J. Biol. Macromol., **25**(1), 3-13 (2025)

Article

Leu606 of human DNA polymerase δ plays a role in discriminating between deoxyribonucleotide and ribonucleotide

[1]Rin Ueno, [1]Shu Ishibashi, Mako Kandabashi, Kentaro Fukata, Teisuke Takita, and Kiyoshi Yasukawa*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

In eukaryotic cells, the enzyme DNA polymerase δ (Pol δ) is involved in the replication of chromosomal DNA. Structurally, it consists of the p125 catalytic subunit and structural subunits p66, p50, and p12. In humans, the active site of Pol δ contains a Leu606 residue that is well conserved in eukaryotic Pol δ. In this study, we examined the role of Leu606 in the discrimination of deoxyribonucleotides and ribonucleotides. Eight single-mutation variants were expressed in *Escherichia coli* and purified from the cells. Using these variants, we measured the incorporation of deoxyribonucleotide (activity A) and ribonucleotide (activity B). Compared with the activity of wild-type Pol δ (set to 100%), the relative activities A and B of the variants ranged from 40% to 180% and 6.1% to 690%, respectively. Furthermore, when the ratio of the relative activity of B to the relative activity of A with respect to that the wild type was set to 100%, the ratios of the eight variants ranged from 16–820%. Among these variants, the incorporation activity of L606G (820%) was the highest, followed by that of L606F (670%) and L606M (570%). These findings indicate that Leu606 plays an important role in discriminating between deoxyribonucleotides and ribonucleotides.

Received July 8, 2024; Accepted August 8, 2024

Key words: human DNA polymerase δ/Pol δ/motif A/ribonucleotide incorporation/steric gate

Introduction

In eukaryotes, DNA polymerases (Pols) α, δ, and ε have been established to play roles in the replication of genomic DNA, among which, Pol α functions as a component of primase complex that initiates the *de novo* synthesis of primers, whereas Pol δ and ε are mainly involved in extending the lagging and leading strands, respectively [1]. Pol δ and ε are characterized by 3′→5′ proofreading exonuclease activity required for high-fidelity DNA synthesis, and Pol δ also plays a role in DNA repair.

Mammalian Pol δ consists of four subunits, namely, the p125 catalytic subunit (encoded by POLD1) and the three structural subunits p50 (POLD2), p66 (POLD3), and p12 (POLD4) [2, 3]. Comparatively, *Saccharomyces cerevisiae* Pol δ consists of three subunits, the catalytic subunit Pol3p (corresponding to p125) and the structural subunits Pol31P and Pol32P (corresponding to p50 and p66, respectively) [4]. The Pol δ

[1]Both authors contributed equally to this work.

*Correspondence author: Kiyoshi Yasukawa.
Phone: +81-75-753-6266
E-mail: yasukawa.kiyoshi.7v@kyoto-u.ac.jp

sequence comprises a Motif A sequence (DXXXLYPS) containing a catalytically essential Asp residue that is conserved in eukaryotic Pols [2–4]. Site-maturation mutagenesis of the Leu612 residue in the Motif A of yeast Pol δ revealed that whereas strains containing the eight variants L612F, G, I, K, M, N, T, and V were viable, those with 11 other variants were non-viable [5]. This mutagenesis also revealed that the mutation rates of yeast strains with Pol δ variants at position 612 were higher than those of strains containing the wild-type enzyme (WT) [5, 6].

To prevent the mis-incorporation of ribonucleotides during DNA synthesis, Pols have a steric gate configuration that results in a steric clash between the 2′-hydroxyl group of an incoming ribonucleotide and a bulky active-site residue [7]. Recent study shows that an amino acid residue in the finger domain senses ribonucleotides by steric hindrance [8]. The steric gate of *S. cerevisiae* Pol δ is Tyr613 and that of human is Tyr607 (Fig. 1). However, Pols incorporate a certain percentage of ribonucleotides irrespective of the presence of the steric gate, and consequently, ribonucleotides can become embedded within genomic DNA [9, 10]. The rates at which ribonucleotides are incorporated by Pol δ during DNA synthesis are estimated to be 2×10^{-4} [11], whereas when ribonucleotides are abundant, the rates can increase up to 2×10^{-3} [12]. In the case of human Pol δ, the rate at which the L606M variant (corresponding to the yeast Pol δ L612M variant) incorporates ribonucleotides has been demonstrated to be 7-fold higher than that of the WT [12], thus tending to indicate that the Leu606 residue is important not only for preventing the mis-incorporation of deoxyribonucleotides but also that of ribonucleotides. However, there is currently limited information available regarding the effects of substituting Leu606 with other amino acids. In this study, to analyze in detail how Leu606 mutations affect the activity of Pol δ, we prepared eight single-site human Pol δ variants (L606F, G, I, K, M, N, T, and V),

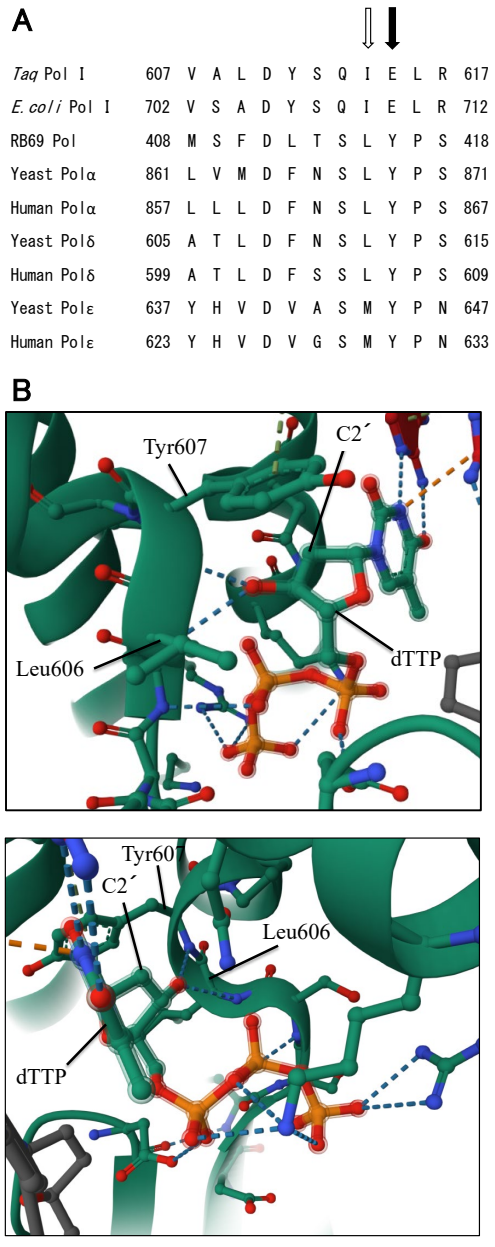


Fig. 1. Structure of human Pol δ. (A) Amino acid alignment of motif A residues. The open arrow indicates the position corresponding to Ler606 of human Pol δ. The closed arrow indicates the amino acid residue functioning as a steric filter. (B) Close-up view of the active site. The structure is based on PDB code 6TNY [3]. dTTP, Leu606, and Tyr607 are shown. The broken line indicates a hydrogen bond.

which were characterized with respect to their deoxyribonucleotide and ribonucleotide incorporation activities.

Materials and Methods

Expression and purification of Pol δ variants

Expression of WT was described previously [11]. Briefly, *E. coli* BL21-CodonPlus(DE3)-RP [F^-, *ompT*, $hsdS_B$ (r_B^- m_B^-) *gal dcm* (DE3)] (Stratagene) was used as a host. pET-POLD4/1, the pET-20b(+) plasmid harboring the genes encoding p12 and p125, and pGBM-POLD2/3, the pGBM2 plasmid harboring the genes encoding p50 and p66, were used for the expression of WT (Fig. 2). For the expression of p125 variants, pET-POLD4/1 harboring the genes encoding the wild-type p12 and p125 variants were constructed by site-directed mutagenesis using the pET-POLD4/1 as a template and the oligonucleotides (Table 1).

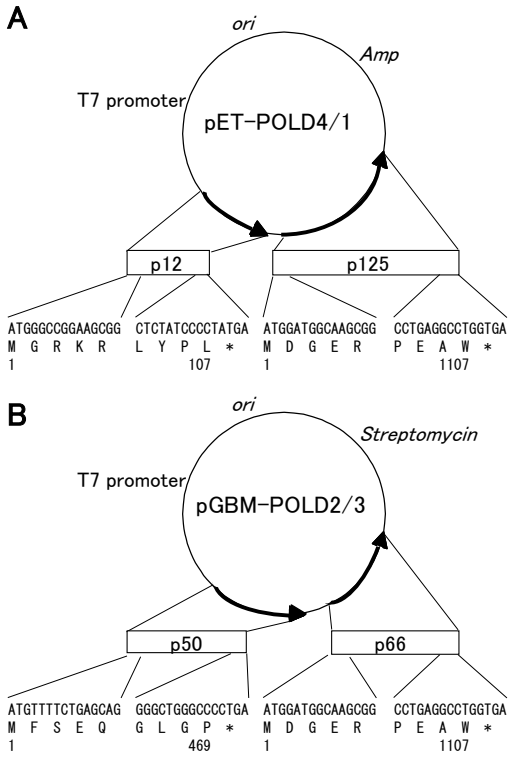


Fig. 2. Structures of expression plasmid. (A) pET-POLD4/1. (B) pGBM-POLD2/3.

Table 1. Oligonucleotide.

Primers	Sequences (5'-3')
pL606I_F	CCTGGACTTCTCCTCGATTTACCCGT CCATCATGA
pL606I_R	TCATGATGGACGGGTAAATCGAGGA GAAGTCCAGG
pL606V_F	CCTGGACTTCTCCTCGGTGTACCCG TCCATCATGA
pL606V_R	TCATGATGGACGGGTACACCGAGGA GAAGTCCAGG
pL606T_F	CCTGGACTTCTCCTCGACGTACCCG TCCATCATGA
pL606T_R	TCATGATGGACGGGTACGTCGAGGA GAAGTCCAGG
pL606F_F	CCTGGACTTCTCCTCGTTTTACCCGT CCATCATGA
pL606F_R	TCATGATGGACGGGTAAAACGAGGA GAAGTCCAGG
pL606M_F	CCTGGACTTCTCCTCGATGTACCCG TCCATCATGA
pL606M_R	TCATGATGGACGGGTACATCGAG GAGAAGTCCAGG
pL606K_F	CCTGGACTTCTCCTCGAAGTACCC GTCCATCATGA
pL606K_R	TCATGATGGACGGGTACTTCGAGG AGAAGTCCAGG
pL606G_F	CCTGGACTTCTCCTCGGGGTACCCG TCCATCATGA
pL606G_R	TCATGATGGACGGGTACCCCGAGGA GAAGTCCAGG
pL606N_F	CCTGGACTTCTCCTCGAATTACCCGT CCATCATGA
pL606N_R	TCATGATGGACGGGTAATTCGAGGA GAAGTCCAGG

WT and variants were prepared as described previously [13]. Briefly, the overnight culture of the BL21(DE3) transformants (10 mL) was carried out in a 10-cm LB broth plate containing 50 μg/mL ampicillin, 20 μg/mL streptomycin, and 30 μg/mL chloramphenicol at 37ºC for 16 h. Then, the culture was added to 25 mL of Terrific Broth containing the same antibiotics described above, and growth was continued at 15ºC for 8 h. Then, the culture was added to 2,000 mL of Terrific Broth containing the same antibiotics described above in a 3-liter flask and incubated at 15°C under vigorous aeration by an air-pump. When OD_{600} reached 0.6, 830 μL of 0.5 M IPTG

was added, and growth was continued at 15ºC for 15 h. After centrifugation at 10,000 × *g* for 5 min, the cells were harvested, suspended with 2 mL of 50 mM HEPES-NaOH buffer (pH 7.5), 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 500 mM NaCl (buffer A) per 1 g cells. To the suspension, 1 mL of 4 mg/mL lysozyme, 100 mM spermidine, 0.25 mM PMSF in buffer A was added per 3 g cells and incubated on ice for 30 min, at 37°C for 1.5 min, and on ice for 30 min. After centrifugation at 20,000 × *g* at 4°C for 20 min, the supernatant was collected.

The supernatant was applied to a HiTrap chelating HP column (GE Healthcare, Buckinghamshire, UK) which had been treated with 0.1 M $NiSO_4$ and then equilibrated with 50 mM HEPES-NaOH buffer (pH 7.5), 10 mM 2-mercaptoethanol, 500 mM NaCl, 10% w/v glycerol (buffer B) containing 5 mM imidazole. After the wash with buffer B containing 5 mM imidazole, the bound Pol δ was eluted with a linear gradient of buffer B containing 5 to 100 mM imidazole. The fractions containing all four Pol δ subunits were mixed, diluted with 50 mM HEPES-NaOH buffer (pH 7.5), 10 mM 2-mercaptoethanol, 10% w/v glycerol (buffer C) containing 400 mM NaCl, and then applied to a HiTrap Heparin HP column (GE Healthcare) pre-equilibrated with buffer C containing 400 mM NaCl. After the wash with buffer C containing 400 mM NaCl, the bound Pol δ was eluted with a linear gradient of buffer C containing 400 to 800 mM NaCl. The purified enzyme solution was stored at −30°C before use. The protein concentration was determined using a BCA kit (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (BSA) as a standard.

SDS-PAGE and estimation of Pol δ concentration

Samples (4 μL) were mixed with the SDS-PAGE sample buffer (0.25 M tris-hydroxymethyl aminomethane (Tris)-HCl buffer (pH 6.8), 50% v/v glycerol, 10% w/v SDS, 5% v/v 2-mercaptoethanol, 0.05% w/v bromophenol blue) (20 μL) and were incubated at 100°C for 10 min. The solution (10 μL) was applied to 10% polyacrylamide gel with a constant current of 40 mA for 40 min. After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 7% acetic acid.

Following electrophoresis, the gel was dried and analyzed using a TyphoonFLA9500 laser scanner (GE Healthcare, Buckinghamshire, UK) in conjunction with the ImageQuantTL program (GE Healthcare). Purity was defined by the following term.

$$\frac{\text{(Intensities of the protein band corresponding to p125, p66, p50, and p12 of Pol δ)}}{\text{(Intensities of the protein band corresponding to all proteins)}}$$

Nucleotide incorporation and degradation assays

To assess nucleotide incorporation, we initially performed a kinase reaction, which was initiated by mixing 16.5 μL of water, 2.5 μL of T4 kinase buffer, 2 μL of 50 μM 20-nt DNA 5′-CACTGACTGTATGCTGAAGA-3′ (P20), 3 μL of [γ-^{32}P]ATP (370 MBq/mL; 111 Bq/pmol), and 1 μL of T4 polynucleotide kinase (Takara Bio, Kusatsu, Japan), followed by incubation at 37°C for 30 min. A P20/T34 hybrid was prepared by mixing 19 μL of water, 25 μL of 2 μM 5′-[^{32}P]-labeled P20, and 6 μL of 50 μM 34-nt DNA 5′-CAGACTCAGCAGCATCTTCAGCATACAGTCAGTG-3′ (T34) and incubating at 75°C for 4 min. The reaction (20 μL) was carried out with 150 nM P20/T34 in 40 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl, 3.75 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 200 μg/mL BSA, and 500 nM Pol δ in the presence or absence of dNTPs and rNTPs at 37°C for the indicated time. Having stopped the reaction by adding an equal volume of

the sample-loading buffer (10 mM EDTA, 90% v/v formamide, 1 mg/ml xylene cyanol FF, 1 mg/mL bromophenol blue) to the reaction solution, 1.5-μL samples were run on 12% denaturing polyacrylamide gels in 89 mM Tris-HCl buffer (pH 8.0), 89 mM boric acid, 2 mM EDTA at 65 W for 2 h. Following electrophoresis, the gel was dried and analyzed using a TyphoonFLA9500 laser scanner in conjunction with the ImageQuantTL program. The amount of each extended product was calculated by multiplying the amount of 5′-[^{32}P]-labeled P20 applied to the gel by the following term:

$$\frac{\text{(Intensities of the band corresponding to all extended products)}}{\text{(Intensity of the band corresponding to 5′-[}^{32}\text{P]-labeled P20 applied to the gel)}}$$

The total number of times of nucleotide incorporation in the reaction solution at time 0–t (N_t) was calculated by the following equation

$$N_t = 14 \times N_{14,t} + 13 \times N_{13,t} + 12 \times N_{12,t} + 11 \times N_{11,t} + 10 \times N_{10,t} + 9 \times N_{9,t} + 8 \times N_{8,t} + 7 \times N_{7,t} + 6 \times N_{6,t} + 5 \times N_5 + 4 \times N_{4,t} + 3 \times N_{3,t} + 2 \times N_{2,t} + 1 \times N_{1,t}$$

where $N_{i,t}$ is the amount (mol) of i-nt extended band in the reaction solution at time t. The nucleotide incorporation was calculated according to the time-course of the total number of times of nucleotide incorporation in the reaction solution.

rGTP incorporation assay

A P20/T34C hybrid was prepared by mixing 19 μL of water, 25 μL of 2 μM P20, and 6 μL of 50 μM 34-nt DNA 5′-CCCCCCCCCCCCCCTCTTC AGCATACAGTCAGTG-3′ (T34C) and incubating at 75°C for 4 min. The reaction (20 μL) was carried out with 200 nM P20/T34C in 40 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl, 3.75 mM $MgCl_2$, 1 mM DTT, 200 μg/mL BSA, and 1 μM Pol δ in the presence of 5.2 μM dGTP, 470 μM [α-^{32}P]rGTP, at 37°C for 2 h. The reaction was stopped by the addition of an equal volume of the sample-loading buffer, followed by 12% polyacrylamide gel electrophoresis as described in the previous section.

Results and Discussion

Location of Leu606 in the active site of human Pol δ

Figure 1A shows the Motif A sequence (DXXXLYPS) of different Pol molecules. The Leu606 amino acid of human Pol δ is a conserved residue among eukaryote Pols. Figure 1B shows a close-up view of the active site. The close proximity of the side chain of Tyr607 to the C2′ of the sugar moiety of dTTP tends to indicate that if rNTP is bound instead of dTTP, steric hindrance may occur between the 2′-hydroxyl group of ribose and the side chain of Tyr607. Leu606 is adjacent to Tyr607. The N atoms of Leu606 and Tyr607 form a hydrogen bond with the 3′-hydroxyl group of ribose. We thus hypothesize that not only Tyr607 but also Leu606 is involved in distinguishing between dNTPs and rNTPs.

Production of recombinant human Pol δ Leu606 variants

We expressed in *E coli* and purified WT human Pol δ and eight Leu606 variants. Figure 3 shows the SDS-PAGE patterns of the Pol δ preparations. The profiles of WT Pol δ and all variants were characterized by four bands with molecular masses of 125, 66, 50, and 12 kDa corresponding to the p125, p66, p50, and p12 subunits, respectively. However, the presence of several other bands indicated that the preparation had not been purified to homogeneity. We suspect that this might be attributable to the fact that p66 readily dissociates from the tetrameric Pol δ during purification, and thus indicates that the resulting monomeric p66 and trimeric

p125/p50/p12 need to be separated from the tetrameric Pol δ. Table 2 shows the purity of Pol δ in the preparations as assessed by our densitometric analysis of the SDS-PAGE gels. Compared with the purities of the WT (95%) and L606G, K, N, T, and V (85%–95%), we obtained relatively lower purities for L606F (51%), L606I (54%), and L606M (67%), which could be associated with structural damage incurred following the substitution of Leu606 with Phe, Ile, or Met.

A major impediment in this study was the lack of a purified recombinant human Pol δ preparation. During the 1990s, the human Pol δ gene was identified and cloned [14–16], and in 2007, recombinant Pol δ was expressed in *E. coli* [13]. Thereafter, recombinant human Pol δ was expressed in baculovirus [17, 18], which was subsequently used for structural analysis [3]. Our findings in the present study would tend to indicate that Pol δ undergoes a certain post-translational modification, which does not occur in *E. coli*, and that this modification might be required for the acquisition of stability.

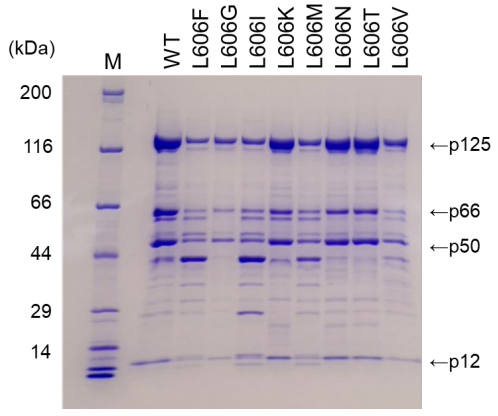


Fig. 3. SDS-PAGE of Pol δ variants under reducing conditions. Coomassie Brilliant Blue-stained 12.5% SDS-polyacrylamide gel showing marker proteins (Protein Markers for SDS-PAGE, Nacalai Tesque) and purified enzyme preparations of WT and variants.

Table 2. Purity of the Pol δ variant preparations

	Purity (%)	Pol δ concentration (μM)[1]
WT	85	14
L606F	51	6.9
L606G	95	10
L606I	54	8.3
L606K	89	13
L606M	67	10
L606N	95	13
L606T	95	15
L606V	95	7.3

[1]Concentration of tetrameric Pol δ

Effects of the mutations at position 606 of Pol δ on its nucleotide incorporation and degradation activities

To examine the nucleotide incorporation activities of Pol δ variants, we established a primer extension system including a [^{32}P]-labeled P20. Figure 4A shows the sequence of the template-primer T34/P20 and Figure 4B shows a time-course analysis of the reaction in the presence of dNTPs, and the absence of rNTPs. Fully extended products of 34 nucleotides (nt) were obtained with all enzymes. Relative to that of the WT Pol δ, we recorded dNTP incorporation rates of between 40% and 180% for the eight variants (Table 3), which tends to indicate that the mutation at position 606 does not have any pronounced effects on dNTP incorporation activity. Figure 4C shows the time-course analysis of the reaction in the presence of both dNTPs and rNTPs. The assessed concentrations of each rNTP were based on their previously reported concentrations in cells [6, 8, 10]. Notably, the results obtained were similar to those obtained in the absence of rNTPs (Fig. 4B), with values for the dNTP incorporation rates of the eight variants ranging from 35% to 140% of that of the WT (Table 3). These results would accordingly tend to indicate that the presence of rNTPs had little, if any effect on the dNTP incorporation of Pol δ.

Using the same system, we subsequently examined the ribonucleotide incorporation activity of Pol δ variants. Figure 4D shows the time-course analysis of the reaction in the presence of rNTPs and the absence of dNTPs. For all enzyme variants, we obtained 1–4 nt extended and 1–5 nt degraded products, thus indicating that compared with the dNTP incorporation activity, rNTP incorporation was markedly low. Relative to that of the WT Pol δ, we recorded rNTP incorporation rates of between 6.1% and 690% for the eight variants (Table 3), Table 3 also presents values for the ratios of the rNTP incorporation activity (activity B) to the dNTP incorporation activity (activity A). Compared with a ratio of the WT, which was set to 1, we obtained values of between 16% and 820% for the eight variants, among which, the highest values were obtained for L606G (820%) followed by those of L606F (670%) and L606M (570%), and the lowest value was obtained for L606I (16%).
We examined the deoxyribonucleotide degradation activity of the Pol δ variants. Figure 4E shows the time-course of the reaction in the absence of dNTPs and rNTPs. For all enzyme variants, we obtained 1–15 nt degraded products,

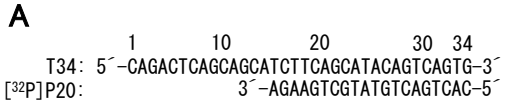
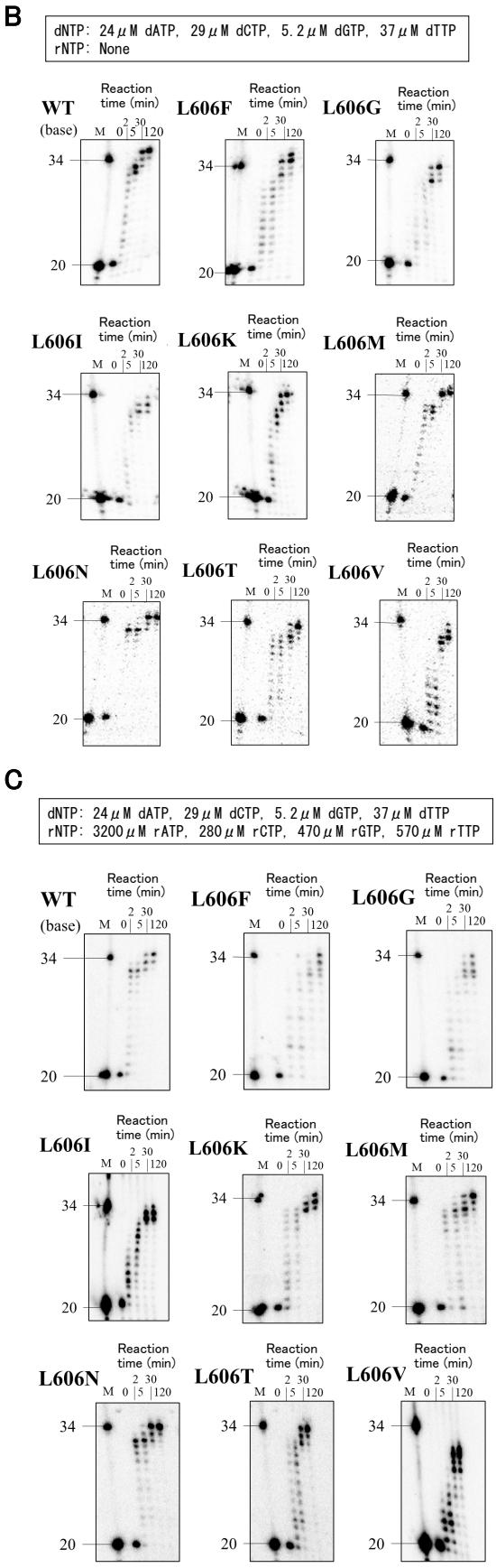


Fig. 4. Nucleotide incorporation and degradation activities of Pol δ variants. (A) Sequence of P20/T34 used as a template-primer. (B–E) Time-course of the reaction products. The reaction was carried out in 40 mM Tris-HCl buffer (pH 7.8), 500 nM Pol δ, 150 nM P20/T34, 3.75 mM $MgSO_4$, 1 mM DTT, 200 μg/mL BSA in the presence (B, C) or absence (D, E) of dNTP and in the presence (C, D) or absence (B, E) of rNTP, at 37ºC for 0, 2, 5 30, and 120 min. Then, the reaction solutions were applied to 12% w/v denaturing polyacrylamide gel. The results of image scanning are shown.



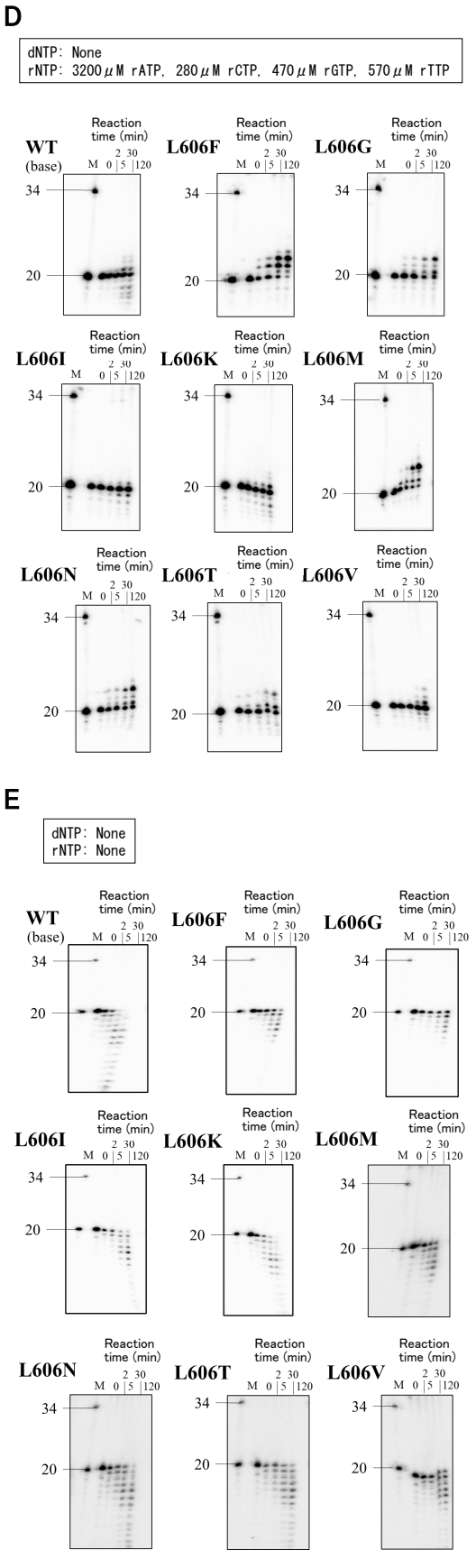


Table 3. Nucleotide incorporation rate of Pol δ variants

Substrate	Nucleotide incorporation rate [nmol/(L min)]			B/A
	dNTP (A)	dNTP and rNTP	rNTP (B)	
WT	480 (1.0)	500 (1.0)	0.428 (1.0)	8.9×10^{-4} (1.0)
L606F	350 (0.73)	290 (0.58)	2.01 (4.9)	6.0×10^{-3} (6.7)
L606G	250 (0.52)	210 (0.42)	1.82 (4.3)	7.3×10^{-3} (8.2)
L606I	190 (0.40)	230 (0.47)	0.026 (0.061)	1.4×10^{-4} (0.16)
L606K	350 (0.73)	290 (0.57)	0.249 (0.58)	7.1×10^{-4} (0.80)
L606M	590 (1.2)	510 (1.0)	2.97 (6.9)	6.0×10^{-3} (5.7)
L606N	860 (1.8)	690 (1.4)	1.87 (4.4)	2.1×10^{-3} (2.4)
L606T	450 (0.95)	450 (0.90)	1.03 (2.4)	2.3×10^{-3} (2.6)
L606V	210 (0.43)	170 (0.35)	0.164 (0.38)	7.8×10^{-4} (0.88)

Value in parenthesis indicates relative value compared to WT.

Table 4. Deoxyribonucleotide degradation activity of Pol δ variants

	Degradation rate [nmol/(L min)]
WT	20 (1.0)
L606F	6.8 (0.34)
L606G	4.0 (0.20)
L606I	12 (0.60)
L606K	16 (0.80)
L606M	6.5 (0.33)
L606N	13 (0.65)
L606T	12 (0.60)
L606V	8.7 (0.44)

Value in parenthesis indicates relative value compared to WT.

with deoxyribonucleotide degradation rates of the variants ranging from 20% to 80% that of the WT (Table 4), again indicating that mutation at position 606 has no appreciable effect on deoxyribonucleotide degradation activity.

To further examine the ribonucleotide incorporation activity, we established a new system based on the template-primer T34C/P20, the sequence of which is shown in Figure 5A. The T34C template contains a 14-nt polyC sequence at the 5′ terminus. In this T34/P20-based system, ^{32}P was labeled with rGTP instead of P20. Figure 5B shows the reaction in the presence of dGTP and rGTP and the absence of dATP, dCTP, dTTP, rATP, rCTP, and rUTP. For all enzyme variants, we obtained 8–14 nt extended products of 28–34 nt. Notably, the intensities of the 28–34 nt bands obtained for the WT and L606I and L606K variants were markedly weaker than those obtained for the other six variants. The rGTP incorporation activities of the other 6 variants were 110–820% of that of WT (Table 5). Among these latter variants, L606F (820%) was found to promote the highest activity of incorporation, followed by L606N (650%) and L606G (480%).

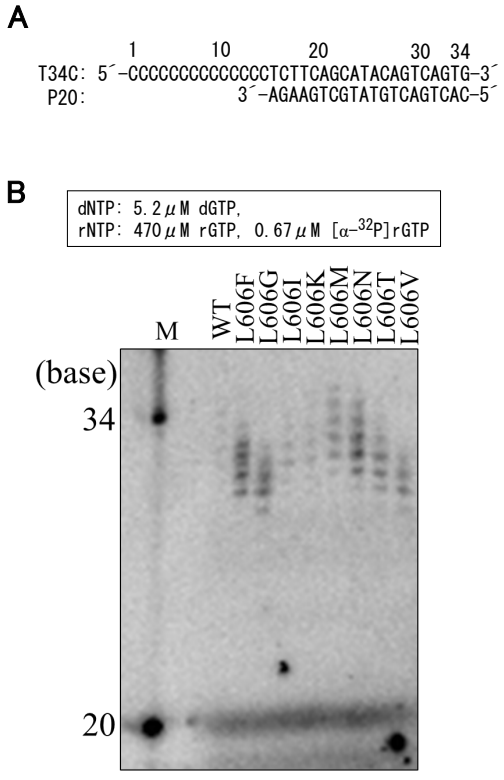


Fig. 5. rGTP incorporation activity of Pol δ variants. (A) Sequence of P20/T34C used as a template-primer. (B) Reaction products. The reaction was carried out in 40 mM Tris-HCl buffer (pH 7.8), 500 nM Pol δ, 150 nM P20/T34C, 3.75 mM $MgSO_4$, 100 mM NaCl, 1 mM DTT, 200 μg/mL BSA, 5.2 μM dGTP, 470 μM [α-^{32}P]rGTP, at 37ºC for 2 h. Then, the reaction solutions were applied to 12% w/v denaturing polyacrylamide gel. The result of image scanning is shown.

Table 5. rGTP incorporation activity of Pol δ variants

	Incorporation rate [nmol/(L min)]
WT	1.7 (1.0)
L606F	14 (8.2)
L606G	8.1 (4.8)
L606I	1.6 (0.9)
L606K	1.7 (1.0)
L606M	4.6 (2.4)
L606N	11 (6.5)
L606T	3.9 (2.3)
L606V	1.8 (1.1)

Value in parenthesis indicates relative value compared to WT

A majority of Pols contains a Glu or Tyr residue within Motif A, which functions as a steric gate (or a steric filter) to minimize rNTP incorporation [7, 19]. Our findings in this study indicate that Leu606 plays an important role in discriminating between deoxyribonucleotides and ribonucleotides. The mutation of Leu606 to Phe or Gly was found to enhance the rate of rNTP incorporation (Figs. 4D and 5, Tables 3 and 5), which we speculate could be attributable to the fact that the mutation shifts the position of Tyr607, thereby reducing steric hindrance with the 2′-hydroxyl group of ribose. An alternative

possibility is that the mutation influences the hydrogen bonding of the N atom of the amino acid residue at position 606 to the 3′-hydroxyl group of ribose, thus altering the position of the ribose.

In yeast pol ε, the steric gate is Tyr645. It was reported that the ribonucleotide incorporation activity of M644L variant was 3-fold lower and that of M644G variant was 11-fold higher than that of the WT pol ε [20]. In contrast to pol δ (this study) and pol ε [20], the mutation of Phe272 human pol β, an amino acid residue adjacent to the steric gate residue Tyr271, to Ala little affected discrimination between deoxyribonucleotides and ribonucleotides [21]. Therefore, the effects of the amino acid residues adjacent to the steric gate residue on the sugar discrimination vary depending on Pol species.

In conclusion, our findings in this study indicate that the Leu606 residue of human Pol δ plays an important role in the distinction of incoming dNTPs and rNTPs. We accordingly speculate that this amino acid is widely conserved in eukaryotic DNA polymerases to minimize the incorporation of rNTPs.

Acknowledgements

We sincerely appreciate Dr. Yuji Masuda of Research Institute of Environmental Medicine, Nagoya University for his extensive support of this work.

References

[1] Nick McElhinny, S.A., Gordenin, D.A., Stith, C.M., Burgers, P.M., and Kunkel, T.A. (2008) *Mol. Cell.* **30**, 137–144.

[2] Prindle, M.J. and Loeb, L.A. (2012) *Environ. Mol. Mutagen.* **53**, 666–682.

[3] Lancey, C., Tehseen, M., Raducanu, V.S., Rashid, F., Merino, N., Ragan, T.J., Savva, C.G., Zaher, M.S., Shirbini, A, Blanco, F.J., Hamdan, S.M., and De Biasio, A. (2020) *Nat. Commun.* **11**, 1109.

[4] Jain, R., Hammel, M., Johnson, R.E., Prakash, L., Prakash, S., and Aggarwal, A.K. (2009) *J. Mol. Biol.* **394**, 377–382.

[5] Venkatesan, R.N., Hsu, J.J., Lawrence, N.A., Preston, B.D., and Loeb, L.A. (2006) *J. Biol. Chem.* **281**, 4486–4894.

[6] Nick McElhinny, S.A., Stith, C.M., Burgers, P.M., and Kunkel, T.A. (2007) *J. Biol. Chem.* **282**, 2324–2332.

[7] Brown, J.A. and Suo, Z. (2011) *Biochemistry* **50**, 1135–1142.

[8] Parkash, V., Kulkarni, Y., Bylund, G.O., Osterman, P., Kamerlin, S.C.L., and Johansson, E. (2023) *Nucleic Acids Res.* **51**, 11225–11238.

[9] Williams, J.S. and Kunkel, T.A. (2014) *DNA repair* **19**, 27–37.

[10] Cerritelli, S.M. and Crouch, R.J. (2016) *Trends Biochem. Sci.* **41**, 434–445.

[11] Williams, J.S. and Kunkel, T.A. (2022) *Annu. Rev. Biochem.* **91**, 133–155.

[12] Clausen, A.R., Zhang, S., Burgers, P.M., Lee, M.Y., and Kunkel, T.A. (2013) *DNA Repair* **12**, 121–127.

[13] Masuda, Y., Suzuki, M., Piao, J., Gu, Y., Tsurimoto, T., and Kamiya, K. (2007) *Nucleic Acids Res.* **35**, 6904–6916.

[14] Chung, D.W., Zhang, J.A., Tan, C.K., Davie, E.W., So, A.G., and Downey, K.M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11197–11201.

[15] Zhang, J., Tan, C.K., McMullen, B., Downey, K.M., and So, A.G. (1995) *Genomics* **29**, 179–186.

[16] Liu, L., Mo, J., Rodriguez-Belmonte, E.M., and Lee, M.Y. (2000) *J. Biol. Chem.* **275**, 18739–18744.

[17] Zhou, Y., Chen, H., Li, X., Wang, Y., Chen, K., Zhang, S., Meng, X., Lee, E.Y., and Lee, M.Y. (2011) *PLoS One* **6**, e22224.

[18] Zhou, Y., Meng, X., Zhang, S., Lee, E.Y., and Lee, M.Y. (2012) *PLoS One* **7**, e39156.
[19] Johnson, M.K, Kottur, J., and Nair, D.T. (2019) *Nucleic Acids Res.* **47**, 10693–10705.
[20] Nick McElhinny, S.A., Kumar, D., Clark, A.B., Watt, D.L., Watts, B.E., Lundström, E.B., Johansson, E., Chabes, A., and Kunkel, T.A. (2010) *Nat. Chem. Biol.* **6**, 774–781.
[21] Cavanaugh, N.A., Beard, W.A., Batra, V.K., Perera, L., Pedersen, L.G., and Wilson, S.H. (2011) *J. Biol. Chem.* **286**, 31650–31660.

Funding
This study was supported in part by Grants-in-Aid for Scientific Research (no. 22H03332 and 23K24590 for T.T. and K.Y.) from the Japan Society for the Promotion of Science, Kyodai Foundation for K.Y., and Koyanagi Foundation for K.Y.

Communicated by Sumioi Ishijima