Distinct Function of Conserved Amino Acids in the Fingers of Saccharomyces cerevisiae DNA Polymerase α*

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Structural differences between class A and B DNA polymerases suggest that the motif B region, a wall of the catalytic pocket, may have evolved differentially in the two polymerase families. This study examines the function of the motif B residues in Saccharomyces cerevisiae DNA polymerase α (pol α). Effects of the mutations were determined by biochemical analysis and genetic complementation of a yeast strain carrying a temperature-sensitive pol α mutant. Many conserved residues were viable with a variety of substitutions. Among them, mutations at Asn-948 or Tyr-951 conferred up to 8-fold higher colony formation in a URA3 forward mutation assay, and 79-fold higher trp1 reversion frequency was observed for Y951P in yeast. Purified Y951P was as accurate as wild type in DNA synthesis but ~6-fold less processive and 22-fold less active in vitro. Therefore, Y951P may increase the frequency of mutant colony formation because of its low level of DNA polymerase activity in yeast. Mutations at Lys-944 or Gly-952 were not viable, which is consistent with the observation that mutants with substitutions at Gly-952 have strongly reduced catalytic activity in vitro. Gly-952 may provide a space for the nascent base pair and thus may play an essential function in S. cerevisiae DNA pol α. These results suggest that class B DNA polymerases have a unique structure in the catalytic pocket, which is distinct from the corresponding region in class A DNA polymerases.

During cell division, a mother cell divides to generate two identical daughter cells, each of which receives a complete genetic complement. DNA replication is a critical step, which duplicates the parental chromosomes prior to cell division in the cell cycle. In eukaryotic cells, at least three DNA polymerases participate in replication of nuclear DNA: DNA polymerases α (pol α), β, and ε, all of which belong to class B (pol α) DNA polymerases (1). The pol α/DNA primase complex plays a key role in the initiation phase of DNA replication; pol δ and pol ε play roles primarily in the elongation phase. During initiation of DNA synthesis, DNA primase synthesizes a short RNA chain (~10-mer), and pol α extends the RNA primer by ~30 deoxyribonucleotides. Pol ε and pol δ are highly processive DNA polymerases that carry out bidirectional DNA synthesis during the elongation phase of DNA replication (2).

Class B family DNA polymerases share structures and catalytic mechanism with class A DNA polymerases such as Taq DNA pol I (3, 4). Motif B is an essential region conserved in class A DNA polymerases. A Lys residue is essential in motif B in Taq pol I (Lys-663) and Klenow fragment (Lys-758) of Escherichia coli pol I (5–7). Phe-667 in Taq (and the equivalent residues in Klenow and T7 pol) are also critical for polymerase activity, as well as discrimination of 2′,3′-dideoxynucleotides (5, 8, 9). Tyr-766 in Klenow fragment (Tyr-671 in Taq pol I) plays a role in fidelity of DNA synthesis (10, 11). Recently, Ponamarev et al. (12) reported that a human pol γ mutant in which a Tyr equivalent to Tyr-766 is changed to Cys has a higher Kcat and lower fidelity than wild type. Interestingly, these aromatic residues seem to interact differently with template (Tyr-671) or substrate (Phe-667) in the open and closed structures, which suggests that they might function as a molecular chaperon (13–16). Lys-663, Phe-667, and Tyr-671 in Taq pol I (and the corresponding residues in other class A DNA polymerases) and Arg-659 in Taq pol I are essential for genetic complementation of a pol I strain of E. coli (7). These motif B residues may constitute the wall of the catalytic pocket together with residues located in other motifs (for example see Refs. 17–22).

Some of the motif B residues are also conserved in DNA polymerases of other classes. The comparable residues in class B polymerases are located in the fingers subdomain and may face toward the polymerase cleft (23–26). Mutations of the essential Lys greatly reduce polymerase activity in human pol α, 629 pol, RB69 pol (27–29), and Klenow fragment (5, 6). Therefore, this motif B Lys may be functionally conserved in class A and B DNA polymerases. In Taq pol I, other motif B residues also play a role in fidelity of DNA synthesis (16, 30–32), although less function is known about the other motif B residues of class B DNA polymerases.

This study analyzes the role of motif B residues in Saccharomyces cerevisiae pol α. Motif B mutants were created using randomized cassette mutagenesis and screened for genetic complementation of temperature-sensitive polI– mutants. Specific mutants of interest were purified and characterized. The results are discussed in terms of the structure-function relationships in DNA polymerase motif B.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Oligonucleotides—Yeast strain S111polI–17, trp1-289 tyr1 ura3–1 ura3–2 ade2–101 gal2 can1 polI–17 (33), and YCplac33, a genomic clone of S. cerevisiae pol α (34), were generously provided by Hiroiuk Araki at the National Institute of Genetics.

Vector Construction—Mutant cassettes were inserted into unique restriction sites upstream and downstream of the motif B region (see Fig. 1). The restriction sites were created using the GeneEditor in vitro.

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‡ The abbreviations used are: pol, DNA polymerase; FOA, fluoroorotic acid; BSA, bovine serum albumin; DTT, dithiothreitol.

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site-directed mutagenesis kit (Promega). Oligonucleotides with silent mutations (underlined) were as follows: +SacII, 5'-CAT GGT GTT GTA CCG CGG GTA TTC TTA GTA CTT GCTG-3'; +XhoI, 5'-TTG GAT TGT GAT TTT GTA AAT GCC AAG G-3'. In this protocol, the +SacII and the +XhoI primers produced new restriction sites, and the -XhoI primer destroyed an XhoI site farther downstream. Prior to random library construction, the SacII-XhoI fragment was replaced with the sequence that has a stop codon (TGA) in place of Lys-944 (non-functional vector).

Preparation of Motif B Cassettes with Randomized Sequence—Random mutagenesis was performed essentially as described (7, 19, 21, 35, 36). Oligonucleotides containing randomized sequence were synthesized by Integrated DNA Technologies (ABI) as follows: 6% RANDOM (antisense strand): 5'-TTG GGT TTT CCG CGG-3'. The linearized vector cassette was ligated to the 3'-terminal nucleotides of the 6% RANDOM (antisense strand). 25 cycles of PCR were carried out in a reaction containing 50 mM Tris-HCl, pH 8.0, 2 mM nucleotides of the 6% RANDOM (antisense strand) oligomers, 5'-GGG TGT TTT CCG CGG GTA TTC TTA GTA CTT GCTG-3' and 5'-AAC TCG AGA TTT TAC GCA AAG C-3', each 5.5 pmol of template DNA of each PCR product. The PCR product (6% random fragment) contains on average 94% wild type nucleotide and 2% each of the other three nucleotides; 6% RANDOM (sense strand): 5'-AAC TCG CTT ATG GGG ATC AG-3'. 

Library Construction—Non-functional vector and 6% random or totally random vector (totally random library plasmid or totally random library plasmid) were introduced into the DH5α (Invitrogen) and DH10bac (Invitrogen) strains. Cells were collected, and the supernatant was loaded onto a nickel-resin column (His-bind Resin WI; Novagen) equilibrated with Buffer A (20 mM Tris-HCl, pH 8.0, 1 M KCl, 5 mM 2-mercaptoethanol, 10% glycerol). The column was washed sequentially with 10 ml of Buffer A, 2 ml of Buffer B (20 mM Tris-HCl, pH 8.0, 1 x KCl, 5 mM 2-mercaptoethanol, 10% glycerol), and 2 ml of Buffer A. Enzyme was eluted with 5 ml of elution buffer (20 mM Tris-HCl, pH 8.0, 2 x KCl, 5 mM 2-mercaptoethanol, 10% glycerol), and 0.5-ml fractions were collected. Peak fractions were dialyzed against 500 ml of dialysis buffer (20 mM Tris-HCl, pH 8.0, 2 x KCl, 5 mM 2-mercaptoethanol, 10% glycerol) for 4 h followed by dialysis buffer II (50% glycerol, 50 mM Tris-HCl, pH 8.0, 2 x 2-mercaptoethanol) for 4 h. Purity was checked by SDS-PAGE. Protein concentration was determined using Bradford reagent.

Plasmid DNA Purification—DNA polymerase was prepared as described (30). For primer extension assays on damaged DNA, a 5'-P-labeled 15-nucleotide oligomer (5'-CAC TGA TCTG TAT TAC-3') was annealed to the 30-mer template (5'-CTC GTC AGC ATC TTC ATC ATA CAG TCA TGA G-3') containing either a cis-syn T-T dimer (38) or undamaged at the underlined position, at a molar ratio 1:2. A 36-mer template (5'-TGT GCG GCA GAT TAC GTC GAA TAC GCA AAC GCA-3') was used with dTTP instead of dGTP, to produce a 3'-P-labeled 28-mer primer (5'-CGC GCC GAA TAC CCG TCA GCA ATT TAC-3') at molar ratio of 1:2. The reactions were performed using the Expand High Fidelity PCR system (Roche Applied Science). The 5'-end labeled primer was extended with the template and its 3'-end labeled products were analyzed by electrophoresis on a 6% polyacrylamide gel. The gel was exposed to X-ray film.

Primer Extension Assay—Primer extension assay was described previously (30). For primer extension assays on damaged DNA, a 5'-P-labeled 15-nucleotide oligomer (5'-CAC TGA TCTG TAT TAC-3') was annealed to the 30-mer template (5'-CTC GTC AGC ATC TTC ATC ATA CAG TCA TGA G-3') containing either a cis-syn T-T dimer (38) or undamaged at the underlined position, at a molar ratio 1:2. A 36-mer template (5'-TGT GCG GCA GAT TAC GTC GAA TAC GCA AAC GCA-3') was used with dTTP instead of dGTP, to produce a 3'-P-labeled 28-mer primer (5'-CGC GCC GAA TAC CCG TCA GCA ATT TAC-3') at molar ratio of 1:2. The reactions were performed using the Expand High Fidelity PCR system (Roche Applied Science). The 5'-end labeled primer was extended with the template and its 3'-end labeled products were analyzed by electrophoresis on a 6% polyacrylamide gel. The gel was exposed to X-ray film.
performed at 37 °C for 60 min in 20 μl containing 2 mM MgCl₂, 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 100 ng/μl BSA, 2 mM DTT, 33 mM pol α, 4 mM 23P-labeled primer-template DNA, and 100 μM dNTP. Reactions were terminated by addition of an equal volume of 2× loading buffer containing 90% formamide, 20 mM EDTA, 0.05% xylene cyanol, and 0.05% bromphenol blue. Reaction products were analyzed by 14% polyacrylamide gel electrophoresis.

Processivity Assay—An oligo(dT)₂₅-oligomer (Amersham Biosciences) was 23P-labeled at its 5′ terminus and annealed to poly(dA) (Amersham Biosciences) at a weight ratio of 1:10. DNA polymerase was incubated at 37 °C for 10 min in 25 μl containing 100 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 50 mM KCl, 100 ng/μl BSA, 2 mM DTT, 300 μM dTTP, 40 μM template annealed to 4 μM primer; enzyme concentration was varied in range of 0.35 to 180 nM to optimize the assay. Reactions were terminated by addition of an equal volume of termination buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.05% xylene cyanol, and 0.05% bromphenol blue) and analyzed by 14% denaturing polyacrylamide gel electrophoresis.

Specific Activity—Polymerase activity was assayed in a 25 μl reaction containing 80 μM potassium phosphate, pH 7.2, 8 mM 2-mercaptoethanol, 200 μg/ml of activated calf thymus DNA, 80 μM each of dATP, dGTP, and dCTP, and [3H]dTTP (18.5 kBq), and 8 μM 32P-labeled primer-template DNA in the presence of equimolar challenge DNA (challenge DNA)

Expression Vector (Non-functional Vector)

Transformation

Making a Replica

Genetic Complementation

Fig. 1. Alignment of motif B of DNA polymerases. Class B shares QNSYGY motif (P-helix in RB69 DNA polymerase), and class A shares RRK/YGYGY motif (O-helix in Taq pol I). Open boxes indicate evolutionarily conserved residues. Sc, S. cerevisiae; Hs, Homo sapiens; RB, RB69; Tgo, Thermococcus gorgonarius; 9N-7, Thermococcus sp 9N-7; E, E. coli; Tg, Thermus aquaticus; Bs, Bacillus subtilis.

Fig. 2. Selection strategy for functional mutants. An oligomer containing 69 nucleotides of 6% randomized sequence was sequenced by PCR. This fragment was digested by restriction enzymes and substituted for the corresponding restriction fragment in the non-functional vector. This random library plasmid was used for transformation of the SIIpol1–17 strain, and functional mutants were selected at 37 °C.

Results

Selection of Functional Mutants from 6% Random Library—Motif B is a conserved motif in class A and B DNA polymerases that forms a wall in the fingers domain and is part of the catalytic pocket (13, 42). The fingers domain includes residues that perform essential functions during enzyme catalysis; however, the ternary structure of motif B varies in different DNA polymerases. For example, in Taq pol I (class A) the single O-helix interacts with the substrate. In RB69 DNA polymerase (class B), the helix P may play the comparable roles, but the second helix N may also be employed for the substrate interaction (29, 42). Furthermore, in the primary structure alignment, a single amino acid seems to be lost in the putative P-helix of class B DNA polymerases (Fig. 1). To analyze functions of motif B residues in a class B DNA polymerase, we
substituted them for the 69 consecutive nucleotides of *S. cerevisiae* DNA pol α using a synthetic nucleotide cassette (Gln-935-Val-957; see Fig. 2).

Motif B mutants were transformed into pol1-17 and cultured at 37 °C (restrictive temperature); at this temperature cell growth depends on expression of the plasmid-borne pol α. 29% of the transformants survived and formed colonies. 140 plasmids were purified and sequenced; 13 expressed wild type pol α, 87 mutants had two to eight amino acid substitutions (Fig. 3,A). Underlined mutants were tested in the forward mutation assay (data not shown). B, active clones from the Gln-940, Asn-948, and Tyr-951 libraries. Number of isolates is indicated on the right. Underlined mutants were tested in the forward mutation assay (Fig. 4, A–C). C, active clones from the Lys-944 and Gly-952 libraries; codon sequences are indicated. Number of isolates is indicated on the right. D, active *Taq* pol I mutants from the Gly-762 library.

To further explore the range of acceptable substitutions at the conserved sites in motif B, five additional plasmid libraries were constructed that include amino acid substitutions of *S. cerevisiae* DNA pol α residues Lys-944, Gly-952, Tyr-951, Asn-948, or Gln-940. All possible variants of these residues were constructed using oligonucleotides with a randomized codon corresponding to the target amino acid. At the non-permissive temperature, all viable clones carried Lys at residue 944 (17 AAA and six AAG codons) and Gly at residue 952 (GGN codon). In contrast, amino acid substitutions at Gln-940, Asn-948, and Tyr-951 were tolerated and were selected as viable mutants (Fig. 3, B and C). We also performed the random substitution assay of Gly-672 in class A *Taq* pol I. Interestingly, samples of the functional clones grown at the non-permissive temperature carried the wild type, Ala, Trp, Arg, or Phe sequence (Fig. 3D).

**URA3 Forward Mutation Assay**—In the class *A Taq* pol I, the conserved amino acids interact with the incoming substrate and are essential for cell growth (7). Mutations at nearby residues increased the error frequency *in vitro* (16, 30, 31). The screen described above identified mutants of Gln-940, Asn-948, and Tyr-951 that support colony formation at 37 °C, complementing the temperature-sensitive phenotype of *S. cerevisiae* pol1-17. However, it seemed possible that substitutions in these highly conserved residues of pol α do not fully complement the pol1-17 deficiency and might be associated with a non-lethal phenotype. If they play any role in pol α, mutations could affect fidelity DNA synthesis and increase spontaneous mutation frequency in *S. cerevisiae*.

This idea was tested by growing yeast at 37 °C for 16 h and subsequently transferring the cultures to plates containing 5-FOA. This assay is a forward mutagenesis assay, in which viable clones acquire mutations in the uracil metabolic path-
way in yeast. Five mutant polymerases (N948H, N948I, N948M, Y951E, and Y951P) were mutants with 3- to 8-fold higher mutation frequency on average than wild type polα (Fig. 4, A–C). The mutation rate/generation (43, 44) was estimated for each mutant as follows: 21 (N948A), 46 (N948H), 36 (N948M), 29 (Y951E), and 33 (Y951P) × 10⁻⁷ corresponding to 3.1-, 6.9-, 3.9-, 4.3-, and 4.9-fold higher mutation rate than wild type (6.7 × 10⁻⁷), respectively (Table I). Recently, Pavlov et al. (45) characterized a Y951A mutant of DNA pol α, which had no apparent phenotype. This observation is consistent with the fact that strain Y951A polα, isolated in this study, had a wild type spontaneous mutation frequency (Fig. 4C). Other mutants isolated in this study were not associated with an increased mutation rate (underlined residues in Fig. 3, A and B), and they were not studied further.

**Mutation Spectra of WT and Y951P**—In vivo and in vitro study, mutant effect have been reported by amino acid substitutions of the Tyr residues in E. coli pol I (class A) and RB69 DNA polymerase (class B) (10, 11, 46, 47). Therefore, we decided to further study the pol α with a substitution at the Tyr-951. The mutation spectrum of wild type and Y951P pol α, which was generated in yeast, was determined by sequencing the *ura3* gene in colonies that survived 5-FOA selection in the presence of the corresponding pol α allele. The mutation spectrum of wild type and Y951P pol α is similar, including predominantly single base substitutions. Wild type pol α produced 23 mutations with 20 single base substitutions, a 27-bp duplication, a single base deletion, and an 868-bp deletion (Table II). No notable sequence motif was flanked by or found within the mutation regions. Y951P generated 20/23 single base substitutions and three large deletions that are flanked by direct repeats of ACT, CCACACCG, or GCCTGCTT, respectively (Table II).

***trp1 Reversion Assay***—If Y951P primarily increases the rate of base substitutions, a mutator phenotype should be also detected in the *trp1* reversion assay in yeast (Fig. 4D). The wild type reversion frequency was 14 × 10⁻⁷ at 37°C, and the Y951P reversion frequency was 1100 × 10⁻⁷ at 37°C, an increase of 79-fold over wild type. Base substitution preference was confirmed by DNA sequencing of 15 sample colonies with wild type or Y951P allele; they exclusively contained T → C base substitutions in the target TAG codon.

**Characterization of DNA Polymerases**—It has been postulated that DNA polymerase mutants generate genome instability because of reduced fidelity during DNA synthesis (47–49) or because of reduced activity (50–53). These possibilities were tested by purifying and characterizing Y951P pol α.

Polymerase fidelity was evaluated using the M13mp2 in vitro forward mutation assay, in which misincorporations are scored as mutations that inactivate the LacZα target sequence (blue-white screening). The mutant frequency of Y951P and wild type pol α was 3.4 × 10⁻⁷, and no significant difference in the mutation spectrum was observed (data not shown). Translesion DNA synthesis activity was also similar for wild type and Y951P pol α (Fig. 5, A and B). These data indicate that Y951P does not have reduced fidelity on natural or damaged DNA templates in vitro.

In contrast, the processivity of Y951P was reduced nearly 6-fold; under conditions of limiting polymerase, wild type incorporates up to ~12 deoxynucleotides, but Y951P incorporated ~two deoxynucleotides (Fig. 5C). Furthermore, the specific activity of Y951P was 22-fold lower than wild type (Table III). The reduced activity was further confirmed by steady-state analysis of single nucleotide incorporation; Y951P was 27-fold less efficient for incorporation of dGMP (Table III).
Fig. 5. Biochemical characterization of wild type and Y951P pol α. A, primer extension analysis using primer only (lane 1), control (lanes 2 and 3), or cis-syn T-T dimer template (lanes 4 and 5). Either wild type (lanes 2 and 4) or Y951P (lanes 3 and 5) was added to the reaction. B, primer extension analysis using primer only (lane 1), control (lanes 2 and 3), Etheno A (EtheA; lanes 4 and 5), abasic site (lanes 6 and 7), O'-Methylguanosine (6MeG; lanes 8 and 9), O'-Methylyimidine (4Met; lanes 10 and 11), or 8-hydroxyguanine (8OHG; lanes 12 and 13). Wild type (lanes 2, 4, 6, 8, 10, and 12) or Y951P (lanes 3, 5, 7, 9, 11, and 13) was added to the reaction. In panels A and B, primer and target positions are indicated by open or closed triangles. C, processivity analysis. 32P-Labeled oligo(dT)16, primer and target positions are indicated by open triangle (0.35 nM) of wild type (lanes 2–7) or Y951P (lanes 8–13) for 10 min. In lane 1, no enzyme was added. The open triangle indicates the primer position.

Table III

| Enzyme                | K_m (μM) | h-inc (μm⁻¹ min⁻¹) | f-inc (mu/mol/min) | f-inc(mutant)/f-inc(WT) | K_D (nM) | K_D (rel) |
|-----------------------|----------|--------------------|------------------|-------------------------|----------|----------|
| WT                    | 1        | 7.0 ± 1.6          | 0.26 ± 0.09      | 0.038 ± 0.02            | 1        | 82       |
| Y951P                 | 1/22     | 140 ± 66           | 0.19 ± 0.04      | (1.4 ± 0.7) × 10⁻³     | 1/27     | 107      |
| G952Y                 | 1/260    | 1000 ± 620         | 0.48 ± 0.21      | (4.7 ± 1.2) × 10⁻⁴     | 1/81     | 1.0      |
| G952A                 | 1/1500   | 1200 ± 250         | 0.14 ± 0.001     | (1.1 ± 0.3) × 10⁻⁵     | 1/3500   | 160      |

*Wild type activity was 28,000 units/mg protein.

Apparent K_m and h-inc were determined by Hanes-Woolf plot for dGTP. The kinetic values are the average of at least triplicate determinations. S.D. are also shown. Incorporation efficiency, f-inc represents K_m/K_D.

K_D was kinetically determined as described under “Experimental Procedures.”

K_D(rel) value is the ratio of the binding affinity of polymerase enzyme for template primer DNA (T/P) to challenged DNA (Single-stranded (ss) or double-stranded (ds) DNA) as described under “Experimental Procedures.”

Using the same template primer, K_D (DNA) for wild type pol α was 82 nM, which was similar to Y951P and human pol α (Table III) (54). Relative binding affinity for single-stranded DNA, double-stranded DNA, and template-primer DNA was also similar for wild type and Y951P pol α (Table III).

Despite the fact that Y951P is deficient in processivity and nucleotide incorporation activity, strains carrying this mutant are viable. This result suggests that the cellular polymerase activity of this mutant is higher than the amount required for cell growth, which is consistent with the report that wild type pol α concentration is in excess of the amount required for cell growth in yeast (55). However, these data suggest that Y951P pol α causes genome instability, because its activity is lower than wild type pol α.

Effect of Mutation at Gly-952—In our original analysis, we were unable to isolate functional mutants at Gly-952. To confirm Gly-952 is essential for pol α function in S. cerevisiae, samples of mutants were expressed and purified, and their specific activity was determined. The mutant proteins had similar chromatographic properties as wild type pol α (data not shown), but the activity of G952E and G952R was undetectable, and the activity of G952A and G952Y was reduced 1500- and 260-fold, respectively (Table III). In the steady-state kinetics of dGMP incorporation, G952A and G952Y were 3500- and 81-fold less efficient DNA polymerases. K_D (DNA) values for G952A were 2-fold higher than wild type. These data demonstrate that Gly-952 plays an essential role in catalytic function of S. cerevisiae DNA pol α.

DISCUSSION

E. coli and Yeast Complementation Systems—This study analyzes the functions of motif B residues in S. cerevisiae pol α (class B polymerase) using mutant DNA polymerases. Motif B was randomized in pol α, and active mutants were isolated.
based on their ability to complement a temperature-sensitive strain of yeast (polI–17). The complementation result was compared with a similar study carried out with class A DNA polymerase Taq pol I and a temperature-sensitive strain of E. coli (recA718 pol A12) (7).

RecA718 pol A12 is complemented by wild type Taq pol I by a mechanism that is not fully understood. Previous studies show that this complementation depends on the polymerase activity of plasmid-born polymerase and requires a minimum of −5% of wild type activity (apparent $f_{\text{app}}$) (7, 36). Our data using the yeast system also suggest that exogenous polymerase activity is important for complementation, i.e. amino acid substitutions at Lys-944 or Gly-952 in S. cerevisiae pol α do not complement (Fig. 3) (discussed below), and the non-complementing Gly-952 mutants have $\sim$1.2% activity than wild type pol α (apparent $f_{\text{app}}$) (Table III). However, as for the E. coli complementation system, it is possible that polymerase activity is not the only requirement for complementation and that the strain background (pol α1–17) might affect the complementation phenotype. For this reason, the biochemical properties of mutant DNA polymerases were also considered, to understand the functions of motif B residues in class B polymerases (Table III).

**Motif B Structures of Families A and B DNA Polymerases**—In a class A DNA polymerase of Taq pol I, four residues (Arg-659, Lys-663, Phe-667, and Tyr-671) that face the incoming nucleotide are evolutionally conserved and functionally important (7). When S. cerevisiae pol α, a class B DNA polymerase, is aligned with class A polymerases, Lys-944 and Tyr-951 correspond to the Lys-663 and Tyr-671 of Taq pol I, respectively; Gln-940 and Asn-948 are conserved class B polymerases that may be the equivalent of Arg-659 and Phe-667 in Taq pol I (Fig. 1). However, this sequence alignment is based only on primary structure. The distance between Lys-944 and Tyr-951 in S. cerevisiae pol α is one residue shorter than the distance between Lys-663 and Tyr-671 in Taq pol I and other class A DNA polymerases (Fig. 1). As a result, the conserved residues do not have the same spatial relationship, and may differ by $-1/4$ helical turn (Fig. 6).

Mutants in human pol α, $\delta 29$ pol, and RB69 pol corresponding to Lys-944 are associated with a large increase in $K_m$ (27–29). In class A DNA polymerases, the corresponding amino acid (Lys-663 in Taq pol I) interacts with the oxygen moiety of the α-phosphate of the incoming dNTP (13) (Fig. 6), and amino acid substitutions increase $K_m$ and decrease $k_{\text{cat}}$ (5, 6). These observations suggest that Lys-944 in S. cerevisiae pol α may also interact with the incoming α-phosphate in vitro. Therefore, if we put the N-terminal Lys, the α phosphate accommodation residue, at the same position in the helix, the C-terminal Tyr and Gly come to the different positions between class A and B DNA polymerases.

**Functions of Tyr-951 in Vitro**—Single nucleotide incorporation kinetics showed that Y951P has a wild type $k_{\text{cat}}$ but a higher $K_m$ than wild type pol α (Table III). Thus, Y951P pol α has a defect in single nucleotide incorporation. Y951P pol α also has a moderate defect in processivity in reactions with a high concentration of dNTP substrate (Fig. 5C); however, there was no significant difference in DNA binding affinity between wild type and Y951P pol α. Kinetic values were based on a single nucleotide insertion that does not include polymerase translocation from N to N+1, i.e. the kinetic values do not reflect the ability of the enzyme to translocate. Considering that $K_m$ and $k_{\text{cat}}$ were near wild type, Y951P might have a defect in translocation in between catalytic cycles.

In the structure of RB69 pol, Tyr-567 (which corresponds to Tyr-951 in S. cerevisiae pol α) wedges in between the nascent base pairs (42, 56). The Pro substitution mutant may introduce a kink at this residue and change the enzyme-base interaction directly or indirectly via an effect on Gly-952 in S. cerevisiae pol α. It is likely that this modification would reduce polymerase activity and processivity.

**Functions of Tyr-951 in Yeast**—In the FOA forward mutation assay in yeast, Y951P pol α showed a moderate increase in the frequency of mutant colony formation (Fig. 4). However, in vitro data did not show that Y951P pol α synthesizes DNA with low fidelity (see Fig. 5 and M13mp2 in vitro forward mutation assay (data not shown)). Therefore the mutator phenotype in yeast may not directly reflect the misincorporation specificity of Y951P pol α but may instead reflect another cellular mechanism. Alternatively, it is possible that the phenotype is because of polI–17, although the polI–17 does not confer mutator phenotype at permissive temperature (data not shown) or at the restriction temperature with most other pol α mutant alleles (Table I).

Among the base substitution errors by URA3 forward mutation assay in yeast, Y951P, but not wild type, generated three large deletions that are flanked by direct repeats (Table II). This result is consistent with other recent reports showing genetic instability in cells with mutations in DNA polymerases or replication-associated proteins (51–53). An increased frequency of direct repeat-associated deletions was also reported with a temperature-sensitive pol α allele in Schizosaccharomyces pombe (52). This type of mutation might not be produced directly by the mutant polymerase, because similar deletions were also induced by low level expression of wild type pol δ (51).

In contrast to the Tyr-951 substitution in S. cerevisiae pol α, mutation at the Tyr in E. coli decreases fidelity DNA synthesis in vivo and in vitro (10, 11, 47, 57). These data suggest that Tyr-951 in S. cerevisiae pol α may not have an identical function to the equivalent Tyr in E. coli pol I; in contrast, Tyr-951 in S. cerevisiae pol α may play a role in catalysis instead of base discrimination.

In this study, no active pol α mutants with altered replication fidelity were isolated at Tyr-951 or other motif B residues. This result does not necessarily imply that motif B does not play a role in accurate DNA synthesis. The lack of the mutator phenotype could simply reflect the fact that pol α does not play a major role in replicative DNA synthesis in yeast. Moderate fidelity reduction might occur in vivo but not be detected because of the presence of active DNA repair systems. In fact, in RB69 DNA polymerase (a class B DNA polymerase), Ala, Ser,
or Thr substitution for Tyr-567 (Tyr-951 in S. cerevisiae) increases base substitution frequency in vitro and reversion and forward mutation in vivo (57). Furthermore, if a mutant has a drastic reduction in activity, it will not complement the mutant host strain and will not produce viable cells. 

Immutable Gly-952—This study also demonstrates that Gly-952 is an essential but poorly characterized catalytic residue in Taq pol I and will not produce viable cells. 952 is an essential but poorly characterized catalytic residue in S. cerevisiae Taq pol I, rather than Tyr-951, is geometrically analogous to Tyr-671 in the related motif in class A DNA polymerases. However, an amino acid replacement would be more similar to class A DNA polymerases than may or may not apply equally to all class B DNA polymerases; alternatively, the amino or carboxyl moieties in the protein backbone may interact with the template DNA.

Results of this study suggest that S. cerevisiae pol α Gly-952, rather than Tyr-951, is geometrically analogous to Tyr-671 in Taq pol I. The conserved Gly-672 in Taq pol I is downstream and may be functionally similar to the non-essential Cys-953 of S. cerevisiae pol α; based on the crystal structure, Taq pol I Gly-672 does not make contact with substrate or DNA (13). To confirm this possibility, active Taq pol I mutants were isolated from random amino acid library at Gly-672 (Fig. 3D). The results show that substitutions for Gly-672 are well tolerated including Ala, Trp, Arg, and Phe. Thus, Taq pol I Gly-672 is not the functional equivalent of Gly-952 in S. cerevisiae pol α.

In summary, this study indicates that the conserved YG sequence in S. cerevisiae DNA pol α is functionally distinct from the related motif in class A DNA polymerases. However, we are not certain whether this conclusion is general, and it may or may not apply equally to all class B DNA polymerases; evidence suggests that some class B DNA polymerases might be more similar to class A DNA polymerases than S. cerevisiae pol α (57, 58). In the accompanying article (56), Gly-952 mutants of pol α are characterized further, and the role of this residue in polymerase fidelity is discussed in detail.

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Distinct Function of Conserved Amino Acids in the Fingers of Saccharomyces cerevisiae DNA Polymerase α

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