contexts and generates multiple single base errors clustered in short patches at a rate that is unprecedented in comparison with other polymerases. The unique error specificity of pol ζ *in vitro* is consistent with Pol ζ-dependent mutagenic specificity reported *in vivo*. This fact, combined with the high rate of single base substitution errors and complex mutations observed here, indicates that pol ζ contributes to mutagenesis *in vivo* not only by extending mismatches made by other polymerases, but also by directly generating its own mismatches and then extending them.

INTRODUCTION

In order to copy the many different types of DNA substrates encountered during replication, repair and recombination, eukaryotes encode multiple DNA polymerases. Among these, DNA polymerase ζ (pol ζ) has a particularly important role in cellular processes that result in mutagenesis (1). Pol ζ is a heterodimer composed of proteins encoded by the *REV3* and *REV7* genes (2). *REV3* encodes the catalytic subunit, which contains a B family DNA polymerase domain at its C-terminus. Genetic studies reveal that cells lacking Rev3p or Rev7p have reduced levels of mutagenesis induced by a variety of physical and chemical agents that damage DNA (1). This strongly implicates pol ζ activity in translesion DNA synthesis. Pol ζ function is also required for the majority of spontaneous mutagenesis in wild-type yeast cells (1,3–7), as well as for mutagenesis associated with transcription (8), with double-strand break repair (9–11), and with defective DNA repair (12–15). Vertebrate pol ζ also modulates base substitution mutagenesis during somatic hypermutation of immunoglobulin genes (16,17).

Unlike B family enzymes such as pol δ and pol ε that have intrinsic 3′ exonuclease activities and are highly accurate (18–20), pol ζ lacks intrinsic 3′ exonuclease activity (2), and cannot proofread any misinsertions it may make. Steady-state kinetic analyses of insertion of individual dNTPs at specific primer–templates indicate that, depending on the mismatch, yeast pol ζ discriminates against dNTP misinsertion by factors of $0.19\text{–}41 \times 10^{-4}$, leading to the suggestion that pol ζ synthesizes DNA with accuracy approaching that of exonuclease proficient pol δ (21). Also, with the exception of a C–C mismatch, pol ζ (21) and pol α (22) exhibit a similar range of dNTP misinsertion efficiencies, suggesting that these two naturally exonuclease-deficient polymerases could have similar base substitution fidelity.

Of importance to understanding the basis of mutagenesis were steady-state kinetic analyses indicating that pol ζ discriminates poorly against the insertion of correct dNTPs onto mismatched primer termini (21,23,24). The promiscuity of pol ζ in mismatch extension greatly exceeds that of pol α, the other non-proofreading B-class enzyme (22,25). Along with reports that pol ζ has a limited ability to insert nucleotides opposite damaged template bases (26), this mismatch extension promiscuity has led to the idea that the role of pol ζ in translesion DNA synthesis is primarily to

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extend mismatches made by other DNA polymerases (1,27). Those other polymerases could include pols α, δ and ε, which have primary responsibility for replicating the undamaged nuclear genome. In fact, among those polymerases examined to date, the promiscuity of pol ζ in mismatch extension is shared only by Y-family enzymes such as pol η (28) and pol κ (27) and by X family enzymes such as pol λ (29) and, for some mismatches, pol β (30,31).The X- and Y-family polymerases are considerably less accurate than the three major replicative polymerases in family B (see further discussions below).

The studies mentioned above, and genetic evidence indicating that pol ζ also participates in forming spontaneous single base insertions and deletions and more complex mutations (5,6,12,32), prompted the present study to define the fidelity of DNA synthesis by *Saccharomyces cerevisiae* pol ζ when copying undamaged DNA templates. Here we do so in reactions containing all four dNTPs in direct competition with each other, using an assay that defines rates for single base errors and detects more complex errors in a variety of sequence contexts, similar to those encountered by pol ζ *in vivo*. This assay has been used previously to describe the fidelity of many other DNA polymerases, allowing direct comparisons to be made for copying the same template bases. Our fidelity studies have been carried out with the *S.cerevisiae* pol ζ heterodimer composed of Rev3p (173 kDa) and Rev7p (29 kDa). Motivated by a recent study (33) demonstrating that the efficiency of translesion synthesis by pol ζ is strongly enhanced in the presence of the PCNA processivity clamp and the single-strand DNA-binding protein complex RPA, we also report the effects of these accessory proteins on the fidelity of pol ζ.

MATERIALS AND METHODS

Enzymes and reagents

S.cerevisiae Pol ζ was purified as described previously (33). RPA, PCNA and RFC were purified from *Escherichia coli* overproduction strains as described previously (34,35). Materials for the fidelity assay were from previously described sources (19,36).

Preparation of complementary DNA substrates

In order to measure the fidelity of DNA synthesis when copying complementary strands of the same DNA sequence, two new M13*lac* DNA substrates were constructed for the M13-based forward mutation assay as follows. Double-stranded M13mp2 DNA was digested with restriction endonuclease HhaI and the fragment spanning nt −83 through +196 (where +1 is the first transcribed nucleotide of the *LacZ* gene) was isolated by electroelution from a 2.0% agarose gel. This fragment was treated with T4 DNA polymerase in a reaction containing dGTP to generate a 275 bp blunt-ended fragment whose ends were bp −81 and +194. Double-stranded M13 DNA was digested with restriction endonuclease AvaI, which cuts once at position 5825 in the

M13 genome. This DNA was similarly treated with T4 DNA polymerase in a reaction containing dGTP to generate a linear, blunt-ended M13 vector. The two blunt-ended fragments were ligated and introduced into competent *E.coli* cells to score plaques having a blue-plaque phenotype. DNA from several blue plaques was analyzed by DNA sequence analysis to determine the sequence and orientation of the *LacZ* insert. Two new vectors, one having an insert in the orientation identical to that found in the original series of M13 *lac* vectors and the other having the same insert in the opposite orientation, were chosen for preparation of two different gapped DNA substrates for fidelity assays. The restriction enzymes used for gap construction were BanII and AvaI. These cut both new constructs to yield a large 6218 bp fragment. This fragment was purified using three cycles of PEG precipitation at 37°C, in order to completely remove the smaller DNA fragments with sticky ends. The 6218 bp primer fragment was then hybridized to complementary single-stranded circular M13lac DNAs, as described previously (36). This generates duplex circular M13lac substrates containing a 461 nt single-stranded DNA gap. These gapped substrates were gel-purified and used in polymerization reactions. One gap contains the (+) strand of the 275 base *lacZ* α-complementation sequence, such that the template being copied (Figure 1) is identical to that used in previous studies of polymerase fidelity that involved the M13mp2 forward mutation assay. The other gap contains the (−) strand as a template, thereby allowing parallel measurements of error rates for synthesis instructed by each of the two complementary DNA strands.

Gap-filling DNA synthesis reactions and product analysis

Reactions (25 μl) contained 20 mM Tris–HCl, pH 7.7, 8 mM MgAc$_2$, 60 mM NaCl, 0.5 mM ATP, 100 μM each dNTPs, 1 mM DTT, 100 μg/ml BSA, 25 fmol (1 nM) gapped DNA, ~1 pmol pol ζ and 0.1% Triton X-100. When included, the amounts of accessory proteins used were 500 fmol PCNA, 200 fmol RFC and 5 pmol RPA. Reaction mixtures without accessory factors were incubated for 45 min at 30°C, and those with accessory factors for 30 min, and DNA products were then analyzed by agarose gel electrophoresis as described previously (36). All reactions filled the gap [data not shown, but for typical result see Figure 3 in Ref. (36)]. DNA products of gap-filling reactions were introduced into *E.coli* cells and plated as described previously (1) to score blue M13 plaques resulting from correct synthesis and light blue and colorless plaques containing polymerization errors. The types of errors were determined by sequencing the *lacZ* α-complementation gene in single-stranded DNA isolated from independent mutant M13 plaques. Error rates were calculated according to following equation: ER = $(N_i/N) \times$ MF)/(D $\times$ 0.6), where N_i is the number of mutations of a particular type, N is the total number of mutants analyzed, MF is the frequency of *lacZ* mutants, D is the number of detectable sites for the particular type of mutation and 0.6 is the probability of expressing a mutant allele in *E.coli* (36). Error rates for phenotypically

silent nucleotide changes were calculated as the number of changes observed divided by the total number of bases analyzed, i.e. number of mutants sequenced times the number of bases in the analyzed region (from position 198, the first nucleotide incorporated, to position −81, see spectra in Figure 1). The statistical significance of differences in pairs of error rates was calculated using the Fisher's exact test (37).

(A) Pol ζ, (+) FWD

```
            G
            G
            G                   A  G      G      CGCG G     G  T     G     CC           CC       A
            CA ACGCAATTAA  TGTGAGTTAG  CTCACTCATT  AGGCACCCCA  GGCTTTACAC  TTTATGCTTC  CGGCTCGTAT
            |                       |                     |                   |
           -80                     -60                   -40                 -20

                                           TTT
                                           T
                                           T
                                           T                G        G                    G        G
    CA          G       G      G        T     CG  G  G  G  G     Δ       TG       GG      T
GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACC ATG ATT ACG AAT TCA CTG GCC
    |               |                     |                                        |
    1              20                    40                                       60

                                                            C
                              G     +A                      G                  GGG
         G    C        C    G      T                        G          C   GGG  G    G
    Δ    G    C    C   T   GC  AGG GG                        G          C   GG  GC   C            T
GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT
         |                           |                                   |
        80                         100                                 120

                                   Δ    Δ
              G              G     Δ  GGG                 GGGGGG 1/29                    G 1/22
             GGG           GGG    G  GGG                  GGGGGG                          G
              G             G      G  G                   GGGGGG                          G
              G    G        G      G  G   G   G           GGGG                            G
    G         C    G    T   G      G  G   G   G           G                              CG
    G    C    G  G GC  TC   G   G  GG  G   G Δ G  G   GG GTG   T   G   TGG  G        GGG  CGAT
CCC CCT TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CGC CCT TCC CAA CAG TT CTCGGGCTA
        |                           |                                   |
       140                         160                                 190        Start
```
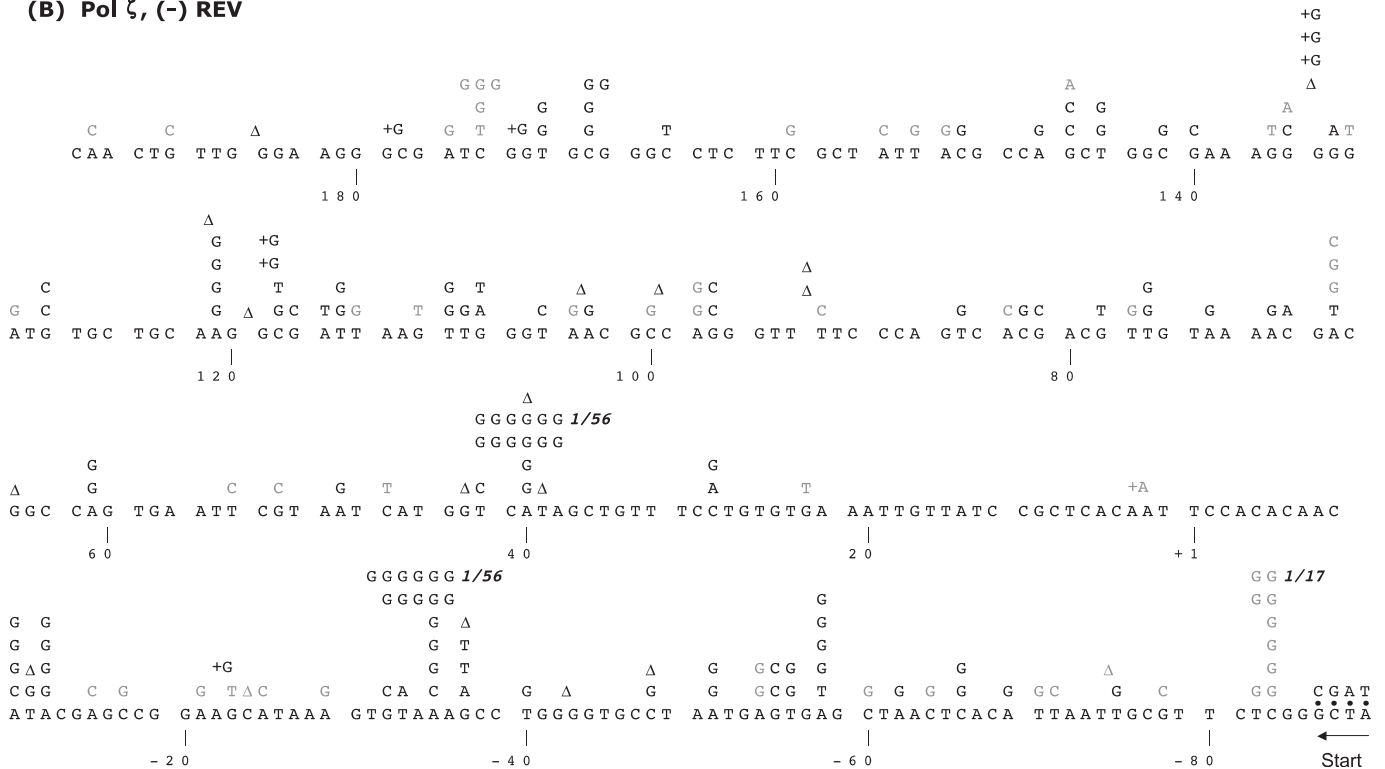
(B) Pol ζ, (−) REV

```
                                                                                          +G
                                                                                          +G
                                                                                          +G
                  GGG         GG                                      A             Δ
                  G    G   G                                    C  G              A
      C    C     Δ      +G   G  T  +GG  G      T         G    C  G  GG    G  C  G  G  C    TC   AT
      CAA CTG TTG GGA AGG GCG ATC GGT GCG GGC CTC TTC GCT ATT ACG CCA GCT GGC GAA AGG GGG
          |                           |                                   |
         180                         160                                 140

            Δ
            G   +G                                                                           C
            G   +G                                                                           G
    C       G   T   G         G  T      Δ     Δ  GC        Δ                        G        G
  G C       G  Δ GC TGG    T  GGA   C  GG   G  GC      C          G   CGC   T  GG  G   GA   T
ATG TGC TGC AAG GCG ATT AAG TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG ACG TTG TAA AAC GAC
          |                           |                                   |
         120                         100                                  80

                               Δ
                               GGGGGG 1/56
                               GGGGGG
      G                        G                    G
  Δ   G        C    C    G  T   Δ C    G Δ          A      T                +A
GGC CAG TGA ATT CGT AAT CAT GGT CATAGCTGTT TCCTGTGTGA AATTGTTATC CGCTCACAAT TCCACACAAC
      |                   |                         |                            |
     60                  40                        20                           +1

                    GGGGGG 1/56                                G                  GG 1/17
                    GGGGG                                      G                  GG
  G  G              G   Δ                                      G                   G
  G  G              G   T                                      G                   G
  G Δ G        +G   G   T       Δ    G  GCG  G           G            Δ            G
CGG  C  G   G TΔC   G   CAC A   G  Δ   G   G  GCG T  GGG G  GC   G  C    GG  CGAT
ATACGAGCCG GAAGCATAAA GTGTAAAGCC TGGGGTGCCT AATGAGTGAG CTAACTCACA TTAATTGCGT T CTCGGGCTA
      |                   |                         |                            |
    -20                 -40                       -60                          -80     Start
```

RESULTS AND DISCUSSION

Fidelity measurements and calculation of error rates

The fidelity of pol ζ with and without accessory proteins was determined for synthesis to fill a single-stranded gap in a circular duplex M13*lac* DNA substrate. The gap contains the *lacZ* α-complementation template sequence (Figure 1)

that when copied correctly leads to a blue M13 plaque phenotype. Polymerization errors are detected as light blue and colorless plaques. Gap-filling reactions were conducted with the pol ζ heterodimer in four conditions: alone, with RPA, with RFC and PCNA, or with RPA, RFC and PCNA. All four reactions filled the gap (data not shown). When the DNA products were analyzed for *lacZ* mutant frequencies,

(C) Pol ζ +PCNA/RFC, (+) FWD

(D) Pol ζ +RPA, (+) FWD

(E) Pol ζ +PCNA/RFC+RPA, (+) FWD

```
                                                      C C
                                                      C C
            T              C                          C                 C
            C              C              C           C                 C          C T
   CA ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA GGCTTTACAC TTTATGCTTC CGGCTCGTAT
      |                    |                    |                    |
     -80                  -60                  -40                  -20


                                     G
                                     G
                                     G
   A              G         G C       A      C  G G    G          A       T         C      G
GTTGTGTGGA ATTGTGAGCG GATAACAATT TCACACAGGA AACAGCTATG ACC ATG ATT ACG AAT TCA CTG GCC
      |                    |                    |                    |
      1                   20                   40                   60


                                                                            Δ
                      T                              C    T    CCC
              C       T                         G         G    G    C    CCC
      T       G   C       GG GC   G Δ    +C          CG         TC   G    C    C
      T  ΔA T  C   A   T  GA GC   CG ΔCC  A     Δ   CG    T      TC   GA   C    C    C         C
GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA CAT
                    |                    |                    |
                   80                  100                  120

   +T                                    T         GGGGGGGG 1/53                  GG 1/18
   +T                                    T     G    GGGGGGGG                       GGG
   +T                     GGG            G         GGGGGGGG                         G
   +T                     GG             G    Δ    GGGGGG                           G
T       G      T  A  TA   G      G   G        TC   Δ    C               G         +C  GG
TT   T     C G      CG GAC TΔG  T  G    TGG  GC CGG GC   G    Δ    C     G  G  +C   C    C    GG   CGAT
CCC CCT TTC GCC AGC TGG CGT AAT AGC GAA GAG GCC CGC ACC GAT CGC CCT TCC CAA CAG TT CTCGGGCTA
         |                    |                                        |           ←
        140                  160                                      190          Start
```

(F) Pol ζ +PCNA/RFC+RPA, (-) REV

```
                      T                                A                          T
              G  TGC     T                    A    C  T  C      G  T              TA
   CAA CTG TTG GGA AGG GCG ATC GGT GCG GGC CTC TTC GCT ATT ACG CCA GCT GGC GAA AGG GGG
                  |                    |                    |
                 180                  160                  140


                              T
                      A       A
C     T    T  G    C          A  A                          T         GT     G          T
ATG TGC TGC AAG GCG ATT AAG TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG ACG TTG TAA AAC GAC
              |                    |                    |
             120                  100                   80

                                   T
                                   C
      G       A           G    C
      G C     A       T   C   GCG                                       C              C
GGC CAG TGA ATT CGT AAT CAT GGT CATAGCTGTT TCCTGTGTGA AATTGTTATC CGCTCACAAT TCCACACAAC
      |                    |                    |                    |
     60                   40                   20                   +1

C
C                     GGGG                        TTTTTTTT 1/50           GGG 1/10
C                     G                           TTTTTTTT                 GGG
C                     GG                     G    TTTTTTTT  T              GGG
C                     GG            +A       G     TTTTT    T               G
C          +G         G  GG         +G       G  G      T    T               G
C C        +G         GCGG   T      Δ    +C  G  G    C  G  T    +A          G
GGGT       +G      G    T  GTGGTGT Δ   Δ    Δ    T   GCG    AGC  G  GGGGC      G   CGAT
ATACGAGCCG GAAGCATAAA GTGTAAAGCC TGGGGTGCCT AATGAGTGAG CTAACTCACA TTAATTGCGT T CTCGGGCTA
         |                    |                    |                    |        ←
        -20                  -40                  -60                  -80        Start
```

Figure 1. Error spectra of single base change by pol ζ alone and with accessory proteins. (**A**) Pol ζ alone with lacZα (+) strand as template in the forward direction: DNA synthesis fidelity assay was performed with gapped M13mp2 substrate that contained a portion of the lacZ gene in the 461 nt gap region. Error changes generated by pol ζ near the M13mp2 lacZ target sequence are shown above the bottom template sequence. The base substitution mutations are shown as the base changed to, single nucleotide deletions are shown as triangles and additions are shown as the plus sign and the base added. The lacZ(+) strand in forward direction is shown, with the transcriptional start site designated as position 1 and the first 53 codons displayed as triplets except the bases introduced during cloning (positions 194–198). The changes colored in gray are phenotypically silent and were found as hitchhikers in the mutants with detectable mutations. The numbers next to the A175G and C196G changes are base substitution error rates at these positions. (**B**) Pol ζ alone with lacZα(−) strand as template in the reverse direction. (**C**) Pol ζ and PCNA/RFC with lacZα (+) strand as template in the forward direction. (**D**) Pol ζ and RPA with lacZα(+) strand as template in the forward direction. (**E**) Pol ζ, PCNA/RFC and RPA with lacZα(+) strand as template in the forward direction. (**F**) Pol ζ, PCNA/RFC and RPA with lacZα(−) strand as template in the reverse direction.

Table 1. Error rates of DNA polymerase ζ with and without accessory proteins

Enzyme			Pol ζ		Pol ζ +PCNA/RFC	Pol ζ +RPA	Pol ζ +PCNA/RFC+RPA	
DNA template strand and orientation			(+) FWD	(−) REV	(+) FWD	(+) FWD	(+) FWD	(−) REV
Mutant frequency			12%	10%	7.9%	7.1%	6.0%	5.2%
No. of mutants analyzed			133	132	116	124	159	133
Error rates ($\times 10^{-5}$)	Frameshift	−1	4.4 (6)	10.0 (16)	5.7 (10)	4.3 (9)	2.8 (9)	1.0 (3)
		+1	0.7 (1)	5.1 (8)	2.3 (4)	1.4 (3)	2.2 (7)	2.0 (6)
	Base substitution	Detectable sites	130 (131)	98 (118)	93 (120)	84 (129)	64 (115)	56 (131)
		Non-detectable sites	140 (51)	150 (55)	80 (26)	120 (43)	74 (33)	49 (18)
Mutant frequency of complex mutations			1.2% (14)	3.0% (39)	0.75% (11)	0.63% (11)	0.26% (7)	0.35% (9)

Notes: The numbers in the parentheses are the corresponding number of mutation events detected. The complex mutations are defined as multiple mutations occurred within 6 bases (Table 3). The single base substitutions/frameshift changes that observed in complex mutations and separated by one or more bases were counted in the error calculation of both complex mutation and base substitution/frameshift events. The tandem mutations were counted in complex mutation only. The base substitutions at non-detectable sites were recovered as 'hitchhikers' in lacZ mutants (shown as gray in Figure 1). For details of the template strand and orientation of the DNA gap substrates see Materials and Methods and Figure 1A–F.

Figure 2. Comparison of pol ζ base substitution error rates with and without accessory proteins for all 12 possible mismatches. Some error rates are reported as less than or equal to values (indicated by an asterisk above the bar).

Table 2. Singe base error rates of pol ζ compared to other DNA polymerases

DNA polymerase	Family	Error rate ($\times 10^{-5}$) Substitution	Deletion	Reference
hpol η	pol Y	3500	240	(47)
hpol κ	pol Y	580	180	(48)
ypol ζ	pol B	130	4.4	This study
ypol α	pol B	9.6	3.1	(50,19)[a]
ypol δ (exo-)	pol B	13	5.7	(20)
ypol ε (exo-)	pol B	24	5.6	(19)
ypol δ	pol B	⩽1.3	1.3	(20)
ypol ε	pol B	⩽2	⩽0.05	(19)

Notes: The abbreviations used in Table 2 are h, human; y, yeast.
[a]Data are from Ref. (50) and recalculated in Ref. (19) based on new detectable sites found.

α-complementation sequence. When copied either by pol ζ alone or by pol ζ plus all accessory proteins, the mutant frequencies were 10 and 5.2%, respectively, similar to values obtained with the (+) strand template (Table 1). DNA sequence analysis of 132 and 133 *lacZ* mutants respectively (Figure 1B and F), from those reactions resulted in the average pol ζ error rates shown in Table 1. Informative comparisons of error rates with the (+) and (−) strand templates are considered in the following discussion of the three major categories of errors made by pol ζ.

Pol ζ has relatively high single base insertion/deletion fidelity

When copying the (+) strand template, yeast pol ζ generated single base insertion and single base deletion errors at average rates of 0.7×10^{-5} and 4.4×10^{-5}, respectively (Table 1). Values when copying the (−) strand template were similar but slightly higher (one-tailed $P = 0.033$ for insertions; $P = 0.056$ for deletions), perhaps reflecting small sequence context effects on insertion/deletion fidelity, since the two templates are complementary rather than identical. Alternatively, small differences in error rates for specific subsets of errors mentioned here and below may partly reflect experimental fluctuation, given that the number of events detected by sequence analysis is sometimes small for certain errors.

the four reactions yielded mutant frequencies of 12, 7.1, 7.9 and 6.0%, respectively. A total of 133, 116, 124 and 159 mutant plaques, respectively, were sequenced to identify the nucleotide changes responsible for the phenotypes. Sequence analysis also revealed phenotypically silent nucleotide changes (light gray in Figure 1) present as hitchhikers in *lacZ* mutants that contain one or more detectable changes. Figure 1A and C–E shows the distribution of single base changes made by Pol ζ when copying the (+) strand template. The mutant frequency data and sequencing results were used to calculate rates for the different types of errors. These rates are summarized in Table 1 and Figure 2, and compared to average single base error rates for other DNA polymerases in Table 2. Also shown in Table 1 are mutant frequencies for more complex sequence changes, as listed in Table 3.

Parallel reactions were performed with the gapped substrate containing the complementary (−) strand of the *lacZ*

Table 3. Complex mutations generated by DNA polymerase ζ

Location	Change
Pol ζ , (+) FWD	
−63 to −57	TTAGCTC to GTAGCTG
−58 to −53	TCACTC to CCCCCT
−15 to 26	T-15A; Δ-11–25; A26C
−13 to 25	Δ-13–25
−9 to 26	Δ-9–26
25 to 26	CA to AT
76 to 80	A76G; Δ78–80
139 to 140	TC to G
161 to 163	AGA to G
161 to 163	AGA to GGG
161 to 163	AGA to GGG
166 to 170	CCCGC to GCCGG
178 to 181	GCCC to TCCT
193 to 194	TC to AG
Pol ζ, (−) REV	
−65 to −61	TCAAT to G
−65 to −61	TCAAT to G
−62 to −56	ATCGAGT to GTCGAGG
−60 to −58	CGA to GGG
−55 to −53	GAG to TT
−54 to −48	AGTAATC to GG
−47 to −45	CGT to GTG
−42 to −41	GG to C
−41 to −39	GTC to CT
−40	T to GG
−38 to −36	CGA to T
−37 to −34	GAAA to TTG
−34 to −33	ΔAT
−34 to −33	AT to GG
−34 to −33	AT to GG
−24 to −21	CGAA to G
−22 to −21	AA to GAG
−17 to −11	CGAGCAT to GGCGCA
−12 to −10	ATA to GTC
22 to 24	AGT to GG
27 to 28	ΔGT
40 to 42	ACT to CC
41 to 52	TCCGAAATGTGA to GCCGAAGTGTGG
52 to 54	GCT to CCC
52 to 87	G52T; Δ53–87
54 to 55	TT to AA
55 to 56	TA to CC
65 to 67	GCA to CC
67 to 70	AGCT to GGCG
73 to 77	ATGTT to GGGTG
83 to 85	ACT to CCG
107 to 109	GGT to TA
108 to 111	GTTG to ATTT
109 to 110	TT to GTG
109 to 110	TT to GTG
117 to 122	GCGGAA to CCGGG
129 to 132	GTAG to AT
136 to 137	+GG
153 to 155	TTA to GTC
Pol ζ + PCNA/RFC, (+) FWD	
−69 to −68	ΔTG
−13 to 25	Δ-13–25
33 to 39	ACAGCTA to GCAGCTG
105	A to TT
111 to 115	CTTAA to TTTAG
126 to 136	GCACATCCCCC to ACCCCTTCCCTT
139 to 141	TCG to CC
149 to 151	GCG to CC
150 to 156	CGTAATA to AGTAATG
165 to 171	GCCCGCA to CCCCCCG
195 to 196	TC to CG
Pol ζ +RPA, (+) FWD	
61 to 64	TGGC to GGGG
71 to 83	Δ71–83

Table 3. *Continued*

Location	Change
79 to 82	GTCG to TTCT
112 to 115	TTAA to CTAG
131 to 136	TCCCCC to ACCCCCT
131 to 137	TCCCCCT to ACCCCCC
156 to 161	AGCGAA to GGCGAG
160 to 161	AA to GG
194 to 196	CTC to TTG
195 to 196	TC to CG
195 to 196	TC to CG
Pol ζ + PCNA/RFC+RPA, (+) FWD	
−56 to −55	ΔAC
61 to 65	TGGCC to GGGCG
111 to 115	CTTAA to TTTAG
152 to 156	TAATA to GAATG
161 to 163	AGA to GGG
178 to 184	GCCCTT to CCCCTTC
195 to 196	TC to CTG
Pol ζ + PCNA/RFC+RPA, (−) REV	
−74 to −69	TAATTA to GAATTT
−73 to −69	AATTA to TATTT
−73 to −69	AATTA to TATTT
−73 to −69	AATTA to TATTT
−73 to −69	AATTA to TAATTT
−69 to −65	ACACT to TCACA
−25 to −20	ACGAA to GCGAAG
42 to 44	TGG to GGT
43 to 44	GG to TT

Notes: Base changes are underlined. Δ, deletions; +, insertions; (+) FWD and (−) REV, substrate strand and orientation. Multiple mutations within 6 bases were counted.

The ability of pol ζ to generate single base insertions and deletions is consistent with genetic studies implicating pol ζ in frameshift mutagenesis *in vivo* (12,38–41). Nonetheless, yeast pol ζ is substantially more accurate than the Y-family DNA polymerases pol η and pol κ (Table 2) or the X family polymerases involved in DNA repair (42), including yeast pol IV (43). In fact, pol ζ insertion/deletion error rates are similar to those for yeast pol α and exonuclease-deficient derivatives of yeast pol δ and pol ε (Table 2). Thus all four eukaryotic family B polymerases interact with undamaged primer–templates in a manner that modulates to similar extents misalignments that contain mismatches with a single unpaired base in the template strand (for deletions) or primer strand (for insertions).

The pol ζ error rate for deletions is marginally higher than for insertions [Table 1, one-tailed $P = 0.063$ for (+) strand template; $P = 0.076$ for (−) strand template], which is similar to observations with other polymerases [reviewed in (44)]. Both types of errors were more frequently observed in repetitive sequences (e.g. 22/31 deletions, 12/15 insertions, Figure 1) as compared with non-iterated sequences. This was also the case when copying the complementary strand substrate (data not shown), and is consistent with the strand slippage hypothesis proposed 40 years ago (45) and reviewed recently in (42). With one exception (higher deletion rate (10×10^{-5}) in the (−) strand template for pol ζ alone than for pol ζ with accessory proteins (1.0×10^{-5}), one-tailed $P < 0.0001$), pol ζ error rates for single base insertions and deletions were similar in the presence of the accessory proteins (Table 1). The limited number of mutants sequenced, and the infrequency of their occurrence, does not exclude that

accessory proteins might differentially influence error rates for specific insertion or deletion errors depending on the type or composition of the misalignment and/or its location. Nonetheless, any effects of RPA, RFC and PCNA on insertion and deletion error rates are small in comparison with the >10 000-fold selectivity against misalignments (i.e. error rates $<10^{-4}$) conferred by pol ζ alone. Thus pol ζ itself is by far the primary determinant of selectivity against single base errors resulting from substrate misalignments occurring when it copies undamaged DNA. Among many different exonuclease-deficient eukaryotic DNA polymerases (46), pol ζ has relatively high insertion/deletion fidelity (Table 2).

Pol ζ generates lacZ mutants with complex multiple sequence changes

Pol ζ generated many *lacZ* mutants that contained more than a single base change. This includes six tandem double base substitutions and one tandem triple base substitution. These were recovered among 265 sequenced *lacZ* mutants [Table 1 and Table 3, Pol ζ (+) FWD and Pol ζ, (−) REV], yielding an error rate for tandem double substitutions of ∼3 × 10^{-5} (estimated by treating them as single detectable base substitutions). This is an extraordinary rate given that forming a tandem double base substitution requires three consecutive rare incorporation events: an initial misinsertion, a second misinsertion from the resulting mismatch and extension of doubly mismatched primer terminus. Pol ζ infidelity for tandem double base substitution errors is only exceeded by pol η (47) and pol κ (48), both of which also have a lower fidelity than pol ζ for single base substitutions (Table 2). Interestingly, tandem double base substitutions, including those unlikely to result from cytosine deamination in photodimers, are reported in the spectra of mutations induced by UV light (49).

Many of the *lacZ* mutants generated by pol ζ during DNA synthesis *in vitro* contained two or more non-tandem but closely spaced sequence changes (Tables 1 and 3). Many of the sequence changes present in these complex *lacZ* mutants are base substitutions, while others are deletion or insertion of one or more nucleotides, and these events are found in various combinations (Table 3). Importantly, the proportion of such mutants among the total number of *lacZ* mutants analyzed, and the overall frequency with which these complex events are generated (Table 1), far exceeds that observed in previous studies of other family B polymerases, including yeast pol α (≤0.06%) (50), pol δ (≤0.05%) (20) and pol ε (≤0.04%) (19). These data lead to two important conclusions. First, they clearly demonstrate that yeast pol ζ can both create and then promiscuously extend primer–templates containing multiple, closely spaced mismatches. Second, they strongly imply that it is indeed pol ζ that is directly responsible for generating complex mutations in yeast that include a single base frameshift combined with nearby base substitutions (5,32,41).

Also observed in the collection of non-single base errors generated by pol ζ alone were deletions of larger numbers of nucleotides (Table 3). Previous studies have shown that two other B family enzymes, pol α (50) and pol δ (20), also delete large numbers of nucleotides. Those deletions involved loss of one of two direct repeats of 2–9 bases plus

the intervening nucleotides. They can be explained by a model (51) in which the first repeat sequence encountered by a polymerase during gap filling is copied, then the primer containing the repeat frays and relocates to the second repeat sequence. This provides a primer terminus adequate for continued synthesis from the misaligned intermediate that contains a loop of unpaired, template strand bases that are eventually deleted. Interestingly, the endpoints of the deletions generated here by pol ζ do not involve obvious direct repeat sequences. Thus, they may be generated by a mechanism that is distinct from large deletions generated by pol α and pol δ. However, the deletions generated by pol ζ are sometimes associated with base substitutions adjacent to the deletion endpoints. Thus, the general misalignment process may be similar, but by virtue of its promiscuous mismatch extension capacity, pol ζ may be able to extend misaligned large deletion intermediates containing less homology at the primer terminus than is required for extension by pol α and pol δ. The capacity to extend such substrates may be relevant to participation of pol ζ in the mutagenic repair of double-strand breaks in DNA (9,10).

Pol ζ exhibits low base substitution fidelity

The single base substitution error rates of pol ζ alone when copying the (+) and (−) strand templates were 130×10^{-5} and 97×10^{-5} (Table 1). These rates represent the number of substitutions generated per phenotypically detectable nucleotide polymerized, i.e. those errors known to result in a M13 plaque color phenotype. The values in Table 1 are average error rates for the 12 possible single base–base mismatches scored in many different sequence contexts. Similar rates (Table 1) were calculated (Materials and Methods) for errors at phenotypically silent locations, recovered as 'hitchhiker' substitutions in *lacZ* mutants.

A comparison of base substitution errors made by yeast pol ζ with those made by other DNA polymerases in the same target shows that pol ζ is much less accurate than the homologous B family yeast polymerases that conduct the bulk of chain elongation during replication of the yeast nuclear genome. Pol δ and pol ε are at least 100-fold more accurate than pol ζ (Table 2). Although this is partly due to their intrinsic proofreading activities (19,20), even exonuclease-deficient derivatives of pol δ and pol ε, as well as naturally exonuclease-deficient pol α, have substantially higher base substitution fidelity than pol ζ. The lower base substitution fidelity of pol ζ must necessarily result from lower average discrimination against dNTP misinsertion, because pol ζ is the only enzyme in the *in vitro* assay. However, the average base substitution fidelity of yeast pol ζ is higher than those for human pol κ and human pol η, both are Y-family DNA polymerases (Table 2). This can be rationalized by structural studies suggesting that Y-family polymerases have larger and more solvent accessible nascent base pair binding pockets than do B family polymerases (52).

Pol ζ has highly unusual base substitution error specificity

Error rates for the 12 different single base–base mismatches made when copying the (+) strand templates are shown in

Figure 2. Similar rates were observed for copying the (−) strand template (data not shown). Many of these error rates differ from steady-state kinetic values for individual dNTP misinsertions, as well as for correct dNTP incorporation onto mismatched primer termini (21). Logically, mismatches resulting from higher misinsertion rates that are also extended with higher efficiencies might be expected to give rise to the highest base substitution error rates. For example, the kinetic results (21) imply that the highest error rate among the 12 mismatches should be for the T-dGMP mismatch. However, the highest base substitution error rate detected in the forward mutation assay is for stable misincorporation of dCMP opposite template A. Notably, this high rate is not due to a single mutational hotspot in the lacZ target because misincorporation of dCMP was detected multiple times at nine distinct template A positions (Figure 1A). The disparity with the steady-state kinetics are likely due to the fact that the kinetic experiments were performed within a single sequence context for each mismatch, while the base substitution error rates determined here using the forward mutation assay include a wide variety of sequence contexts (Figure 1), with error rates highly dependent on sequence context (discussed below).

The present data reveal that average pol ζ error rates vary over 30-fold, depending on the mismatch. Unexpectedly, two aspects of pol ζ error specificity differ from all other polymerases characterized to date, including pol ζ's B family homologs pol α (50), pol δ (20) and pol ε (19). Pol ζ is the only polymerase whose error rate for the A-dCMP mismatch exceeds that of the 11 other mismatches (Figure 2), and it is the only enzyme whose error rate for the C-dCMP mismatch is second highest among all mismatches. The error rates for these and other mismatches are only reduced by ≤3-fold in the presence of the accessory proteins, implying that these error signatures should be useful for discerning the contribution of pol ζ to base substitution mutagenesis *in vivo*. For example, the unusually high relative rate for the C–C mismatch, and the fact that the third highest rate is for the G-dGMP mismatch, both suggest that pol ζ generates C–G to G–C transversions *in vivo*. Strong support for this prediction comes from the study of a yeast strain harboring a point mutation in the active site of pol δ that results in replicative stress (53). This strain has a spontaneous mutator phenotype that includes a high mutation rate for C–G to G–C transversions. Importantly, this rate is reduced by ≥12-fold upon inactivation of Rev3, just as predicted by the error specificity of pol ζ in Figure 2.

Strong sequence context effects on pol ζ base substitution error rates

Pol ζ base substitutions are distributed non-randomly within the *lacZ* target sequence (Figure 1). For example, at template position 175 (Figure 1A), the error rate for incorporation of dCMP opposite A is 3.5% (1/29) (Figure 1A). An even higher rate of 4.5% (1/22) is observed for incorporation of dCMP opposite C at C196, a phenotypically silent location. Thus, in some sequence contexts, pol ζ is among the least accurate of DNA polymerases, rivaling the remarkable infidelity of the Y-family polymerases. The types and locations of sites of

most frequent substitutions shown in Figure 1 are distinct for those of pol η (47) and other DNA polymerases like pol β (54) when copying the same template. They are not limited to one specific sequence or to one mismatch. Initial examination of the local sequence environments of the most versus least frequent sites did not reveal an obvious pattern that could explain the distribution.

Pol ζ error rates are highly asymmetric

Inspection of the error specificity of pol ζ reveals two types of asymmetric misincorporation. First, error rates for reciprocal mismatches of the same base composition are not the same. For example, the error rate for misincorporation of dCMP opposite template A is 11-fold higher than for misincorporation of dAMP opposite template C (Figure 2, one-tailed $P < 0.0001$). Other asymmetries of this type are seen in Figure 2 and were also observed for copying the (−) strand template (data not shown). Thus the ability of pol ζ to discriminate against misincorporation depends not only on the base composition of the mismatch, but also mismatch symmetry with respect to which base is in the template or incoming as a dNTP. This is consistent with structural studies showing different polymerase side chain interactions with the template base and the incoming dNTP.

A second type of asymmetry involves differences in error rates for the two mismatches that could theoretically explain a base substitution arising during replication of double-stranded DNA *in vivo*. For example, the error rate for incorporating dCMP opposite A175 in the (+) template is 3.5%, but the error rate for incorporating dGMP opposite T175 in the complementary (−) template is ≤0.13%, a difference of at least 27-fold. Additional examples of this type of asymmetry are seen in different sequence contexts, resulting in an average error rate for misincorporation of dCMP opposite template A that is 8-fold higher than for misincorporation of dGMP opposite template T (Figure 2, one-tailed $P < 0.0001$). These asymmetries and the variations in error rates by mismatch and sequence context may be useful for assigning a role for pol ζ in specific mutagenic transactions *in vivo*.

Accessory proteins have modest effects on single base substitution fidelity

The overall average base substitution fidelity of pol ζ was increased by ~2 fold in the presence of the accessory proteins (Table 1). When individual single base mismatches (Figure 2) or individual template locations (Table 1 and Figure 1) are compared, the accessory proteins either have no effect or they increased (e.g. C-dCMP mismatch) or decreased (G-dATP) base substitution fidelity by a few-fold. The one exception is at template nucleotide −69 in the (−) strand template, where the accessory proteins increased the rate of A-dATP errors (Figure 1B and F). The results suggest that the accessory proteins may modulate the fidelity of Pol ζ either up or down, in a mismatch and/or sequence-specific manner. Similar effects of accessory proteins have also been observed previously with two highly accurate B family enzymes, RB69 DNA polymerase (55,56) and yeast pol δ (57). The present study shows that the effects

of accessory proteins are small compared to the level of discrimination imparted by the polymerase. In other words, the pol ζ heterodimer itself is by far the prime determinant of fidelity for both single base substitutions and single base-insertion/deletions errors. In the future, it will be interesting to test whether other accessory proteins influence the fidelity of pol ζ. Candidate proteins include Rev1p, which is implicated by genetic evidence to participate in pol ζ-dependent mutagenesis *in vivo* (1), and the 9-1-1-checkpoint clamp, which has been shown recently to physically interact with Pol ζ and is partially required for its contribution to spontaneous mutagenesis in yeast (7).

Dual roles for pol ζ in mutagenic synthesis

Based on steady-state kinetic analysis, it has been suggested previously that pol ζ is a highly accurate DNA polymerase whose primary role in mutagenesis is mismatch extension (21). The present study demonstrates that pol ζ actually has low average fidelity for single base substitutions, and very low fidelity in specific sequence contexts. Such low fidelity, and the similarities in *in vitro* error signatures and *in vivo* mutagenesis described above, strongly implicates pol ζ in both nucleotide misinsertion and mismatch extension during mutagenic synthesis *in vivo*. It is important to note that this idea does not exclude the current 'two polymerase model' for TLS (58,59). A large number of structurally diverse intermediates can be envisioned for both spontaneous and damage-induced mutagenesis. It may well be that the relative contribution of misinsertion and mismatch extension by pol ζ versus other low fidelity polymerases, several of which are also promiscuous for mismatch extension (27,28,48) may depend on the nature of the mutagenic intermediate that must be created and then extended. Consistent with a role for pol ζ in both misinsertion and mismatch extension during TLS is the demonstration that accessory proteins stimulate pol ζ to perform TLS at rates comparable to copying undamaged DNA (33). That study clearly indicates that pol ζ can insert nucleotides opposite lesions and then extend those insertions to complete bypass. Moreover, *in vivo* results on the bypass of abasic sites, T–T (6-4) photoadducts and T–T *cis–syn* cyclobutane dimers, have led to the suggestion that pol ζ, not pol δ, is responsible for insertion during TLS when pol η does not perform this function (41,60).

It is currently unknown whether pol ζ error rates during TLS are similar to, lower, or higher than those observed here for copying undamaged DNA. Nonetheless, it is particularly interesting that pol ζ has higher base substitution fidelity when copying undamaged DNA than does pol η. During complete bypass of a *cis–syn* thymine–thymine dimer, pol η generates base substitution errors at the 3′ T at rates that are high, and similar to those observed for copying the equivalent undamaged T (61). However, yeast pol ζ can also clearly conduct complete TLS reactions, inefficiently without accessory proteins (2,26) and efficiently when the accessory proteins are present (33). Is pol ζ less accurate during lesion bypass than seen here with undamaged templates? If not, how is damage-induced mutagenesis *in vivo* suppressed by pol η, an enzyme with very low intrinsic fidelity, whereas damage-induced mutagenesis is promoted by

pol ζ, a polymerase with at least 10-fold higher base substitution fidelity (Table 2). Among several possibilities, it may be that TLS errors made by pol η are subjected to error correction mechanisms to a greater extent than are errors made by pol ζ. For example, if pol η conducts bypass at the replication fork (27,62), those mismatches may be proofread by pol δ or pol ε or corrected by mismatch repair (63), a process that may be physically coupled to the replication fork (64,65). However, if pol ζ were to participate in TLS during gap-filling DNA synthesis after the fork has moved on (66–68), those mismatches might not be as efficiently proofread or repaired by mismatch repair, resulting in mutagenesis. The potential of pol ζ to replace pol δ as a TLS gap-filling replicase in order to avoid replication fork stalling could explain why pol ζ is responsible for the majority of both spontaneous and damage-induced mutagenesis observed *in vivo*.

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