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Eukaryotic DNA polymerase amino acid sequence required for 3′ → 5′ exonuclease activity

(yeast DNA polymerase II/mutator phenotype/proofreading/protein domain/DNA replication)

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ABSTRACT **We have identified an amino-proximal sequence motif, Phe-Asp-Ile-Glu-Thr, in *Saccharomyces cerevisiae* DNA polymerase II that is almost identical to a sequence comprising part of the 3′ → 5′ exonuclease active site of *Escherichia coli* DNA polymerase I. Similar motifs were identified by amino acid sequence alignment in related, aphidicolin-sensitive DNA polymerases possessing 3′ → 5′ proofreading exonuclease activity. Substitution of Ala for the Asp and Glu residues in the motif reduced the exonuclease activity of partially purified DNA polymerase II at least 100-fold while preserving the polymerase activity. Yeast strains expressing the exonuclease-deficient DNA polymerase II had on average about a 22-fold increase in spontaneous mutation rate, consistent with a presumed proofreading role *in vivo*. In multiple amino acid sequence alignments of this and two other conserved motifs described previously, five residues of the 3′ → 5′ exonuclease active site of *E. coli* DNA polymerase I appeared to be invariant in aphidicolin-sensitive DNA polymerases known to possess 3′ → 5′ proofreading exonuclease activity. None of these residues, however, appeared to be identifiable in the catalytic subunits of human, yeast, or *Drosophila* α DNA polymerases.**

Class B DNA polymerases† are related through a series of conserved amino acid sequences that occur in the order IV, II, VI, III, I, V, with region I being the most highly conserved (2). Regions II, III, I, and V are implicated in DNA polymerase functions (3–8). The location of the 3′ → 5′ exonuclease activity domain has been sought through amino acid sequence similarity with the apparently unrelated *E. coli* DNA polymerase I. Crystallographic and mutational evidence has determined that the 3′ → 5′ exonuclease active site of *E. coli* DNA polymerase I is composed of a group of carboxylate residues (D355, E357, D424, and D501) clustering around two metal ions that coordinate the 3′-terminal phosphate and a second group of residues (L361, F473, and Y497) located around the terminal base and ribose positions (9, 10). It has been proposed that residues D355 and E357 are conserved in the left part of region IV of aphidicolin-sensitive DNA polymerases (11–13). Similarly, residues D424, Y497, and D501 were suggested to be conserved in the right part of, and distal to, region IV (4, 12, 13). Mutation to alanine of three of the relevant carboxylate residues in bacteriophage φ29 DNA polymerase leads to exonuclease-deficient DNA polymerase activity (13). Though related to other aphidicolin-sensitive DNA polymerases, φ29 DNA polymerase does not have a well-conserved region IV (2) and may not be a good test case for alignments involving this region. With coliphage T4 DNA polymerase, which does have a well-conserved region IV (2, 11, 12), mutation to alanine of residues D189 and E191, predicted to correspond to the *E. coli* polymerase residues D355 and E357, does not lead to exonuclease deficiency, calling into question the concept of a generally conserved 3′ → 5′ exonuclease active site (14).

We present evidence that another amino acid sequence motif, typically occurring about 65 residues amino-proximal to region IV, corresponds to the *E. coli* DNA polymerase I sequence containing residues D355 and E357. Mutation of the corresponding carboxylate residues in *Saccharomyces cerevisiae* DNA polymerase II‡ resulted in the predicted exonuclease-deficient polymerase activity *in vitro* and spontaneous mutator phenotype *in vivo*. In addition, our amino acid sequence alignments support the earlier proposals (4, 12, 13) that *E. coli* DNA polymerase I residues D424, Y497, and D501 are also conserved in aphidicolin-sensitive DNA polymerases, with the important exception of α DNA polymerases.

MATERIALS AND METHODS

Yeast Strains. CG379 (*MATα his7-2 ade5-1 leu2-3,-112 trp1-289 ura3-52 CAN1*), AMY52-3D (*MATa ura3-52 leu2-1 lys1-1 ade2-1 his1-7 hom3-10 trp1-289 can1*), and BJ3501 (*MATα pep4::HIS3 prbΔ1.6 his3-Δ200 ura3-52 can1 gal7*) were used.

Plasmid Construction. The genetic code for the amino acid sequence FDIET was altered to that for FAIAT by site-directed mutagenesis (16) using the synthetic oligonucleotide 5′-ATGGCATTTGCTATAGCAACCACGAAG-3′ (corresponding to nucleotides 1450–1476) and a 2.1-kilobase (kb) *Pvu* I–*Eco*RI fragment of *POL2* DNA (17) cloned into M13mp18. The DNA was sequenced to verify the desired alteration and cloned as a 2.1-kb *Hpa* I–*Eco*RI DNA fragment into the *Nru* I–*Eco*RI interval of plasmid YIp5, creating the 6.7-kb plasmid YIpJB1.

Construction of *pol2-4* Strains. The allele of *POL2* in which the genetic code for the amino acid sequence FDIET is altered to that for FAIAT is named *pol2-4*. Yeast cells were transformed to Ura$^+$ with YIpJB1 DNA linearized using *Bam*HI to target integration to the *POL2* locus (18). Southern blotting of DNA from transformants showed the expected 12-kb *Hpa* I DNA fragment when probed with ^{32}P-labeled *POL2* 0.9-kb *Hin*dIII fragment, whereas wild-type yeast gave the expected 5.3-kb *Hpa* I fragment. Integrants of BJ3501

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†DNA polymerases with sequence similarity to *Escherichia coli* DNA polymerase I are class A, and those with sequence similarity to eukaryotic DNA polymerase α are class B (1). We also refer to class B DNA polymerases as "aphidicolin-sensitive" DNA polymerases. The term "α-type" is not used, since it might cause confusion with the different classification of DNA polymerases as α, β, γ, δ, or ε.

‡*S. cerevisiae* DNA polymerases II and III are comparable to mammalian DNA polymerases ε and δ, respectively (15). We retain the names *S. cerevisiae* DNA polymerases II and III, since homology—rather than mere analogy—with mammalian DNA polymerases ε and δ has not been demonstrated. *S. cerevisiae* DNA polymerase I is, however, also referred to as *S. cerevisiae* DNA polymerase α.

were maintained in medium lacking uracil. For phenotypic measurements, integrants of CG379 and AMY52-3D were grown on 5'-fluoroorotic acid to select for excision of *URA3* (19) and reconstitution of the original genetic configuration as determined by Southern blotting and DNA·DNA hybridization. Yeast DNA from the resulting derivatives was amplified by the polymerase chain reaction using primers 5'-ATTCCAATCAGTTATTCGAGGCCAG-3' (nucleotides 1175–1199) and 5'-CACCATTGAAGGTGGATATAA-CAGT-3' (complement of nucleotides 1729–1705) and sequenced using primer 5'-GTAGAAGCGCCACTTCATCG-3' (complement of nucleotides 1669–1650) to determine whether the nucleotide sequence was that expected for the *POL2* or *pol2-4* alleles.

Spontaneous Mutation Rates. Spontaneous mutation rates were measured as reversion of auxotrophs to prototrophy using the Leningrad test (20) or as forward mutation to canavanine resistance using the method of the median (20). In the former case, yeast cells grown in YPDA medium (26) to stationary phase were washed, suspended in water at densities of $\approx 10^6$ to 10^7 cells per ml, and applied to plates using a multipronged replicator. For each determination, 500–600 compartments were replicated onto plates containing synthetic medium either lacking or containing a limiting amount of the required amino acid. Plates were incubated for 5–10 days at 30°C, and spontaneous reversion rates were calculated by the P_0 method.

Enzyme Purification. Strain BJ3501 and its YIpJB1 integrant expressing the *pol2-4* allele were grown in YPDA medium. Cells were disrupted and subjected to S-Sepharose and Mono Q column chromatography as described (17, 21), except that the S-Sepharose column was eluted with buffer containing 0.5 M KCl. The DNA polymerase II peak from Mono Q was purified by Mono S column chromatography. DNA polymerase II was distinguished from other polymerase activities by its characteristic elution positions from Mono Q and Mono S columns (21) and by assay under conditions specific for DNA polymerase II using high salt, low pH, and poly(dA)·oligo(dT) as template·primer (17). The 3' → 5' exonuclease activity of DNA polymerase II is described in Hamatake *et al.* (21). Following the S-Sepharose step, DNA polymerase II is largely free of nonintrinsic nuclease activity and the ratio of polymerase to exonuclease remains approximately constant (21). This enabled us to measure exonuclease conveniently in a single-stranded DNA nuclease assay by the release of acid-soluble radioactivity as described (21), except that reactions contained 0.1 M KCl and the DNA substrate was prepared as follows. Nicked, gapped calf thymus DNA (100 μg) was radiolabeled in a reaction containing 50 μCi of [α-^{32}P]dTTP (1 Ci = 37 GBq), 10 units of *E. coli* DNA polymerase I Klenow fragment, 50 mM Tris·HCl (pH 7.5), 7 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM of each deoxynucleotide triphosphate, and 0.1 mg of bovine serum albumin in a volume of 1 ml. Following incubation at 37°C for 2 hr, the DNA was purified twice by gel filtration on Sephadex G-50 and heat-denatured before use.

Amino Acid Sequence Alignment. We used the University of Wisconsin Genetics Computer Group BESTFIT program on a Vax computer on sequences obtained from the National Biomedical Research Foundation and Swissprotein data

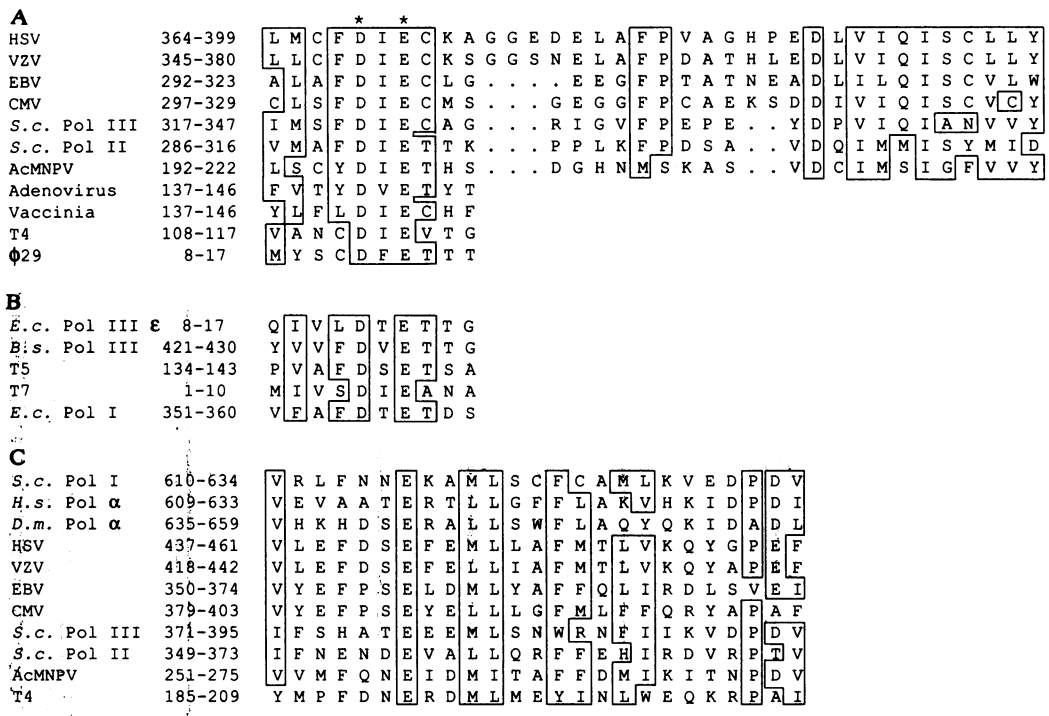

FIG. 1. Alignments of DNA polymerase amino acid sequences proposed to contain conserved 3' → 5' exonuclease active site residues. The single-letter code is used. Numbers refer to amino acid residues. Boxed are prominent identities or similarities scoring at least 0.5 on the Dayhoff matrix as used in ref. 24. (*A*) Conserved region, beginning 54–109 residues amino-proximal to region IV (as defined in ref. 2), of aphidicolin-sensitive DNA polymerases known to possess an associated 3' → 5' exonuclease activity. Asterisks mark invariant Asp or Glu residues proposed in this paper to be 3' → 5' exonuclease active site residues. Sequences are aligned with *E. coli* DNA polymerase I and related enzymes in *B* as we propose in the text. (*B*) Sequence comprising part of the exonuclease active site, including residues D355 and E357, of *E. coli* DNA polymerase I aligned with sequences from related enzymes. (*C*) Left part of region IV of aphidicolin-sensitive DNA polymerases, previously proposed (11–13) to be similar to the 3' → 5' exonuclease active site sequences in *B*. Sequences in *C* and *B* are aligned as suggested in refs. 11–13. [In ref. 13, the left part of region IV was proposed for most polymerases, but not for *S. cerevisiae* DNA polymerase I (see text), whereas for *A. californica* nuclear polyhedrosis virus and adenovirus DNA polymerases, the sequences given in *A* were proposed.] Pol, DNA polymerase; HSV, herpes simplex virus; VZV, varicella zoster virus; EBV, Epstein–Barr virus; CMV, human cytomegalovirus; AcMNPV, *A. californica* nuclear polyhedrosis virus; *S.c., S. cerevisiae; E.c., E. coli; B.s., Bacillus subtilis; H.s., Homo sapiens; D.m., Drosophila melanogaster; E. coli* Pol III ε, ε subunit of *E. coli* DNA polymerase III.

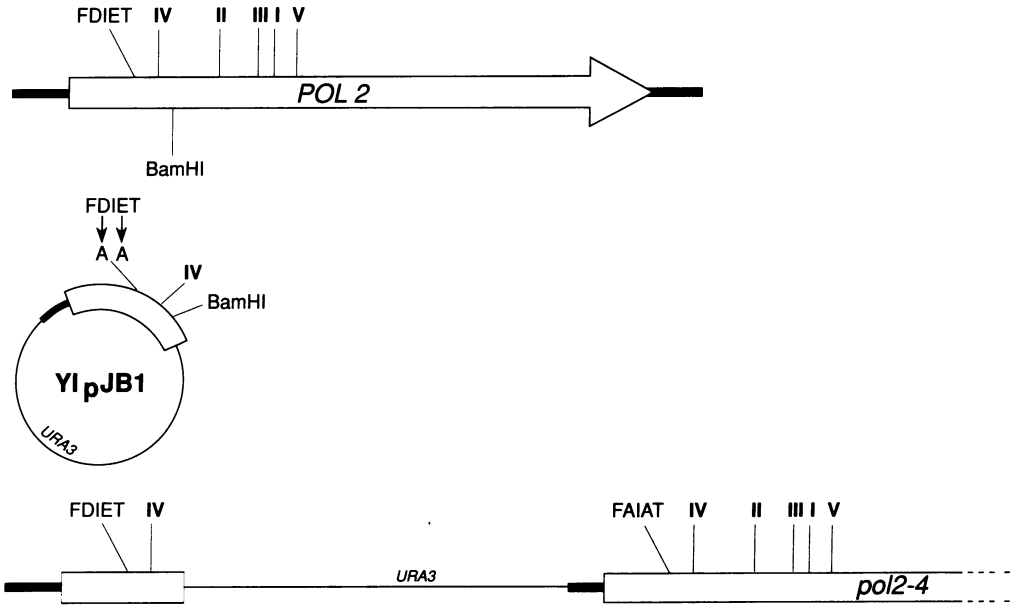

FIG. 2. Structures of *POL2*, plasmid YIpJB1, and plasmid integrant. (*Top*) *S. cerevisiae POL2* gene (open arrow) and the locations of DNA encoding the amino acid sequence FDIET, conserved regions (defined in ref. 2) of aphidicolin-sensitive DNA polymerases (bold Roman numerals), and the *Bam*HI site. Thick lines, flanking yeast DNA. (*Middle*) Plasmid YIpJB1 used to construct *pol2-4*, containing part of *POL2* with the code for the amino acid sequence FDIET changed to that for FAIAT (open box). Fine line, vector DNA containing *URA3* gene. (*Bottom*) Integration of YIpJB1 at the *POL2* locus, resulting in duplication of the 5′ region of *POL2* and formation of *pol2-4*, which contains the code for the mutated amino acid sequence FAIAT. Excision of the plasmid by homologous recombination (not shown) restores the original genomic DNA configuration leaving either *POL2* or *pol2-4*, depending on whether the crossover occurs downstream or upstream, respectively, of DNA encoding the FDIET or FAIAT amino acid sequences.

bases. Aphidicolin-sensitive DNA polymerase sequences were aligned with herpes simplex virus (type I, strain Angelotti) DNA polymerase sequence to produce the alignments shown, with the following exceptions: yeast DNA polymerase II was aligned with yeast DNA polymerase III, Epstein–Barr virus, and *Autographa californica* nuclear polyhedrosis virus DNA polymerases; adenovirus DNA polymerase was aligned with herpes simplex virus DNA polymerase residues 300–1235, except that adenovirus residues 435–445 were aligned visually; T4 residues 108–117 were aligned visually, and other T4 sequence alignments are from refs. 2, 11, and 14. PRD1 DNA polymerase was aligned with ϕ29 DNA polymerase; alignment of ϕ29 DNA polymerase is based on ref. 13. Class A DNA polymerase amino acid sequences were aligned with *E. coli* DNA polymerase I sequence and/or alignments were taken from refs. 12, 13, 22, and 23.

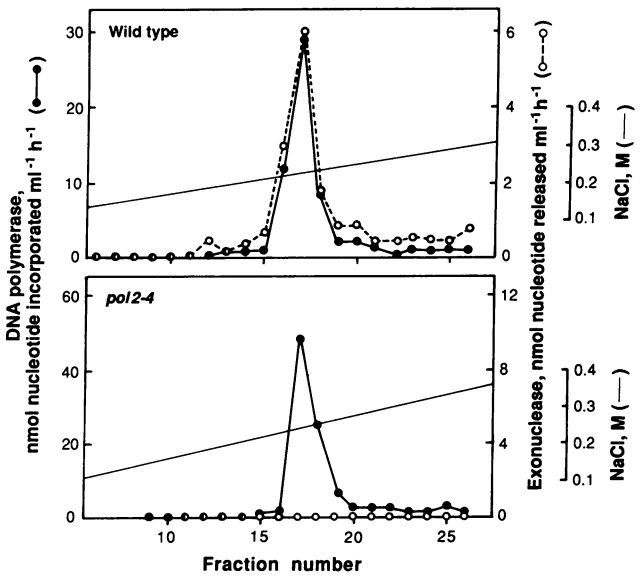

FIG. 3. Mono S column chromatography of wild-type and mutant yeast DNA polymerase II. DNA polymerase II was partially purified from 12 liters of wild-type BJ3501 cells grown to OD_{600} = 2.6 or 24 liters of *pol2-4* cells grown to OD_{600} = 1.4. Data points are the means of three determinations.

RESULTS AND DISCUSSION

Conserved Amino Acid Sequence Amino-Proximal to Region IV. Computer-generated alignments of the amino acid sequence of yeast DNA polymerase II with the sequences of yeast DNA polymerase III and herpes viral family DNA polymerases identified a conserved region beginning 54–82 residues amino-proximal to region IV (Fig. 1*A*). This region, previously identified in the latter set of DNA polymerases (25, 26), was not found in human, yeast, or *Drosophila* α DNA polymerases. The FDIET/C motif (using the single-letter code) occurring in this region was one of the most highly conserved sequences in yeast DNA polymerases II and III and herpes viral family DNA polymerases, FDIE

Table 1. Spontaneous mutator phenotype of *pol2-4* strains

Parent strain	Marker gene	Spontaneous mutation rate ($\times 10^8$)		Ratio
		POL2	*pol2-4*	*pol2-4/POL2*
CG379	*ade5-1*	17 ± 9.8	730 ± 63	43
CG379	*his7-2*	0.20 ± 0.30	4.7 ± 1.4	24
CG379	*CAN1*	17 ± 1.4	86 ± 7.1	5
AMY52-3D	*leu2-1*	22 ± 1.9	400 ± 18	18
AMY52-3D	*hom3-10*	220 ± 31	1900 ± 50	9
AMY52-3D	*his1-7*	1.5 ± 0.9	46 ± 7.6	31

Spontaneous mutation was measured as reversion of auxotrophs to prototrophy or, in the case of *CAN1*, as forward mutation to canavanine resistance. Numbers are the means (±SD) of three determinations using three independent isolates. *pol2-4* genotypes were confirmed by DNA sequencing.

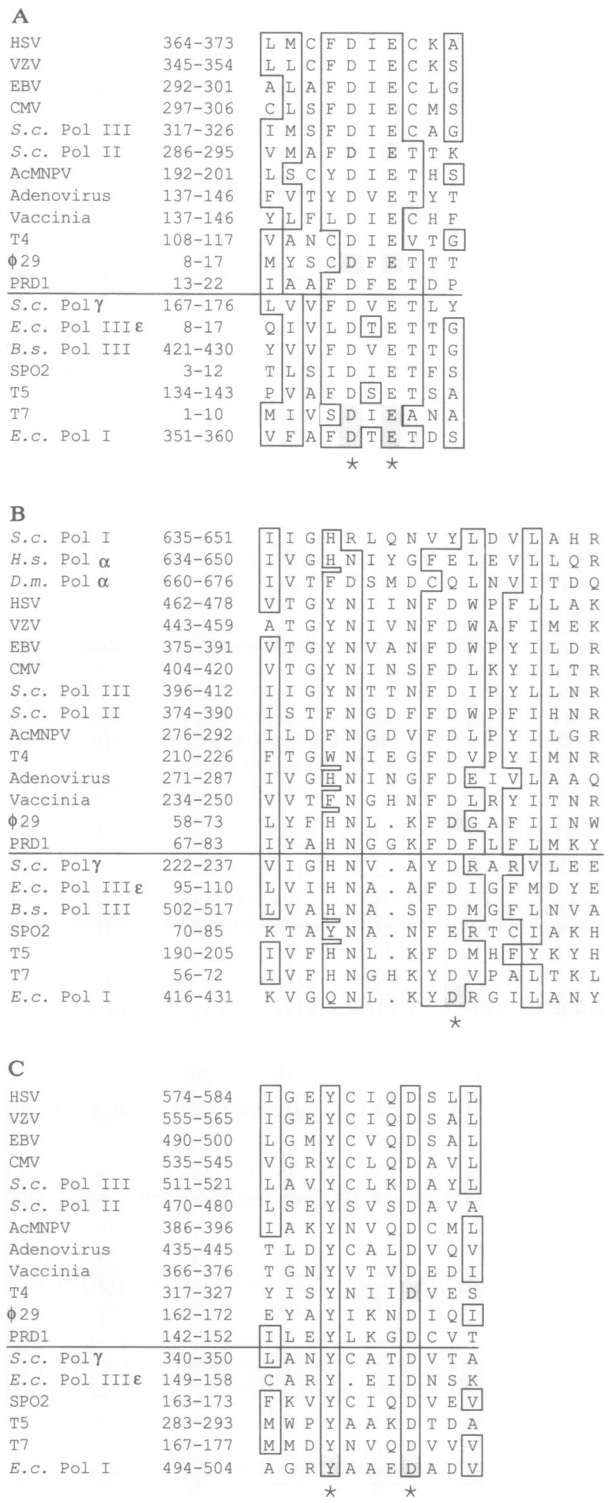

FIG. 4. Multiple amino acid sequence alignments of three regions proposed to comprise the 3′ → 5′ exonuclease active site. (*A*) Alignment proposed in this paper between *E. coli* DNA polymerase I sequence containing exonuclease active site residues D355 and E357, other class A enzymes, and region of aphidicolin-sensitive DNA polymerases amino-proximal to region IV. (*B*) Alignment, essentially as proposed in refs. 12 and 13, between the *E. coli* DNA polymerase sequence containing D424, other class A polymerases, and the right part of region IV of aphidicolin-sensitive DNA polymerases. (*C*) Alignment, essentially as proposed in refs. 4, 12, 13, and 14, between the *E. coli* DNA polymerase sequence containing Y497 and D501, other class A polymerases, and region of aphidicolin-sensitive DNA polymerases between regions IV and II. Asterisks mark residues of the 3′ → 5′ exonuclease active site of *E. coli* DNA polymerase I. Boxed are prominent identities and similarities scoring at least 0.5 on the Dayhoff matrix. Horizontal lines separate aphidicolin-sensitive DNA polymerases (above line) from *E. coli* DNA polymerase I and related enzymes (below line). Residues are screened where their mutation has resulted in exonuclease-deficient DNA polymerase: residues 355, 357, 424, 497, and 501 of *E. coli* DNA polymerase I (9, 10); residues 5 and 7 of T7 polymerase (30); residues 12, 14, and 66 of ϕ29 polymerase (13); residues 290 and 292 of yeast DNA polymerase II (present work); residue 324 of T4 polymerase (ref. 14; exonuclease was determined for the double mutant of residues 191 and 324). See legend to Fig. 1 for abbreviations.

being the only tetrapeptide sequence absolutely conserved in this set of polymerases. Similar motifs, similarly located relative to region IV, were identified by computer alignment in *A. californica* nuclear polyhedrosis virus, adenovirus, and vaccinia virus DNA polymerases (Fig. 1*A*).

We realized that the yeast DNA polymerase II sequence VmAFDiET strikingly resembled the sequence VfAFDtET of *E. coli* DNA polymerase I (lower-case letters represent nonidentical residues), which contains the D355 and E357 3′ → 5′ exonuclease active site residues (Fig. 1*B*). Moreover, a feature common to the aphidicolin-sensitive polymerases aligned in Fig. 1*A* and the *E. coli* DNA polymerase I-related enzymes aligned in Fig. 1*B* is that the Asp and Glu corresponding to the *E. coli* D355 and E357 are the only invariant residues. In comparison, the left part of region IV, in which these exonuclease active site residues were previously thought to be conserved (11–13), does not contain an invariant Asp residue corresponding to critical (10) *E. coli* DNA polymerase I residue D355 (Fig. 1*C*). We proposed, then, that the FDIET sequence (residues 289–293) of yeast DNA polymerase II, rather than any sequence in the left part of region IV, corresponded to the *E. coli* DNA polymerase I sequence that includes the 3′ → 5′ exonuclease active site residues D355 and E357.

Mutation of the FDIET Sequence Motif of DNA Polymerase II. In experiments similar to those of Derbyshire *et al.* (9), we altered *POL2*, the gene determining DNA polymerase II, to include the code for the D290 → A and E292 → A mutations. A yeast strain expressing the mutant allele, named *pol2-4*, was created by targeted integration of plasmid YIpJB1 (Fig. 2). We partially purified DNA polymerase II in the single polypeptide form that retains the intrinsic 3′ → 5′ exonuclease activity (21, 27–29). Following Mono S column chromatography, the wild-type strain displayed the expected peak of polymerase activity coincident with a 3′ → 5′ exonuclease peak, assayed as single-stranded DNA nuclease (Fig. 3). The *pol2-4* mutant yeast yielded an amount of polymerase activity similar to that of the wild-type strain, but no coincident peak of exonuclease activity was detected. A trace of nuclease, present in all fractions assayed and equivalent to 0.9% of the peak of the wild-type activity, limited estimation of the extent of the reduction in exonuclease to about 100-fold. Essentially the same profiles were obtained when 3′ → 5′ exonuclease activity was measured by the more specific terminal mismatch excision assay (ref. 21; unpublished observations). Although we have not ruled out other formal possibilities, such as a structural disorganization of the mutant enzyme caused by the amino acid substitutions, the observed exonuclease deficiency is strikingly consistent with the assignment of active site residues predicted from the amino acid sequence alignments. In the case of *E. coli* DNA polymerase I, crystallographic analysis of the mutant protein showed no alteration in protein structure other than the expected changes at the mutation sites (9).

Spontaneous Mutator Phenotype. The presumed *in vivo* role of 3′ → 5′ exonucleases of eukaryotic DNA polymerases is to edit out incorrectly incorporated nucleotides, and elimination of this function is expected to result in a spontaneous mutator phenotype. Consistent with this, the YIpJB1 integrant of strain

CG379 displayed about a 40-fold increase in spontaneous reversion of *ade5-1* (data not shown). Following growth on medium containing 5-fluoroorotic acid to select for excision of the integrated plasmid, 83% of the derivatives retained the mutator phenotype. A 0.55-kb region of the yeast chromosomal DNA normally encoding the FDIET amino acid sequence was amplified and sequenced. Yeast DNA amplified from four of the nonmutators had the DNA sequence encoding FDIET, whereas DNA from four of the mutator strains had the sequence encoding FAIAT, showing that the mutator phenotype segregated with the *pol2-4* genotype. The same procedure produced similar results with strain AMY52-3D, where mutagenesis was measured as reversion of *his1-7* (data not shown). Spontaneous mutagenesis rates, measured at six loci in strains CG379 and AMY52-3D, were observed to increase 5- to 43-fold in the *pol2-4* derivatives (Table 1). *pol2-4* mutants, however, displayed no significant defects in growth at 30°C or 37°C or in sensitivity to UV or methyl methanesulfonate (unpublished observations), indicating that the DNA polymerase II 3′ → 5′ exonuclease has no essential role in cell growth or in the repair of DNA damage.

Conserved 3′ → 5′ Exonuclease Active Site. We have presented evidence for the apparent conservation of the *E. coli* DNA polymerase I 3′ → 5′ exonuclease active site residues D355 and E357 in a region of aphidicolin-sensitive DNA polymerases different from that previously proposed (11–13). However, the results of our multiple amino acid sequence alignments concur with previous observations (4, 12, 13) that *E. coli* DNA polymerase I residues D424, Y497, and D501 appear to be conserved in the right part of, and distal to, region IV. The proposed alignments are shown in Fig. 4. Five 3′ → 5′ exonuclease active site residues of *E. coli* DNA polymerase I appear to be absolutely conserved in the aphidicolin-sensitive DNA polymerases—except for α DNA polymerases, which are discussed below. (We did not locate conserved residues corresponding to *E. coli* residue L361 or F473.) This argues that class B and class A DNA polymerases are in fact related, as has been suggested (31).

Exonuclease Active Site Residues Are Not Identified in α DNA Polymerases. No example of the sequence DXE (where X is any amino acid), corresponding to *E. coli* DNA polymerase I residues 355–357, is conserved in alignments of the amino portions of human, yeast, and *Drosophila* α DNA polymerases (ref. 2; Hirose and Matsukage, personal communication). Strikingly, an Asp residue proposed to correspond to *E. coli* DNA polymerase I residue D424 is invariant in aphidicolin-sensitive DNA polymerases known to possess 3′ → 5′ proofreading exonuclease activity yet occurs in none of the three α DNA polymerases aligned in Fig. 4*B*. (Human DNA polymerase α has a Glu in position 643, which might potentially substitute for the Asp.) A previous proposal that some of these 3′ → 5′ exonuclease active site residues are conserved in human and yeast α DNA polymerases was based on different amino acid sequence alignments (13). Thus, in ref. 13, 3′ → 5′ exonuclease active site residues were assigned to the left and right parts of region IV of the human enzyme but to sequences amino-proximal to region IV, as defined in refs. 2, 14, 26, and 32, in the yeast enzyme. Furthermore, the yeast DNA polymerase α sequence matched to the YX_3D motif of *E. coli* DNA polymerase I residues 497–501 was given in ref. 13 as YQVCE but should read YQVTCE (residues 720–725). Although a sequence with the same spacing of aromatic and Asp residues as the YX_3D motif is conserved close to region II of α DNA polymerases (14)—residues 810–814 (FIVPD) of the yeast enzyme, residues 801–805 (YIVPD) of the human enzyme, and residues 824–828 (YIVPD) of the *Drosophila* enzyme—these sequences were not aligned by the BESTFIT program with the YX_3D motif conserved in other aphidicolin-sensitive polymerases (unpublished observation). This corroborates an earlier observation that the region containing the YX_3D motif, referred to as region A, is not conserved in α DNA polymerases (3). It thus appears that none of the five proposed exonuclease active site residues can be identified in the catalytic subunits of α DNA polymerases. This may explain why the catalytic subunits of yeast and human α DNA polymerases, purified from overproducing strains, are devoid of detectable 3′ → 5′ proofreading exonuclease activity (ref. 33; W. C. Copeland and T. S.-F. Wang, personal communication).

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