Mutagenesis of human DNA polymerase λ: essential roles of Tyr505 and Phe506 for both DNA polymerase and terminal transferase activities

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ABSTRACT

DNA polymerase (pol) λ is homologous to pol β and has intrinsic polymerase and terminal transferase activities. However, nothing is known about the amino acid residues involved in these activities. In order to precisely define the nucleotide-binding site of human pol λ, we have mutagenised two amino acids, Tyr505 and the neighbouring Phe506, which were predicted by structural homology modelling to correspond to the Tyr271 and Phe272 residues of pol β, which are involved in nucleotide binding. Our analysis demonstrated that pol λ, Phe506Arg/Gly mutants possess very low polymerase and terminal transferase activities as well as greatly reduced abilities for processive DNA synthesis and for carrying on translesion synthesis past an abasic site. The Tyr505Ala mutant, on the other hand, showed an altered nucleotide binding selectivity to perform the terminal transferase activity. Our results suggest the existence of a common nucleotide-binding site for the polymerase and terminal transferase activities of pol λ, as well as distinct roles of the amino acids Tyr505 and Phe506 in these two catalytic functions.

INTRODUCTION

DNA polymerase (pol) λ is a nuclear enzyme that has been assigned, based on sequence homology, to the family X polymerases, comprising pol β, pol μ and terminal transferase (TdT) (1). The gene encoding the novel pol λ was cloned and mapped to mouse chromosome 19 and to human chromosome 10. Furthermore, isolation of endogenous pol λ from calf thymus has been described (2). Pol λ has 32% amino acid identity to pol β and contains all four pol β structural subdomains, named fingers, palm, thumb and the 8 kDa 5’-deoxyribose phosphate lyase (dRP lyase) domain. However, the high homology of pol λ with pol β is exclusively confined to the C-terminal half of the protein (amino acids 244–575) containing the catalytic site. Pol λ contains an intrinsic dRP lyase activity and can substitute for pol β in in vitro base excision repair (3). Both the human and the calf thymus enzymes were able to synthesise DNA on a template containing abasic (AP) sites with the same efficiency as on undamaged DNA, thus suggesting a potential role of pol λ in translesion synthesis (2,4). Moreover, it has recently been demonstrated that human pol λ interacts functionally and physically with proliferating cell nuclear antigen (PCNA). This interaction stabilises the binding of pol λ to the primer template, thus increasing its affinity for the hydroxyl primer and its processivity in DNA synthesis, without affecting the nucleotide incorporation rate. PCNA also stimulated efficient synthesis by pol λ across an AP site leading to elongation past the lesion (4).

Beside pol β and pol λ, other members of polymerase family X are TdT and pol μ (5). Interestingly, whereas pol β does not possess terminal transferase activity, both pol λ and pol μ are able to add nucleotides to the 3’-end of a single-stranded (ss)DNA (6,7). These different properties of pol β and pol λ might reflect a different architecture of the active site. Sequence alignments of the members of the polymerase X family showed two long motifs of conserved residues, which have been shown to constitute the dNTP-binding site in the crystal structures of pol β and TdT (8–13). The first motif is LYFTGS in human pol β (amino acids 271–275) and human pol λ (amino acids 504–509) and LGWTGS (amino acids 447–452) in human TdT. Essential roles in dNTP binding and catalysis have been shown for the pol β residues Y271 and F272 (12,14,15). These residues correspond to Y505 and F506 of human pol λ and to G449 and W450 of human TdT. Additional important amino acids involved in nucleotide binding by pol β are R254 and R258 (8), the latter being adjacent to the F272 side chain in the crystal structure of pol β complexed with its substrates. Interestingly, homology

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modelling revealed that the putative nucleotide-binding pocket of pol λ has a more hydrophilic character with respect to that of pol β, since the pol β residue R258 is replaced in pol λ by the moderately hydrophobic residue I492, which lies close to F506.

Since it is not known whether the same residues of pol λ are responsible for both the template-dependent (i.e. DNA polymerase) and template-independent (i.e. terminal transferase) activities, we have generated three pol λ mutants: Y505A, F506G and F506R. While the first two (Y505A and F506G) allowed us to test the effects of the lack of the corresponding aromatic side chains, the F506R mutant was generated to test the effect, if any, of replacement of the Phe ring with a positively charged chain within the hydrophobic nucleotide-binding pocket of pol λ. We have analysed the effects of these mutations on both the DNA polymerisation and terminal transferase activities of pol λ. Our data suggest a common nucleotide-binding site for both the DNA polymerase and terminal transferase functions of pol λ and that the Y505A and F506G/R mutations affect these two activities differently.

MATERIALS AND METHODS

Chemicals

[3H]dTTP (30 Ci/mmol), [3H]dCTP (18 Ci/mmol), [3H]dUTP (19 Ci/mmol), [3H]dATP (73 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were from Amersham. Unlabelled dNTPs, tetrahydrofuran (dSpacer) were from Glen Research. Activated calf thymus DNA was prepared as described (16). Whatman was the supplier of the GF/C filters.

Nucleic acid substrates

The d73mer, either undamaged or containing the synthetic AP site and the corresponding primers were chemically synthesized and purified on denaturing polyacrylamide gels (4). The sequence of d73mer is 5'-[3H]dATP (73 Ci/mmol) and [γ-32P]ATP (3000 Ci/mmol) were from Amersham. Unlabelled dNTPs, poly(dA) and oligo (dT)12–18 were from Roche Molecular Biochemicals. The tetrahydrofuran (dSpacer) was from Glen Research. Activated calf thymus DNA was prepared as described (16). Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka.

Enzymatic assays

DNA polymerase assay. Pol λ activity on poly(dA)-oligo(dT)10:1 was assayed in a final volume of 25 μl containing 50 mM Tris–HCl (pH 7.0), 0.25 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 0.5 mM MnCl2, 0.2 μM poly(dA)-oligo(dT)10:1 (3'-OH ends), 50 nM pol λ and 5 μM [3H]dTTP (5 Ci/mmol), unless otherwise indicated in the figure legends. All reactions were incubated for 15 min at 37°C unless otherwise stated and the DNA precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described (16). For denaturing gel analysis, the reaction mixture included 50 mM Tris–HCl (pH 7.0), 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM MnCl2. Enzymes, unlabelled dNTPs and template were as indicated in the figure legends. For the processivity assay, the reactions were performed in the presence of 40-fold molar excess of poly(dA)-oligo(dT) as a trap (4) over the labelled template.

Terminal transferase assay. Pol λ terminal transferase activity on a single-stranded 66mer oligonucleotide was assayed in a final volume of 25 μl containing 50 mM Tris–HCl (pH 7.0), 0.25 mg/ml BSA, 1 mM DTT, 0.5 mM MnCl2, 0.2 μM of single-stranded 66mer (3'-OH ends). Pol λ and [3H]dNTPs (10 Ci/mmol) were added as indicated in the figure legends. All reactions were incubated for 15 min at 37°C unless otherwise stated and the DNA precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described (16).

Steady-state kinetic data analysis

For kinetic analysis, enzymatic activity was measured in the presence of 25 nM pol λ wt and Y505A and 75 nM pol λ F506G and F506R and increasing concentrations of DNA or nucleotide substrate. For dTTP incorporation, concentrations tested were 0.1, 0.2, 0.75, 1.25, 5, 10 and 25 μM. For DNA
substrate utilisation, poly(dA)-oligo(dT) concentrations used (as 3'-OH ends) were 10, 20, 40, 80 and 200 nM. Enzymatic activity was measured at the 16 min time point, which corresponded to the mid-point of the linear phase of the reaction, as determined by time-dependent incorporation experiments. The \( K_m \) and \( V_{max} \) values were calculated by plotting the initial velocity dependence on substrate concentration and fitting the data to the Michaelis-Menten equation in the form:

\[
v = \frac{k_{cat}E_0}{1 + \frac{K_m}{[S]}}
\]

where \( k_{cat}E_0 = V_{max} \).

For quantification of the reaction products after separation on sequencing gels, the products were calculated from the values of integrated gel band intensities \( I_T / I_{T-1} \), where \( T \) is the target site, the template position of interest, and \( I_T \) is the sum of the integrated intensities at positions \( T, T+1 \ldots T+n \).

All the intensity values were normalised to the total intensity of the corresponding lane to correct for differences in gel loading. An empty lane was scanned and the corresponding value subtracted as background.

**RESULTS**

Substitutions of Y505 and F506 have different effects on the polymerase activity of DNA polymerase \( \lambda \)

Human pol \( \lambda \), either wild-type or the Y505A, F506G or F506R mutant, was titrated in DNA polymerase assays with a 5'-end-labelled 17:73 primer/template DNA oligonucleotide substrate. All the reactions were performed in the presence of Mn\(^{2+}\), which has been shown to be the optimal divalent cation for pol \( \lambda \) (21), and the products were resolved by sequencing gel analysis. As shown in Figure 1A, both the F506 pol \( \lambda \) mutants (lanes 1–6) displayed a strongly reduced activity compared to the wild-type (lanes 10–12). On the other hand, the pol \( \lambda \), Y505A mutant (lanes 7–9) showed only a moderate reduction in catalytic activity at low enzyme concentrations (compare lanes 8 and 9 to lanes 11 and 12), but nearly identical activity at high concentrations (compare lane 7 with lane 10).
These results indicated a highly distributive mode of synthesis by pol λ, as already reported (4). Both the pol λ wt and the Y505A mutant showed a strong stop site around positions +42 to +45 (giving a product of ~59±62 nt, indicated by the arrow), corresponding to a G-rich sequence. The kinetic parameters for nucleotide incorporation and DNA binding were determined for the four enzymes as described in Materials and Methods, and are summarised in Table 1. The pol λ F506G and F506R mutants showed a 50- to 100-fold decrease in the apparent \( k_{\text{cat}} \) value with respect to the wild-type. Interestingly, the F506G mutant had nearly 30-fold lower \( K_m \) values for both the nucleotide and the DNA substrates with respect to the wild-type enzyme, resulting in similar efficiencies of substrate utilisation (\( k_{\text{cat}}/K_m \) values). On the other hand, the F506R substitution did not have any significant effect on the apparent \( K_m \) value for the nucleotide substrate with respect to pol λ wt, but showed a 27-fold decrease in the \( K_m \) value for the 3'-OH primer end with respect to the wild-type enzyme. As a result, the F506R mutant displayed a 77-fold reduction in nucleotide utilisation efficiency (\( k_{\text{cat}}/K_m \) values), whereas the efficiency of DNA substrate utilisation was reduced only 4-fold. The pol λ Y505A mutant, on the other hand, showed only a 3-fold reduction in its apparent \( k_{\text{cat}} \) value, with nucleotide and DNA substrate utilisation efficiencies nearly identical to pol λ wt.

**The DNA polymerase λ F506G and F506R mutants show reduced processivity with respect to both the wild-type and the Y505A mutant**

Human pol λ has been shown to interact with PCNA, which stimulates its processivity (4). In order to test the responsiveness of the mutant enzymes to PCNA, pol λ wt and the Y505A, F506G and F506R mutants were tested in the absence or presence of increasing concentrations of human PCNA, under distributive conditions. As shown in Figure 1B, PCNA stimulated synthesis by pol λ wt and the Y505A mutant in a very similar manner (compare lanes 1–5 with lanes 6–10). On the other hand, the pol λ F506G and F506R mutants were severely impaired in their polymerase activity compared to the wild-type, synthesising products of 10–15 nt in both the
The DNA polymerase λ F506G mutant has a reduced ability to bypass an abasic site

Pol λ has been shown to be able to synthesise DNA across an AP site lesion on the template strand (2,4). Pol λ wt and the Y505A and F506G mutants were titrated under standing conditions, using a 18/73mer primer/template DNA oligonucleotide substrate. The 18mer primer was annealed to nucleotides 26–44 of the 73mer template, thus leaving a single-stranded 5′-tail of 25 nt available for synthesis (see Materials and Methods for details). The 73mer oligonucleotide contained a single synthetic AP site (tetrahydrofuran moiety) in place of the G at position 25. Thus, upon annealing of the 18mer primer, an abasic site resulted at position +1 of the template. As shown in Figure 3A, in the presence of all four dNTPs pol λ wt (lanes 1–3) and the Y505A mutant (lanes 4–6) were able to synthesise a full-length product starting from the lesion. On the other hand, the F506G mutant aborted DNA synthesis after incorporation of one nucleotide (lanes 7–9), corresponding to the position of the AP site. These results suggested that the F506G mutant, albeit able to incorporate one nucleotide in front of the lesion, was severely impaired in its ability to elongate the resulting primer end. Next, pol λ wt and the F506G mutant were tested on the same 18/73mer substrate with the lesion, in the presence of increasing concentrations of all four dNTPs. As shown in Figure 3B, while the wild-type enzyme was able to catalyse both incorporation in front of the lesion and subsequent elongation (lanes 2–9) independent of the dNTP concentration, the F506G mutant could only add one nucleotide at the position corresponding to the AP site and was unable to further elongate the nascent DNA chain, even at high dNTPs concentrations (lanes 10–17). When total DNA synthesis (calculated from the intensities of the bands) was plotted as a function of the nucleotide substrate concentration (Fig. 3C), pol λ wt showed an apparent maximal rate of elongation of 0.32 min⁻¹, whereas the F506G mutant exhibited a rate of 0.05 min⁻¹.

The terminal transferase activity of DNA polymerase λ is reduced in the mutant Y505A and abolished in the mutants F506G/R

We have recently shown that pol λ has intrinsic terminal transferase activity, with a preference for pyrimidine nucleotides (7). This is also evident from the time–course experiments shown in Figure 4A, where the terminal transferase activity of pol λ wt was tested with a 66mer ssDNA oligonucleotide as substrate, in the presence of different radioactively labelled deoxynucleotides. Pol λ incorporated all three pyrimidine nucleotides (dTTP, dCTP and dUTP) with higher efficiency than the purine dATP, in accordance with previous data (7). Next, the pol λ mutants Y505A, F506G and F506R were compared under the same conditions to pol λ wt in the presence of labelled dTTP. As can be seen from Figure 4B and C, the Y505A mutant showed a

<table>
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<tr>
<th>Substrate</th>
<th>Wild-type</th>
<th>Y505A</th>
<th>F506R</th>
<th>F506G</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTTP</td>
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<td>11.0</td>
<td>9.7</td>
<td>0.5</td>
</tr>
<tr>
<td>μM</td>
<td>(≥ 1)</td>
<td>(≥ 0.1)</td>
<td>(≥ 0.2)</td>
<td>0.1</td>
</tr>
<tr>
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<td>9.6</td>
<td>2.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>μM</td>
<td>(≥ 2)</td>
<td>(≥ 0.1)</td>
<td>(≥ 0.5)</td>
<td>n.d.</td>
</tr>
<tr>
<td>3'-OH</td>
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<td>8.7</td>
<td>82</td>
<td>10</td>
</tr>
<tr>
<td>nM</td>
<td>(≥ 0.7)</td>
<td>(≥ 5)</td>
<td>(≥ 6)</td>
<td>(≥ 4)</td>
</tr>
<tr>
<td>nM</td>
<td>(≥ 0.1)</td>
<td></td>
<td></td>
<td>(≥ 1)</td>
</tr>
<tr>
<td>nM</td>
<td>(≥ 0.01)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

n.d., not determined.

*Table 1. Kinetic parameters for the DNA polymerisation reaction catalysed by DNA polymerase λ wild-type and the Y505A, F506R and F506G mutants*
reduced ability to incorporate dTTP, compared to the wild-type enzyme, whereas the mutants F506G and F506R showed almost no detectable activity. These results indicated that the two pol λ residues Y505 and F506 are important for polymerisation in both the DNA polymerase and terminal transferase activities.

The DNA polymerase λ Y505A mutant specifically reduces pyrimidine nucleotide utilisation by the terminal transferase activity

Pol λ wt and pol λ Y505A were compared in a terminal transferase assay for their ability to incorporate dTTP or dATP in the presence of either Mn²⁺ or Mg²⁺ as the metal activators. Both metals were tested since it is known that the nature of the divalent ion can influence the nucleotide specificity of polymerases. In particular, pol λ has recently been shown to utilise both Mg²⁺ and Mn²⁺, but with very different efficiencies (18). As shown in Figure 5A and B, the terminal transferase activity of pol λ, either wild-type or the mutant, showed a clear preference for Mn²⁺ as the activating metal. However, in the presence of both Mn²⁺ and Mg²⁺, the pol λ Y505A mutant showed reduced dTTP incorporation with respect to pol λ wt, whereas the incorporation of dATP was comparable for the two enzymes. In order to better understand the molecular basis for this effect, a detailed kinetic and thermodynamic analysis was performed for the incorporation of pyrimidine (dTTP and dUTP) and purine (dATP) nucleotides by the terminal transferase activity of pol λ wt and the Y505A mutant. The results are summarised in Table 2. The apparent rate of incorporation (k_{cat}) of pol λ wt was 4- and 3-fold higher for dTTP and dUTP, respectively, than for dATP, thus explaining the observed preference for pyrimidine nucleotides as substrates for the terminal transferase activity. Pol λ wt and the Y505A mutant. The results are summarised in Table 2. The apparent rate of incorporation (k_{cat}/K_{m} values) with respect to the wild-type, but a 3- to 4-fold reduction in the incorporation efficiencies of dTTP and dUTP. This effect was mainly due to differences in the K_{m} values for the pyrimidine nucleotides of the pol λ Y505A mutant with respect to pol λ wt. Interestingly, comparison of the kinetic parameters for dTTP and dUTP
incorporation for the DNA polymerisation activity reported in Table 1 with the corresponding values for the terminal transferase activity reported in Table 2 revealed that the difference between pol \( \lambda \) wt and the Y505A mutant for pyrimidine utilisation was specific for the terminal transferase activity.

**DISCUSSION**

In mammalian cells, four members of the pol X family have been identified so far: pol \( \beta \), TdT, pol \( \lambda \) and pol \( \mu \) (5). Pol \( \beta \) performs template-directed incorporation of nucleotides, whereas TdT adds nucleotides to the 3'-end of a DNA strand, in a template-independent manner. Strikingly, pol \( \lambda \) and pol \( \mu \) possess both template-directed polymerisation and terminal transferase activities (7,17). Crystal structures are available for pol \( \beta \) and TdT (8–13). Based on these structures and on mutagenesis studies, the residues important for nucleotide binding and catalysis have been identified. Sequence alignments with the other members of the polymerase X family showed that these residues belong to highly conserved domains. One of the conserved dNTP-binding domains is shown in Figure 6. Its sequence is LYFTGS in human pol \( \beta \) (amino acids 270–275) and human pol \( \lambda \) (amino acids 504–509) and LGWTGS (amino acids 447–452) in human TdT. In pol \( \beta \), the Y271 residue has been shown to constitute the floor of the dNTP-binding pocket and was proposed to contribute to stabilisation of the enzyme-primer complex by engaging a hydrogen bond with the primer base (12). It is interesting to note that Y271 is conserved in pol \( \lambda \) (corresponding to Y505), but not in TdT, which bears a glycine at this position (G447). This suggests that the function of this residue is different for template-directed polymerases and for TdT. The adjacent amino acid (F272 in pol \( \beta \)) plays an essential role in catalysis. When pol \( \beta \) adopts its 'closed' conformation prior to catalysis, the phenyl ring of F272 disrupts the salt bridge between D192 and R258, freeing D192 to coordinate the nucleotide binding metal ion (12). Interestingly, the pol \( \beta \) residue R258 is not conserved in pol \( \lambda \), whose corresponding amino acid is I492. However, in a structural model of the pol \( \lambda \) structure, based on the known structures of pol \( \beta \) and TdT, the F506 side chain has been predicted to be in close contact with...
the I492 residue, similarly to the F272 and R258 side chains in pol β. A F272L mutation has been described, which greatly increased the error rate of pol β (15). The F272 residue of pol β corresponds to W449 in human TdT. In the crystal structure of murine TdT, the aromatic ring of residue W450 was shown to be parallel and stacked with the base ring of the incoming nucleotide and also to make contacts with its sugar moiety (13). These observations suggest an important role of an aromatic residue at this position for both template-dependent and template-independent DNA polymerases.

According to this prediction, the pol λ F506G/R mutants analysed in the present work showed a 50- to 100-fold reduction in the apparent catalytic rate ($k_{cat}$) for template-dependent nucleotide incorporation (Table 1) and virtually undetectable terminal transferase activity with respect to pol λ wt. Interestingly, we found a strong decrease in the processivity of the pol λ F506G/R mutants, which dissociated after every two to four incorporation events (Fig. 2A), together with a concomitant increase in the apparent affinity of the mutated enzymes for the primer-template (Table 1). Since processivity is defined as the number ($n$) of incorporation events per dissociation event, $n = k_{pol}/k_{off}$, where $k_{pol}$ is the true polymerisation rate and $k_{off}$ is the rate of dissociation of the enzyme from the template, one possibility is that the F506G/R mutations lower both the dissociation rate ($k_{off}$) and the catalytic rate ($k_{pol}$), so that $k_{pol} = 2–4 k_{off}$, hence $n = 2–4$. The alternative possibility is that these mutations increased the $k_{off}$ rate of the enzyme from the DNA template. However, this would be in contrast to the observed increase in the affinity for the DNA template (Table 1). Moreover, in such a case we would have expected to see at least a partial rescue of the processivity upon addition of PCNA, since it has been shown that PCNA lowers the $k_{off}$ rate of pol λ for dissociation from the template. The experiments shown in Figures 1B and 2B, however, clearly indicated no effect of PCNA on the processivity of the F506G/R mutants.

The ability of a particular polymerase to bypass a non-instructional lesion like an AP site depends on its ability to adopt a less constrained conformation at the nucleotide-binding site (19), which usually preferentially accommodates the templating base and the complementary nucleotide. We have compared the AP translesion capacity of pol λ wt and the Y505A and F506G mutants under standing start conditions. The F506G mutant showed an impaired

Figure 4. The DNA polymerase λ Y505A and F506G/R mutants have reduced terminal transferase activity. (A) Time course of the terminal transferase activity of pol λ wt (0.2 μM) on a single-stranded 66mer oligonucleotide substrate. Terminal transferase activity was measured under the conditions described in Materials and Methods, in the presence of 10 μM labelled dTTP (triangles), dCTP (circles), dUTP (rhombi) or dATP (squares). (B) Time course of the terminal transferase activity of pol λ wt (0.2 μM, white boxes), Y505A (0.2 μM, grey boxes), F506R (0.4 μM, striped boxes) or F506G (0.4 μM, black boxes) on a single-stranded 66mer oligonucleotide substrate. Terminal transferase activity was measured under the conditions described in Materials and Methods, in the presence of 10 μM labelled dTTP. (C) Titration of pol λ wt (white boxes), Y505A (grey boxes) F506R (striped boxes) and F506G (black boxes) on a single-stranded 66mer oligonucleotide substrate. Terminal transferase activity was measured under the conditions described in Materials and Methods, in the presence of 10 μM labelled dTTP.
capacity to catalyse elongation past the lesion under these conditions (Fig. 3A). The apparent maximal rate of translesion synthesis was calculated as 0.32 min⁻¹ for the wild-type enzyme and 0.05 min⁻¹ for the F506G mutant. Comparison with the $k_{cat}$ values listed in Table 1 for normal DNA synthesis showed that pol λ wt had a ~34-fold reduction in its catalytic rate, due to the presence of an abasic site at position +1. On the other hand, the F506G mutant showed only a 4-fold reduction in reaction rate, due to the presence of an abasic site at position +1. On the other hand, the F506G mutant showed only a 4-fold reduction in reaction rate. The mutant enzyme can catalyse only the first step of the AP site bypass process, namely incorporation in front of the lesion without dissociating. Subsequent elongation of the resulting primer end, on the other hand, causes a major pausing of the enzyme. Due to its severely impaired catalytic efficiency and reduced processivity, the F506G mutant likely dissociates from the template during this pausing phase before having the chance to incorporate one additional nucleotide, resulting in virtually no elongation beyond the AP site. It is interesting here to note that the Y505A mutation had no detectable effect on the bypass ability of pol λ, however, as summarised in Table 1, both the apparent $K_m$ and $k_{cat}$ for nucleotide incorporation of the pol λ Y505A mutant were decreased with respect to the wild-type enzyme. This phenotype was very similar to that observed for the corresponding Y271A mutant of pol β (14), and might suggest a perturbation of the nucleotide-binding pocket.

Clear evidence for a role of the pol λ Y505 residue in nucleotide binding specificity comes from the results on the effects of this mutation on the terminal transferase activity of pol λ. As shown in Figure 4 and Table 2, the pol λ Y505A mutant had a reduced ability to catalyse the terminal addition of pyrimidine deoxynucleotides to single-stranded DNA, but showed similar efficiency to the wild-type enzyme when purine deoxynucleotides were used as substrates. The apparent catalytic rates ($k_{cat}$ values) for the wild-type pol λ terminal transferase activity (Table 2) were much lower than the corresponding rates for template-dependent nucleotide incorporation (Table 1). This was likely due to the very high distributivity of the terminal transferase reaction, which usually adds only a few nucleotides for each DNA binding event. Thus, at steady-state, the limiting rate of the reaction is

![Image](https://academic.oup.com/nar/article-abstract/31/23/6916/2904386)

**Figure 5.** The DNA polymerase λ Y505A mutation specifically reduces the efficiency of pyrimidine utilisation by its terminal transferase activity. (A) Dependence of the terminal transferase activity of pol λ wt (0.2 μM, open symbols) or Y505A (0.2 μM, open symbols) on Mn²⁺ as the activating metal ion. Terminal transferase activity was measured under the conditions described in Materials and Methods, in the presence of 10 μM labelled dTTP (triangles) or dATP (circles). (B) Dependence of the terminal transferase activity of pol λ wt (0.2 μM, filled symbols) or Y505A (0.2 μM, open symbols) on Mg²⁺ as the activating metal ion. Terminal transferase activity was measured under the conditions described in Materials and Methods, in the presence of 10 μM labelled dTTP (triangles) or dATP (circles).

**Table 2.** Kinetic parameters for the incorporation of pyrimidine and purine deoxynucleotides by the terminal transferase activity of DNA polymerase λ wild-type and the Y505A mutant

<table>
<thead>
<tr>
<th></th>
<th>dATP $K_m$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min⁻¹ μM⁻¹)</th>
<th>dTTP $K_m$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min⁻¹ μM⁻¹)</th>
<th>dUTP $K_m$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min⁻¹ μM⁻¹)</th>
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<tr>
<td>wt</td>
<td>25±(2)</td>
<td>0.12±(0.01)</td>
<td>0.005±(0.0006)</td>
<td>14±(3)</td>
<td>0.08±(0.01)</td>
<td>0.0057±(0.0004)</td>
<td>37±(3)</td>
<td>0.066±(0.01)</td>
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<tr>
<td>Y505A</td>
<td>32±(3)</td>
<td>0.09±(0.007)</td>
<td>0.003±(0.0002)</td>
<td>60±(4)</td>
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<td>0.0015±(0.0003)</td>
<td>96±(4)</td>
<td>0.06±(0.01)</td>
<td>0.0006±(0.0001)</td>
</tr>
</tbody>
</table>

The DNA polymerase λ Y505A mutation has been aligned to murine pol λ (lamdahus), human pol β (betahu), human TdT (tdthu) and murine TdT (tdtmus). The two residues considered in this study have been highlighted in yellow and the respective substitutions made by site-directed mutagenesis are indicated in red, below the human pol λ sequence. The Trp residue in the TdT sequence, which is functionally homologous to the Phe of pol λ and pol β, is highlighted in green. Light blue shading was used to box all the other amino acids conserved in at least three different enzymes. The identity within these nine amino acids is 100% between human and mouse pol λ, 100% between human and mouse TdT, 77.8% between pol λ and pol β, 66.7% between TdT and pol λ and 44.4% between TdT and pol β.
indeed dissociation of the enzyme from the DNA substrate, hence $k_{\text{on}} = k_{\text{off}}$.

In summary, the results presented here suggest essential functional roles in both the template-dependent (i.e. DNA polymerase) and template-independent (i.e. terminal transferase) activities of $\text{pol} \ \lambda$ for two highly conserved residues in the nucleotide-binding pocket of the $X$ family polymerases. The F506 residue appears to have a critical role in catalysis, whereas the Y505 residue seems to be critical for the specificity of nucleotide incorporation by the $\text{pol} \ \lambda$ terminal transferase activity.

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