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The Frameshift Infidelity of Human DNA Polymerase λ

IMPLICATIONS FOR FUNCTION*

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DNA polymerase λ (Pol λ) is a member of the Pol X family having properties in common with several other mammalian DNA polymerases. To obtain clues to possible functions *in vivo*, we have determined the fidelity of DNA synthesis by human Pol λ. The results indicate that the average single-base deletion error rate of Pol λ is higher than those of other mammalian polymerases. In fact, unlike other DNA polymerases, Pol λ generates single-base deletions at average rates that substantially exceed base substitution rates. Moreover, the sequence specificity for single-base deletions made by Pol λ is different from that of other DNA polymerases and reveals that Pol λ readily uses template-primers with limited base pair homology at the primer terminus. This ability, together with an ability to fill short gaps in DNA at low dNTP concentrations, is consistent with a role for mammalian Pol λ in non-homologous end-joining. This may include non-homologous end-joining of strand breaks resulting from DNA damage, because Pol λ has intrinsic 5′,2′-deoxyribose-5-phosphate lyase activity.

Maintenance of genomic information relies on a variety of processes resulting in faithful DNA repair and replication. DNA polymerases are key players in most of these processes. It is not surprising, therefore, that the discovery of several novel DNA polymerases of unknown function has challenged the existing paradigms of replication and repair. Besides a number of translesion synthesis polymerases that constitute the family Y (1), additional DNA polymerases have been found in the previously identified families A, B, and X (2, 3). For example, DNA polymerase (Pol)[1] λ is a member of family X that was discovered only 3 years ago (4–6). Sequence comparisons have shown that it has 33% amino acid identity and shares the same domain organization with the best studied member of family X, DNA Pol β, an enzyme crucial in base excision repair (7). Members of the family X also include terminal transferase (TdT) (8), Pol μ (9), and Pol σ (10). Despite sharing a significant sequence similarity, the small, monomeric enzymes of this family possess remarkably different properties. For example, TdT is a template-independent polymerase involved in antigen receptor diversification during V(D)J recombination (11, 12). In contrast Pol β is a moderately faithful, template-dependent enzyme (13, 14) that, in addition to its polymerase activity, possesses a 5′, 2′-deoxyribose-5-phosphate (dRP) lyase activity (15, 16). Finally Pol μ, likely involved in non-homologous end-joining (NHEJ) (17), is a largely template-dependent polymerase that is also endowed with some template-independent polymerization activity (9). Unlike Pol β, Pol μ is highly error-prone (Refs. 9 and 18).[2]

Early studies concluded that Pol λ is predominantly expressed in testis in stages of spermatogenesis coincident with meiotic recombination (4). The generation of knock-out mice has, as yet, not confirmed the involvement of Pol λ in this or any other process (19). Although the biological role of Pol λ is currently unknown, insights into its possible cellular functions come from studies of its biochemical properties. Unlike TdT, Pol λ is a template-dependent DNA polymerase (20, 21). Like most other members of family X, Pol λ lacks intrinsic 3′→5′ exonuclease activity (5, 20) and, therefore, cannot proofread any errors it generates. Like Pol β, Pol λ is distributive on an "open" template-primer (*e.g.* a template hybridized with a single primer), but it is processive when filling short gaps (*e.g.* 1–5 nucleotides) in DNA if the 5′ end of the gap contains a phosphate group (20). Moreover, Pol λ has an intrinsic dRP lyase activity, and it can replace Pol β to conduct single-nucleotide base excision repair *in vitro* (22). These properties make Pol λ a suitable candidate to perform some form of DNA repair *in vivo*. Interestingly, Pol λ has high affinity for dNTPs (20), which suggests its possible involvement in DNA transactions occurring under low concentrations of DNA precursors.

Another biochemical property that has been useful in understanding the *in vivo* function of a DNA polymerase is its fidelity. Because the error specificity of a DNA polymerase reflects the way it interacts with its substrates, investigation of error specificity may offer clues as to the DNA transactions the enzyme is able to conduct. For example, the low fidelity and the mutational specificity of DNA polymerases η and ι, both members of family Y, suggest that they participate in somatic hypermutation of immunoglobulin genes (reviewed in Ref. 14), a process responsible for generation of high affinity antibodies. Additional experimental results support this hypothesis (14, 23, 24). Similarly, non-templated additions are a signature of synthesis by TdT, implicating TdT in V(D)J recombination, essential for diversification of immunoglobulin genes (25). Also, analysis of the fidelity of human Pol γ, responsible for the replication and repair of the mitochondrial genome, showed that its mutational specificity is consistent with the nature of mutations associated with aging and disease (26). This finding

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[1] The abbreviations used are: Pol, polymerase; TdT, terminal deoxynucleotidyltransferase; dRP, 5′, 2′-deoxyribose 5-phosphate; NHEJ, nonhomologous end-joining; DSB, double-strand break; BRCT, breast cancer susceptibility gene 1 C terminus.

[2] J. F. Ruiz, K. Bebenek, M. Garcia-Diaz, L. Blanco, and T. A. Kunkel, unpublished data.

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TABLE I
Summary of sequence changes generated by Pol λ

The total number of plaques scored was 6880; among these, 1447 were mutant.

Total mutants sequenced	103
Total bases sequenced	41,921
Total sequence changes	253
Single-base deletions	190
Single-base substitutions	38
Two-base deletions	9
Single-base additions	7
Other changes[a]	9

[a] Other changes included deletions of a larger number of nucleotides and complex changes.

supports the hypothesis that accumulation of Pol γ errors leads to mitochondrial dysfunction. Based on the logic that an error signature can be informative regarding possible functions, we present here a detailed analysis of the fidelity of Pol λ and discuss the implications for its possible function *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—All materials for the fidelity assays were from sources described previously (27). The expression and purification of full-length Pol λ was described previously (20).

Forward Mutation Assay—This assay scores errors in the *lacZ* α gene in M13mp2 during synthesis to fill a 407-nucleotide gap (27). Reaction mixtures (25 μl) contained 1 nM gel-purified M13mp2 gapped DNA substrate, 50 mM Tris-HCl (pH 8.5), 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 μg of bovine serum albumin, 4% glycerol, and 10 μM each of dATP, dGTP, dCTP, and dTTP. Polymerization reactions were initiated by adding Pol λ (300 nM) or Pol β (20 nM), incubated at 37 °C for 1 h, and terminated by adding EDTA to 15 mM. Reaction products were analyzed by agarose gel electrophoresis as described (27). Correct synthesis produces M13mp2 DNA that yields dark blue phage plaques upon introduction into an *Escherichia coli* α-complementation strain and plating on indicator plates. Errors are scored as light blue or colorless mutant phage plaques. DNA from independent mutant clones was sequenced to define the *lacZ* mutation. Because most of the mutant clones generated by Pol λ contained both phenotypically detectable and silent changes, the error rates are described as the number of observed mutations divided by the number of nucleotides sequenced. Error rates for Pol β were calculated as described (27).

Short Gap Frameshift Reversion Assay—Construction of the DNA substrate in which the 6-nucleotide gap contains a portion of the *lacZ* α-complementation sequence modified by the introduction of a TTTT sequence has been described (28). Because of the additional nucleotides, the resulting template encodes a colorless M13 plaque phenotype. Frameshift mutations that restore the reading frame result in blue plaques. Gap-filling reaction mixtures (20 μl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 μg of bovine serum albumin, 4% glycerol, 1.6 nM gapped DNA, 500 μM each of dATP, dGTP, dCTP, and dTTP, 400 units of T4 DNA ligase, and 100 nM Pol λ or 50 nM Pol β. After a 1-h incubation at 37 °C, reactions were terminated by adding EDTA to 15 mM; the products were separated on an agarose gel. The covalently closed circular DNA products were electroeluted from gel slices, and the DNA was precipitated with ethanol. DNA products were introduced into *E. coli* by electroporation and followed by plating as described (27).

RESULTS

Fidelity of Human Pol λ in the Forward Mutation Assay—An earlier study indicated that the fidelity of Pol λ was similar to that of Pol β for single-base substitution errors at a TGA codon in the *lacZ* gene (20). To see if a more comprehensive view of the fidelity of Pol λ offered clues to its function, we used the M12mp2 forward mutation assay that detects a broad range of substitution, deletion, and addition errors in a large number of sequence contexts (Table I). This assay requires the enzyme to fill in a 407-nucleotide gap in an M13mp2 double-stranded circular DNA substrate. Pol λ was able to conduct complete gap-filling, as determined by DNA product analysis in an agarose gel (not shown, but see Fig. 3 in Ref. 27, for a typical result). The gap-filled DNA products were introduced into *E. coli* cells and plated, and the plates were scored for total and mutant M13mp2 plaques (see "Experimental Procedures"). As a control, we carried out reactions with human Pol β. DNA synthesis by Pol β generated *lacZ* mutants at a frequency of 3.5%, consistent with previously reported values (28). Using the same reaction conditions, Pol λ generated mutants at a 6-fold higher frequency (21%, as observed in several independent determinations), indicating that Pol λ is less accurate than Pol β.

Error Specificity—We sequenced DNA isolated from 103 independent *lacZ* mutants to determine the error specificity of Pol λ. Single-base substitution, deletion, and insertion errors were observed (Table I) and they were distributed throughout the template DNA present within the gap (Fig. 1). In addition, a few multiple-base changes were observed. The calculated error rates for the different types of errors made by Pol λ are presented in Table II, and Fig. 2 shows average single-base error rates in comparison to other exonuclease-deficient mammalian DNA polymerases examined with this same assay and template. These data reveal that Pol λ has the lowest single-base deletion fidelity of any of these DNA polymerases, and that its average single-base deletion error rate exceeds its single-base substitution error rate. Further, they show that Pol λ is, on average, 32-fold less accurate for single-base deletions than is homologous Pol β.

In contrast, the overall base substitution error rate of Pol λ is closer to what was observed for Pol β. Pol λ generated both transitions and transversions (Fig. 1, Table III), with the main error (13 of 38) being formation of a T·dGMP mispair (error rate of 14×10^{-4}). The preference for this mispair has also been observed previously with other polymerases (29, 30). Absent from the Pol λ spectrum (Fig. 1) are T to G transversions at template base 103. This error is the most prominent feature of Pol β base substitution error spectra (28, 31) and has been suggested to result from a dislocation mechanism (13, 32). According to this mechanism, a correct insertion may occur on a transiently misaligned template-primer, followed by realignment to create a mispair that is extended to create a base substitution. Although we have no indication of dislocation mutagenesis by Pol λ at this particular site, the specificity of 22 of the 38 Pol λ-generated base substitutions shown in Fig. 1 is consistent with the possibility that some of these could result from dislocation.

The Frameshift Error Specificity of Pol λ—To consider possible mechanisms for the high single-base deletion error rate of Pol λ, we analyzed the sequence contexts in which these deletions occurred. The majority of −1 base deletions occurred at iterated nucleotides (Fig. 1). Deletion of iterated pyrimidines was more frequent than deletions of iterated purines, which could reflect weaker stacking interactions between adjacent template pyrimidines than between adjacent template purines. Deletion of nucleotides in repetitive sequences could result from classical template-primer slippage, a signature of which is an increase in error rate with an increasing number of repeat units in the repetitive sequence (33). Therefore, we looked at the relationship between the error rate and homopolymeric run lengths for the single-base deletions generated by Pol λ. The results (Fig. 3) indicate a 6-fold increase in error rate for deletions in a two-nucleotide homopolymeric run as compared with the rate of deletions of non-iterated bases. Interestingly, no additional increase in error rate is observed as the run length increases further. This is in contrast to the results with Pol β, where deletion rates are lower (in Fig. 3, note difference in scale on *y* axis), and there is a more regular increase in error rate with increasing run length.

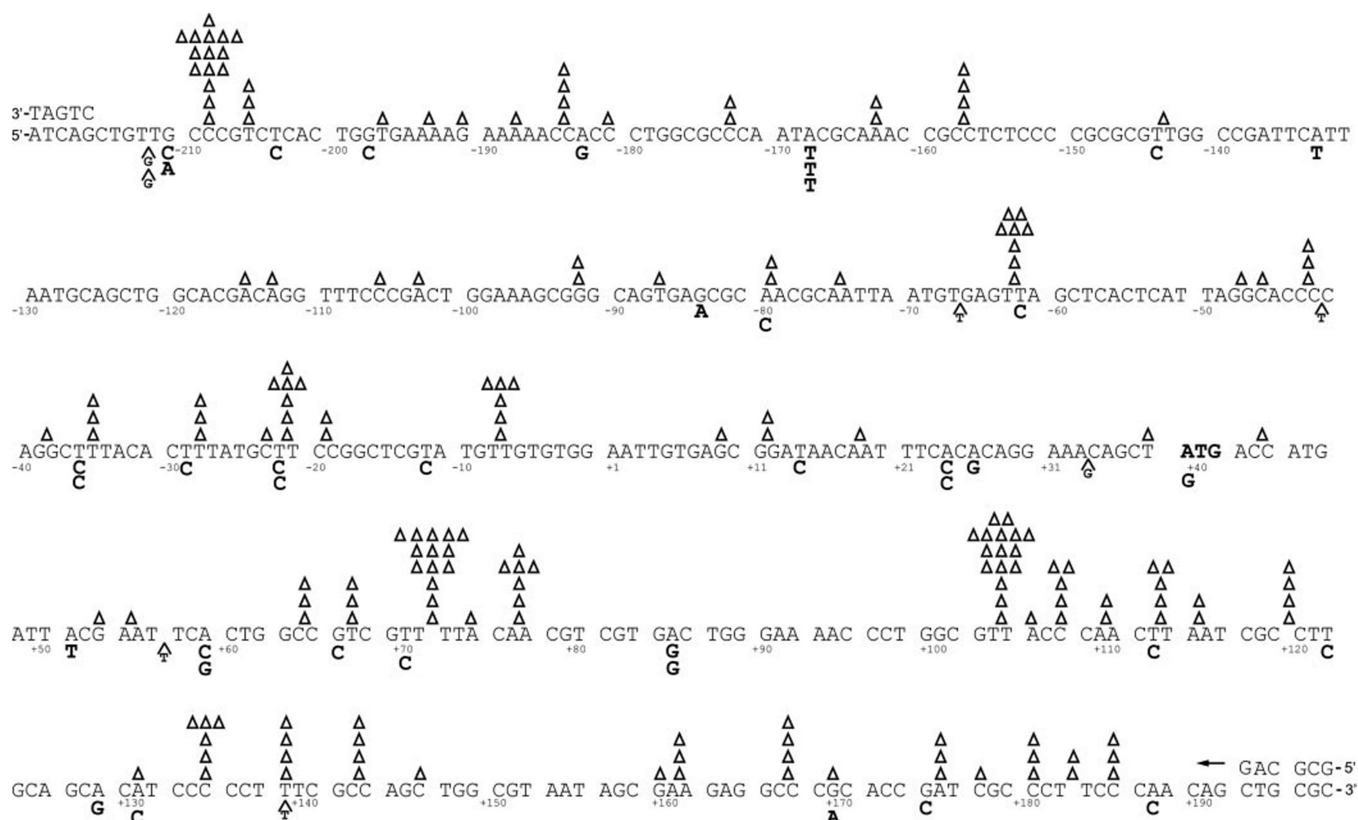

FIG. 1. **Single base error spectrum of human DNA Pol λ.** The 407 template nucleotides within the single-strand gap of the M13mp2 substrate DNA are shown as 5 lines of the template sequence, with nucleotide +1 as the first transcribed nucleotide of the *LacZ* α-complementation region. Base substitutions are indicated by *letters* below the line of the target sequence. Deletion of a base is depicted by an *open triangle* above the line of the sequence, whereas addition of a base is indicated below the line of the sequence by "/\" immediately above the added base. The *arrow* indicates the direction of synthesis.

TABLE II
Pol λ error rates in the forward assay

The error rates were calculated as described under "Experimental Procedures."

Mutation	Error rate × 10^{-4}	
	Pol λ	Pol β
Total frameshifts	49	1.5
−1 base frameshifts	45	1.4
−2 base frameshifts	2.1	<0.06
+1 base frameshifts	1.7	0.05
Base substitutions	9.0	2.3

Low Frameshift Fidelity during Short Gap Filling Synthesis—A number of observations with other DNA polymerases reveal a correlation between single-base frameshift error rates and the processivity of DNA synthesis (see Ref. 34). This has led to the notion that a misaligned intermediate is more likely to form when a polymerase dissociates and/or reassociates with the template-primer. We have reported previously that Pol λ is distributive when copying an open template-primer such as the 407-nucleotide gap used in the forward mutation assay but is more processive when filling a 5-nucleotide gap in which the 5′ end is phosphorylated (20). Therefore, we tested whether the single-base deletion fidelity of Pol λ might be higher when processively filling a short gap. The assay (28) detects single-base deletions (as dark blue revertant plaques) within a 6-base gap containing the template sequence 5′-CTTTTA (see "Experimental Procedures"). In this assay, the frequency of Pol λ-generated revertants is also high, over 100-fold higher than that of Pol β (Table IV). Thus, even when filling a short gap, Pol λ produces deletions at an unusually high rate. DNA sequence analysis confirmed that 39 of 40 dark blue revertants lacked one of the four Ts, whereas only one resulted from a deletion of the template A. This result is consistent with the Pol λ specificity revealed in the forward assay (Fig. 1).

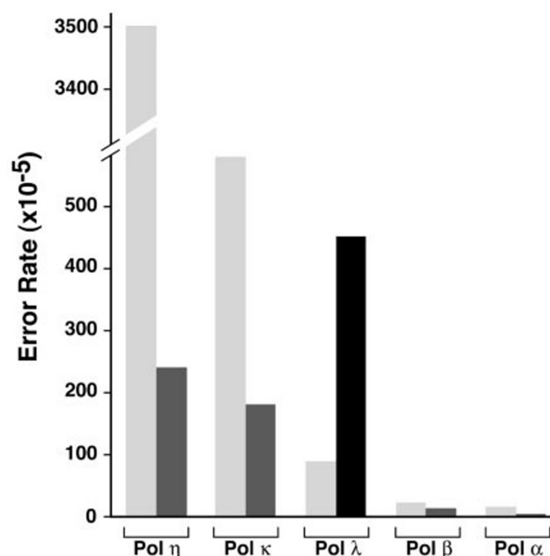

FIG. 2. **Error rates for Pol λ compared with other mammalian DNA polymerases.** Base substitution error rates are represented by *light gray bars*; frameshift rates are depicted by *dark gray bars*, except for the frameshift error rate of Pol λ, which is represented by a *black bar*.

DISCUSSION

All mammalian DNA polymerases that we had examined previously in the forward mutation assay generated base sub-

TABLE III
Pol λ base substitution error rates

In parentheses are the number of changes and the error rate, respectively, at position 103.

Mutation	Pol λ		Pol β	
	Mutants	Error rate $\times 10^{-4}$	Mutants	Error rate $\times 10^{-4}$
Transitions	23	5.5	25	1.4
T → C	13	14	5	1.0
G → A	3	3.1	9	2.2
Transversions	15	3.6	29	1.0
T → G[a]	0	≤1.0	4 (22)	1.0 (118)

[a] T → G transversions excluding errors at position 103.

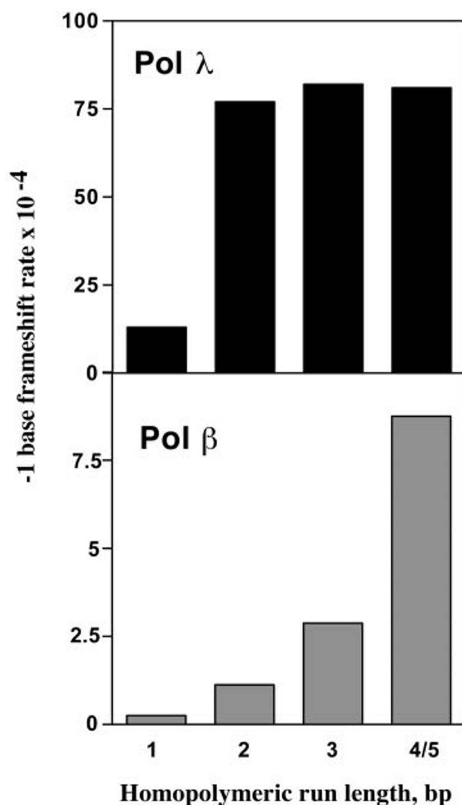

FIG. 3. **Single-base deletion rates as a function of homopolymeric run length.** Error rates are the number of observed single-base deletions (from Fig. 1) divided by the total number of template nucleotides present in runs of the length indicated in the figure among the 103 sequenced *lacZ* clones generated by Pol λ. The template sequence contains 204, 116, 57, and 30 non-iterated nucleotides and nucleotides in runs of 2, 3, and 4 + 5, respectively. Error rates for Pol β were calculated by considering only the phenotypically detectable changes (27).

TABLE IV
Pol λ fidelity during short gap-filling synthesis

Enzyme	Frameshifts			Base substitutions
	Total plaques	Revertants scored	Mutant frequency $\times 10^{-4}$	Mutant frequency[a] $\times 10^{-4}$
Pol λ exp. I	61,000	1815	298 (230)[b]	9
Pol λ exp. II	240,000	8046	340	
Pol β	51,000	13	2.5	10

[a] Results from Ref. 20.
[b] Pol λ reversion frequency from reactions conducted under the same conditions (10 μM dNTPs, 2.5 mM $MgCl_2$) as the forward assay. The value is an average of three independent determinations.

stitutions at a higher average rate than they generated frameshift errors. In contrast, the data presented here (Fig. 2) reveal that human Pol λ is unusual in that its average single-base

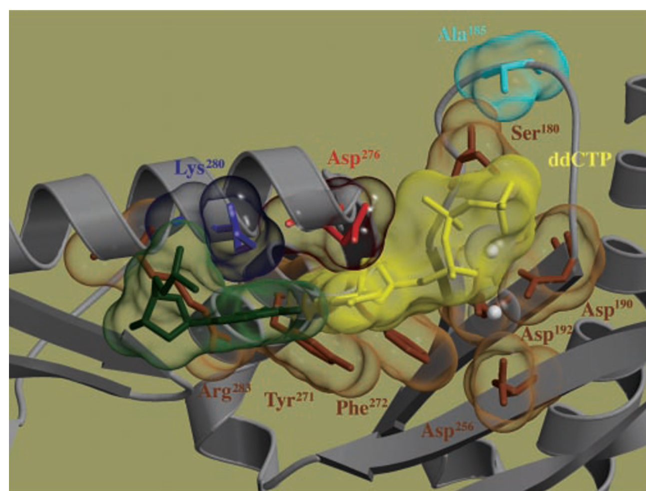

FIG. 4. **The binding pocket for the nascent base pair at the active site of Pol β.** Pol β residues that interact with the templating base (colored *green*) and the incoming nucleotide (colored *yellow*) that are conserved in Pol λ are colored *brown*. Those residues that are not conserved are depicted in *cyan*, *dark blue*, and *red* for Ala-185, Lys-280, and Asp-276, respectively. The figure was created based on the structure of Pol β in ternary complex with gapped DNA and the incoming ddCTP (Protein Data Bank accession number 1BPY) using Molscript (57), GRASP (58), and Raster3D (59).

frameshift error rate is very high and exceeds its average rate of base substitutions. This finding has interesting functional and mechanistic implications. Pol λ has been implicated in base excision repair because, like Pol β, it possesses a dRP lyase activity in its 8-kDa domain, it shows a preference for filling short-gap substrates with a 5′-phosphate, and it can substitute for Pol β in a single-nucleotide base excision repair reaction *in vitro*. Despite these shared features and similar base substitution fidelity, the average single-base deletion fidelity of Pol λ is over 30-fold lower than that of Pol β (Table II). In fact, the average −1 base deletion error rate of Pol λ is even higher than those of polymerases of the Y family, which are renowned for their infidelity. Because of the increase in the single-base deletion rate for runs as compared with non-iterated nucleotides, some of these deletions may reflect classical strand slippage (33). However, the relatively high Pol λ deletion rate for non-iterated nucleotides (Fig. 3) and the lack of a further increase in rate with increasing run length suggests the involvement of additional mechanisms and/or unusual interactions of Pol λ with its substrates. Possibilities include misalignment in the polymerase active site (35–39), nucleotide misinsertion followed by primer relocation (32, 40), or some other mechanism. The fact that the relationship between single-base deletion rates and homopolymeric run length are different for Pol λ compared with Pol β (Fig. 2) or with other polymerases, including the Y family members Pol η and Pol κ (41, 42), suggests that Pol λ interacts with the DNA substrate differently than those other enzymes. The high deletion error rates and lack of an increase in rate for runs of three or more as compared with a run of two imply that Pol λ is relatively efficient at utilizing misaligned DNA substrates stabilized even by only one correct base pair at the primer terminus.

The higher frameshift rate of Pol λ as compared with Pol β indicates differences in the interactions that control the proper alignment of the template-primer and the incoming dNTP. These differences were not anticipated because, as shown in Fig. 4, many of the Pol β residues that comprise the nascent base pair binding pocket are conserved in Pol λ. Among these is Arg-283 (Arg-517 in Pol λ), which contributes to the fidelity of Pol β by providing interactions that stabilize the templating

base. Mutations at this residue result in Pol β with greatly reduced base substitution (43) and frameshift fidelity (44). In fact, the single-base frameshift error rates measured for the Arg-283 → Ala derivative of Pol β are similar to those observed for wild-type Pol λ. Thus, although the presence of an arginine residue at this position in Pol λ (Arg-517) suggests that the function of this side chain is likely to be conserved, it is nonetheless possible that slight differences in the orientation of this residue could affect the interactions that control the alignment of the templating base with the incoming dNTP, contributing to the low frameshift fidelity of the enzyme. In addition, the ability to form and/or to utilize misaligned template-primers could be affected by or linked to any of the three residues in the nascent base pair binding pocket of Pol β that are not conserved in Pol λ: Ala-185, Lys-280, and Asp-276 (Fig. 4). Ala-185 (Lys-422 in Pol λ) is located in proximity to the phosphates of the incoming nucleotide, and Lys-280 (Arg-514 in Pol λ) and Asp-276 (Ala-510 in Pol λ) stack with the templating base and the base of the incoming dNTP, respectively (Fig. 4). Interestingly, the presence of an alanine residue at the position corresponding to Asp-276 in Pol β is believed to contribute to the high affinity of Pol λ for dNTPs (20).

The frameshift fidelity of Pol λ does not increase when processively filling a short gap (Table IV), suggesting that misaligned frameshift intermediates may be efficiently formed and/or stabilized within the active site of the enzyme and extended even when it is conducting processive synthesis. In this respect, Pol λ resembles Pol β. It has been suggested that during short gap synthesis, the polymerase domain of Pol β may dissociate from and then reassociate with the template-primer, allowing its misalignment, while the enzyme remains tethered to the DNA by the binding of the 8-kDa domain to the 5′ end of the gap (28). Pol λ, the processive synthesis of which in a short gap is also facilitated by the presence of a 5′-phosphate on the downstream duplex, may behave in a similar way. The recent solution structure of the 8-kDa domain of Pol λ (45) has confirmed the conservation of the residues involved in 5′-phosphate binding within a positively charged DNA binding groove. This positively charged surface in the 8-kDa domain of Pol λ is significantly larger than in Pol β. It has been suggested that this larger electrostatic surface may allow Pol λ to more stably bind the 5′-phosphate, thus limiting strand displacement synthesis (20).

Is the propensity of Pol λ to use misaligned template-primers related to its physiological function? One possibility is that Pol λ may be involved in NHEJ, which along with homologous recombination, is one of two pathways responsible for the repair of double-strand breaks (DSBs) in eukaryotic cells (46, 47). During NHEJ, broken DNA ends can be aligned using minimal base pairing (microhomology), creating imperfect duplexes with short gaps that need to be filled by a DNA polymerase. By virtue of its ability to use substrates with minimal homology at 3′ ends (Fig. 3), human Pol λ may perform this reaction. Two other members of the Pol X family have already been implicated in DNA end-joining reactions, Pol μ and TdT. TdT is involved in and restricted to repair of DSBs during V(D)J recombination, whereas Pol μ, which has a much wider tissue distribution (9), has been suggested to function in a general NHEJ pathway (17, 18, 48). Pol μ associates with Ku, a key component of the end-joining reaction, and forms a stable complex on DNA in the presence of Ku and ligase IV/XRCC4, another core NHEJ factor. Such a complex is essential for an efficient end-joining reaction involving alignment of ends, gap filling, and ligation (17, 49). Consistent with this role, Pol μ can promote the formation and extension of misaligned primer-templates (Ref. 18).[2] The present study suggests that Pol λ, with its unusually high rate for misalignment-mediated errors and its preference for short gap substrates, also appears to be well suited to participate in NHEJ. The possible involvement of Pol λ in this process is further suggested by genetic (50) and biochemical studies (51) indicating that in yeast, end-joining depends upon the function of DNA Pol IV, a close homologue of human Pol λ. In a recent study, Heidenreich *et al.* (52) showed that NHEJ contributes to mutagenesis in non-replicating diploid yeast cells. They suggested that this mutagenic process might not involve a DNA polymerase. Nevertheless, we find it intriguing that the majority of mutations observed in that study were single-base deletions in short homopolymeric runs. This specificity is characteristic of Pol λ and thus suggests the involvement of Pol IV. The end-joining activity of Pol IV requires the presence of its N-terminal BRCT domain (50, 51). It has been shown that through the BRCT domain, Pol IV interacts with Dnl4, a subunit of the Dnl4-Lif1 complex (51), which is the homologue of the human ligase IV-XRCC4 complex. Thus, the BRCT domain may be involved both in the recruitment of the polymerase and in interactions that couple the gap-filling and the ligation steps. Pol λ, like Pol IV, Pol μ, and TdT also has an N-terminal BRCT domain, which appears to be a common feature of polymerases involved in NHEJ. Finally, a recent study has revealed that Pol λ has TdT activity and the capacity to elongate RNA primers (53), both of which could perhaps be related to its functions *in vivo*.

In yeast, the majority of DSBs are repaired by homologous recombination. However, in higher eukaryotes, repair of DSBs is mostly dependent on the NHEJ pathway (47). Homologous recombination is restricted to late S and G_2 phases of the cell cycle, whereas NHEJ is the predominant repair mechanism during G_0, G_1, and early S phases (54). It is worth noting that while the cellular dNTP pools are highest during S and G_2 phases, they are lowest in G_0 (55), which is consistent with the hypothesis that DNA Pol λ may be involved in DNA transactions occurring when the concentration of precursors is low (20). Hence, Pol λ is a good candidate to function throughout the cell cycle in DNA transactions that involve NHEJ. Its intrinsic dRP lyase activity (22) implies that Pol λ may be useful for repair of DSBs with certain types of damaged DNA ends, *e.g.* those resulting from abortive processing by base excision repair enzymes of clustered DNA lesions composed of abasic sites, oxidized bases, and strand breaks caused by ionizing radiation (56).

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