Biochem. Biophys. Res. Commun.

Kinetic analysis of reverse transcriptase activity of bacterial family A DNA polymerases

Kiyoshi Yasukawa [a,*], Atsushi Konishi [a], Mayu Shinomura [a], Eriko Nagaoka [b], Shinsuke Fujiwara [b,c]

[a]*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*
[b]*Department of Bioscience, Graduate School of Science and Technology, Kwansei-Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan*
[c]*Research Center for Environmental Bioscience, Graduate School of Science and Technology, Kwansei-Gakuin University, 2-1 Gakuen, Sanda, Hyogo 669-1337, Japan*

***** Corresponding author. Fax: +81-75-753-6265.
E-mail address: yasukawa@kais.kyoto-u.ac.jp (K. Yasukawa)

Abbreviations: K4pol, family A DNA polymerase from *Thermotoga petrophila* strain K4; M1pol, family A DNA polymerase from *Thermus thermophilus* strain M1; MMLV, Moloney murine leukemia virus; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Some bacterial thermostable, wild-type or genetically engineered family A DNA polymerases have reverse transcriptase activity. However, difference in reverse transcriptase activities of family A DNA polymerases and retroviral reverse transcriptases (RTs) is unclear. In this study, comparative kinetic analysis was performed for the reverse transcriptase activities of the wild-type enzyme of family A DNA polymerase ($M1pol_{WT}$) from *Thermus thermophilus* M1 and the variant enzyme of family A DNA polymerase ($K4pol_{L329A}$), in which the mutation of Leu329→Ala is undertaken, from *Thermotoga petrophila* K4. In the incorporation of dTTP into poly(rA)-p$(dT)_{45}$, the reaction rates of $K4pol_{L329A}$ and $M1pol_{WT}$ exhibited a saturated profile of the Michaelis-Menten kinetics for dTTP concentrations but a substrate inhibition profile for poly(rA)-p$(dT)_{45}$ concentrations. In contrast, the reaction rates of Moloney murine leukemia virus (MMLV) RT exhibited saturated profiles for both dTTP and poly(rA)-p$(dT)_{45}$ concentrations. This suggests that high concentrations of DNA-primed RNA template decrease the efficiency of cDNA synthesis with bacterial family A DNA polymerases.

Keywords: family A DNA polymerase; Moloney murine leukemia virus; reverse transcriptase; template-primer; *Thermotoga petrophila*; *Thermus thermophilus*.

1. Introduction

In cDNA synthesis, an elevated reaction temperature is desirable because it reduces RNA secondary structure and nonspecific binding of the primer. Reverse transcriptases (RTs) from Moloney murine leukemia virus (MMLV) and Avian myeloblastosis virus (AMV) have been used in cDNA synthesis. MMLV RT and AMV RT are not thermostable. To realize the cDNA synthesis reaction at high temperature, their thermal stabilities were improved by eliminating the RNase H activity [1], random mutation [2], and site-directed mutagenesis [3,4]. cDNA synthesis with thermostable RTs are carried out at around 50°C.

Some bacterial or archaeal, wild-type or genetically engineered DNA polymerases have RNA-dependent DNA polymerase (reverse transcriptase) activity [5–8]. One of the advantages of such DNA polymerases over retroviral RTs are that the DNA polymerases are stable even at around 90°C, enabling direct RT-PCR in a single tube [6–8]. Figure 1A shows domain structure of DNA polymerases. The proofreading 3’-5’ exonuclease domain confers high fidelity. Bacterial family A DNA polymerases, such as from *Thermus thermophilus* and *Thermus aquaticus*, do not have the 3’-5’ exonuclease domain and exhibit reverse transcriptase activity. Bacterial family A DNA polymerases, such as from *Thermotoga petrophila* and *Thermotoga maritime* have the 3’-5’ exonuclease domain and do not exhibit reverse transcriptase activity. Archaeal family B DNA polymerases, such as *Thermococcus kodakarensis* and *Pyrococcus furiosus*, have the 3’-5’ exonuclease domain and exhibit reverse transcriptase activity. However, family B DNA polymerases are not suitable in cDNA synthesis because the reaction stops when a uracil-containing template is incorporated [9].

Figure 1B shows the modeled structure of Family A DNA polymerase (K4pol) from *Thermotoga petrophila* K4. Nine residues (Tyr326, Leu329, Gln384, Lys387, Phe388, Met408, Asn422, Tyr438, and Phe451) are predicted to be involved in DNA/RNA distinction. Figure 1C and D show that the steric hindrance with the 2'-hydroxyl group of ribose in K4pol might be removed by the mutation of Leu329→Ala. Previous site-directed mutagenesis study revealed that six variants (Y326A, L329A, Q384A, F388A, M408A, and Y438A) exhibited the reverse transcriptase activity [10]. However, the threshold PCR cycle numbers needed to reach the constant fluorescent intensity in the direct RT-PCR with these variants were higher by 5–10 cycles than that in the conventional RT-PCR using MMLV RT in cDNA synthesis and family B DNA polymerase from hyperthermophilic archaeon *Thermococcus kodakaraensis* in the subsequent PCR [10]. This indicates that cDNA synthesis with these variants is less efficient than that with MMLV RT. In this study, to address this issue, we made kinetic analysis of the reverse transcriptase activities of the wild-type enzyme of family A DNA polymerase (M1pol$_{WT}$) from *T. thermophilus* M1 and the variant enzyme of K4pol (K4pol$_{L329A}$) in which the mutation of Leu329→Ala is undertaken. The results have shown that high concentrations of DNA-primed RNA template decrease their reverse transcriptase activities.

2. Materials and methods

2.1. Expression and purification of recombinant form of K4pol$_{L329A}$

K4pol$_{L329A}$ was prepared as described previously [10]. Briefly, *E. coli* strain

BL21(DE3) codon plus [*F*$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*$^+$ *Tet*^{r} *galλ* (DE3) *endA Hte* [*argU proL Cam*^{r}]] (Agilent Technologies) was transformed with the pET-21a plasmid harboring the K4pol$_{L329A}$ gene. The K4pol$_{L329A}$ was expressed in the soluble fraction of the transformants. After the heat treatment of the soluble fraction at 85°C for 30 min, the enzyme was purified. The enzyme concentration was determined by the method of Bradford [11] using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan) with bovine serum albumin (Nacalai Tesque) as standard.

2.2. Expression and purification of recombinant form of MMLV RT

Recombinant MMLV RT was prepared as described previously [3]. Briefly, *E. coli* strain BL21(DE3) was transformed with the pET-22b plasmid harboring the gene encoding the C-terminally His$_6$-tagged MMLV RT. The MMLV RT was expressed in the soluble fraction of the transformants, from which active enzyme was purified.

2.3. Expression and purification of recombinant form of M1pol$_{WT}$

T. thermophilus strain M1 was isolated from a hot spring (73°C) at Kagoshima (Japan) and identified as *T. thermophilus* based on 16S rDNA sequence. Amino acid sequence of DNA polymerase and nucleotide sequence of 16S rDNA from M1 show the highest identity to those of *T. thermophilus* HB8 (99% and 98%, respectively). DNA polymerase gene (*M1pol*) of M1 was amplified by PCR using oligonucleotide primers (5’-AAAAAAACATATGGAGGCGATGCTTCCGCT-3’ and 5’-AAGAATTCTAACCCTTGGCGGAAAGCCAGT-3’) and M1 chromosomal DNA as a template. PCR was performed using 1 unit of KOD Plus polymerase (Toyobo, Osaka, Japan) under following condition (3 min at 94°C, followed by 30 cycle of 15 s at 94°C, 30 s at 50°C, 3 min at 68°C). The amplified DNA was digested with restriction enzymes *Nde*I and *Eco*RI, and the obtained DNA was inserted into the pET-28a digested with the same enzymes. The resultant plasmid was designed as pET-M1pol. BL21(DE3) codon plus was transformed with pET-M1pol. The transformants were aerobically grown at 30°C in 300 ml of LB medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl; adjusted to pH 7.3 with NaOH) containing 20 μg/ml kanamycin and 30 μg/ml chloramphenicol to OD_{660} of 0.4. IPTG was then added to the final concentration of 1 mM. The cells were further incubated at 30°C for 4 h and were harvested by centrifugation (8,000 × g, 10 min). The cells were resuspended in 7 ml of buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, pH 7.9) and disrupted by sonication. The lysate was then separated by centrifugation (8,000 × g, 10 min). The obtained supernatant was heated at 75°C for 30 min and centrifuged again (8,000 × g, 10 min). The supernatant was applied to a Ni^{2+}-TED Sepharose column equilibrated with buffer A. Next, the column was washed several times with the same buffer. The N-terminally His_6-tagged *T. thermophilus* M1 DNA polymerase ($M1pol_{WT}$) was eluted with buffer B (20 mM Tris-HCl, 500 mM NaCl, 100 mM imidazole, 0.1% Triton X-100, pH 7.9). The active fractions were pooled and dialyzed against a buffer containing 20 mM Tris-HCl, 500 mM NaCl, pH 7.9.

2.4. Nucleotide sequence accession number

The nucleotide sequences of DNA polymerase and 16S rDNA from *T.*

thermophilus M1 reported in this study have been submitted to the DDBJ nucleotide sequence database under the accession numbers AB744210 and AB744654, respectively.

2.5. DNA-dependent DNA polymerase assay

DNA-dependent DNA polymerase activity to incorporate dNTP into gapped M13mp2 DNA was examined as described previously [12,13]. Briefly, the 6,789-bp fragment was obtained by the digestion of the 7,196-bp double-strand circular M13mp2 DNA with *Pvu*II and the precipitation by 6% w/v polyethylene glycol-8000, 550 mM NaCl. The gapped DNA was prepared by the annealing of the 7,196-base single-strand circular M13mp2 DNA (140 μg in 10 mM Tris-HCl, pH 8.0) with the heat-denatured 6,789-bp fragment (150 μg in 10 mM Tris-HCl, pH 8.0). The reaction (15 μL) was carried out with 670 nM $K4pol_{L329A}$ and 57 μg of gapped DNA in 50 mM bicine-KOH, 1 mM $Mn(CH_3COO)_2$, 115 mM CH_3COOK, 8% glycerol at pH 8.2 (buffer B) at 70ºC. The reaction products (13 μL) were applied to 0.8% agarose gel, followed by staining with ethidium bromide (1 μg/ml).

2.6. Reverse transcriptase assay

Reverse transcriptase activity to incorporate dTTP into poly(rA)-$p(dT)_{45}$ was examined, based on the previous method using poly(rA)-$p(dT)_{15}$ [3,4]. Briefly, 2.0 mM poly(rA)-$p(dT)_{45}$ (T/P) (the concentration is expressed as that of $p(dT)_{45}$) was prepared by mixing 300 μL of 2.5 mM $p(dT)_{45}$ (Sigma-Aldrich Japan, Ishikari, Japan) and 75 μL of 100 mM poly(rA) (the concentration is expressed as that of rA) (GE Healthcare, Buckinghamshire, UK) followed by the incubation at 70ºC for 15 min and at room temperature for 30 min. [^{3}H]dTTP (1.85 Bq/pmol) was prepared by mixing 860 μL of water, 100 μL of [^{3}H]dTTP (1.52 TBq/mmol) (GE Healthcare), and 40 μL of 100 mM dTTP (GE Healthcare). The reaction was carried out with various concentrations of dTTP and T/P in buffer B at 50ºC for $K4pol_{L329A}$ and $M1pol_{WT}$, or in 25 mM Tris-HCl, 50 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, pH 8.3 at 37ºC for MMLV RT.

3. Results

3.1. DNA- and RNA-dependent DNA polymerase activities of $K4pol_{L329A}$

Recombinant $K4pol_{L329A}$, MMLV RT, and $M1pol_{WT}$ were expressed in *E. coli* and purified from the soluble fractions of the cells. Figure 2A shows the result of SDS-PAGE analysis of purified enzyme preparations. The preparations of $K4pol_{L329A}$, MMLV RT, and $M1pol_{WT}$ yielded a single band with a molecular mass of 101, 75, and 90 kDa, respectively. Figure 2B shows the result of DNA-dependent DNA polymerase assay. In this assay, the 407-base gap of the 7,196-bp double-strand circular M13mp2 DNA is filled by the reaction with DNA polymerase. The reaction product with $K4pol_{L329A}$ exhibited the band corresponding to the gap-filled DNA, but did not exhibit the one corresponding to the gapped DNA. Figure 2C shows the result of reverse transcriptase assay. The amounts of [^{3}H]dTTP incorporated increased with increasing time in the reaction with $K4pol_{L329A}$, $M1pol_{WT}$, and MMLV RT. But they did not increase with increasing time in the reaction with the wild-type K4pol (K4pol$_{WT}$). This indicates that the mutation of Leu329→Ala does not abolish the DNA-dependent DNA polymerase activity and creates reverse transcriptase activity in K4pol.

3.2. Inhibitory effect of T/P toward the reverse transcriptase activities of K4pol$_{L329A}$ and M1pol$_{WT}$

Figure 3A shows the reaction rates of K4pol$_{L329A}$, M1pol$_{WT}$, and MMLV RT for reverse transcriptase reaction at various dTTP concentrations. A saturated profile of the Michaelis-Menten kinetics were obtained, and the K_m and k_{cat} values were determined to be 280 ± 80 μM and 1.7 ± 0.3 s^{-1}, respectively, for K4pol$_{L329A}$, 230 ± 70 μM and 1.0 ± 0.2 s^{-1}, respectively, for M1pol$_{WT}$, and 250 ± 60 μM and 35 ± 6 s^{-1}, respectively, for MMLV RT. This indicates that the k_{cat} values of K4pol$_{L329A}$ and M1pol$_{WT}$ at 50ºC were 5 and 3 %, respectively, of that of MMLV RT at 37ºC while their K_m values were similar.

Figure 3B shows the reaction rates of K4pol$_{L329A}$, M1pol$_{WT}$, and MMLV RT for reverse transcriptase reaction at various T/P concentrations. A substrate inhibition profile was obtained for K4pol$_{L329A}$ and M1pol$_{WT}$: the reaction rates reached the maximum at 10 μM T/P, decreased with increasing T/P concentration (10–100 μM), and reached 50% of the maximum at 100 μM T/P. On the other hand, a saturated profile of the Michaelis-Menten kinetics was obtained for MMLV RT: the K_m and k_{cat} values were determined to be 5.6 ± 0.8 μM and 29 ± 6 s^{-1}, respectively.

3.3. Temperature dependence of the reverse transcriptase activity of K4pol$_{L329A}$

Considering that RNA is stable up to around 65ºC, the optimal reaction temperature for cDNA synthesis reaction with thermostable DNA polymerases is thought to be around 65ºC. However, poly(rA)-p(dT)$_{45}$ dissociates at 65ºC. To estimate the reverse transcriptase activity of K4pol$_{L329A}$ at 65ºC, the reaction rates of K4pol$_{L329A}$ to incorporate dTTP into T/P were measured at various temperatures (Fig. 4). They increased with increasing temperature from 37 to 52ºC. Inset shows Arrhenius plot on the assumption that the observed reaction rates correspond to V_{max}. Linear relationship was held between the natural logarithm of V_{max} and $1/T$. In this plot, $\ln(V_{max})$ value of 6.4 (the V_{max} value of 600 nM s^{-1}) was estimated at 65ºC, and the k_{cat} value at 65ºC was calculated to be 12 s^{-1}. Considering that the k_{cat} values of MMLV RT obtained in this study were 35 and 29 s^{-1}, the reverse transcriptase activity of K4pol$_{L329A}$ at around 65ºC might be comparable to that of MMLV RT at 37ºC.

4. Discussion

The mechanism of the template-primer-mediated inhibition of reverse transcriptase activity is unclear. Because K4pol$_{L329A}$ has the 3'-5' exonuclease domain but M1pol$_{WT}$ does not (Fig. 1), it is thought that the 3'-5' exonuclease domain is not critical for the inhibition. Bacterial family A DNA polymerases and retroviral RTs share a common structure: both groups comprise the fingers, palm, and thumb domains. Crystal structures of *E. coli* DNA polymerase [14], *T. aquaticus* DNA polymerase [15], human immunodeficiency virus type 1 (HIV-1) RT [16], and MMLV RT [17] revealed that the palm domains are similar but the fingers and thumb domains are different between the two groups [18]: particular amino acid residues in α-helix of the fingers domain are involved in nucleotide binding in family A DNA polymerases while those in β-sheet of the fingers domain are involved in RTs; and the sites in the thumb domain which interact with the minor groove of the primer-template are different in the two groups. There might be a possibility that the binding of template-primer with particular sites in the fingers or thumb domain of family A DNA polymerase is involved in the inhibition.

The template-primer-mediated inhibition of reverse transcriptase activity suggests that high concentrations of DNA-primed RNA template decrease the efficiency of cDNA synthesis with bacterial family A DNA polymerases. We speculate that in RT-PCR, this inhibition affects the quantification, but does not much affect the detection of a target RNA. In clinical diagnosis, various nucleic acid tests are used for the detection of a target RNA from pathogens. Sauter and Marx generated *T. aquaticus* DNA polymerase variant, L322M/L459M/S515R/I638F/S739G/E773G/L789F, with increased reverse transcriptase activity by random mutation [19]. They showed that 0.1–5 ng of a target RNA can be quantified by the direct real-time RT-PCR with this variant enzyme in a single tube format [19]. This does not contradict our result because the concentration of T/P is as high as 100 μM (about 3 μg/μL) in this study. To achieve the efficient synthesis of cDNA using bacterial family A DNA polymerases with reverse transcriptase activity, optimization is required for the concentration of T/P.

How are the template-primer-mediated inhibition of reverse transcriptase activities of K4pol and M1pol removed? If particular region involved in the T/P-mediated inhibition is identified, site-directed mutagenesis might be effective. Mayanagi et al. reported the structure of the ternary complex of family B DNA polymerase from hyperthermophilic archaeon *Pyrococcus furiosus*, proliferating cell nuclear antigen

(PCNA), and DNA [20]. Shimada et al. reported that cold inducible RNA helicase from hyperthermophilic archaeon *Thermococcus kodakaraensis* is critical for its adaptation to cold temperature [21]. There is a possibility that molecules that interact with nucleic acids, such as sliding clump, a ring-shaped protein that slides on DNA, and RNA helicase, an enzyme that unwinds single-strand paired RNA, might be effective to remove the inhibition. Removal of the inhibition will open up the wide application of bacterial family A DNA polymerase in quantitative RT-PCR in a single tube format.

Acknowledgments

This study was supported in part (K.Y.) by Grants-in-Aid for Scientific Research (nos. 19580104 and 21580110) from the Japan Society for the Promotion of Science and Daiwa Securities Health Foundation.

References

[1] G.F. Gerard, R.J. Potter, M.D. Smith, K. Rosenthal, G. Dhariwal, J. Lee, D.K. Chatterjee, The role of template-primer in protection of reverse transcriptase from thermal inactivation, Nucleic Acids Res., 30 (2002) 3118–3129.

[2] B. Arezi, H. Hogrefe, Novel mutations in Moloney murine leukemia virus reverse transcriptase increase thermostability through tighter binding to template-primer, Nucleic Acids Res., 37 (2009) 473–481.

[3] K. Yasukawa, M. Mizuno, A. Konishi, K. Inouye, Increase in thermal stability of Moloney murine leukaemia virus reverse transcriptase by site-directed

mutagenesis, J. Biotechnol., 150 (2010) 299–306.

[4] A. Konishi, K. Yasukawa, K. Inouye, Improving the thermal stability of avian myeloblastosis virus reverse transcriptase α-subunit by site-directed mutagenesis, Biotechnol. Lett., 34 (2012) 1209–1215.

[5] T.W. Myers, D.H. Gelfand, Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase, Biochemistry, 30 (1991) 7661–7666.

[6] H. Shandilya, K. Griffiths, E.K. Flynn, M. Astatke, P.J. Shih, J.E. Lee, G.F. Gerard, M.D. Gibbs, P.L. Bergquist, Thermophilic bacterial DNA polymerases with reverse-transcriptase activity, Extremophiles, 8 (2004) 243–251.

[7] R. Kranaster, M. Drum, N. Engel, M. Weidmann, F.T. Hufert, A. Marx, Biotechnol. J., 5 (2010) 224–231.

[8] N.J. Schőnbrunner, E.H. Fiss, O. Budker, S. Stoffel, C.L. Sigua, D.H. Gelfand, T.W. Myers, Chimeric thermostable DNA polymerases with reverse transcriptase and attenuated 3’–5’ exonulease activity, Biochemistry, 45 (2006) 12786–12795.

[9] S.J. Firbank, J. Wardle, P. Heslop, R.K. Lewis, B.A. Connolly, Uracil recognition in archaeal DNA polymerases captured by X-ray crystallography, J. Mol. Biol., 381 (2008) 529–538.

[10] S. Sano, Y. Yamada, T. Shinkawa, S. Kato, T. Okada, H. Higashibata, S. Fujiwara, Mutations to create thermostable reverse transcriptase with bacterial family A DNA polymerase from *Thermotoga petrophila*, J. Biosci. Bioeng., 113 (2012) 315–321.

[11] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., 72, 248–254 (1976).

[12] K. Bebenek, T.A. Kunkel, Analyzing fidelity of DNA polymerases, Methods Enzymol., 262 (1995) 217–232.

[13] V. Barrioluengo, M. Álvarez, D. Barbieri, L. Menéndez-Arias, Thermostable HIV-1 group O reverse transcriptase variants with the same fidelity as murine leukaemia virus reverse transcriptase, Biochem. J., 436 (2011) 599–607.

[14] L.S. Beese, V. Derbyshire, T.A. Steitz, Structure of DNA polymerase I Klenow fragment bound to duplex DNA, Science, 260 (1993) 352–355.

[15] S.H. Eom, J. Wang, T.A. Steitz, Structure of *Taq* polymerase with DNA at the polymerase active site, Nature, 382 (1996) 278–281.

[16] L.A. Kohlstaedt, J. Wang, J.M. Friedman, P.A. Rice, T.A. Steitz, Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor, Science, 256 (1992) 1783–1790.

[17] D. Das, M.M. Georgiadis, The crystal structure of the monomeric reverse transcriptase from Moloney murine leukemia virus, Structure, 12 (2004) 819–829.

[18] T.A. Steitz, DNA polymerases: Structural diversity and common mechanism, J. Biol. Chem., 274 (1999) 17395–17398.

[19] K.B. Sauter, A. Marx, Evolving thermostable reverse transcriptase activity in a DNA polymerase scaffold, Angew. Chem. Int. Ed., 45 (2006) 7633–7635.

[20] K. Mayanagi, S. Kiyonari, H. Nishida, M. Saito, D. Kohda, Y. Ishino, T. Shirai, K. Morikawa, Architecture of the DNA polymerase B-proliferating cell nuclear antigen (PCNA)-DNA ternary complex, Proc. Natl. Acad. Sci. USA, 108 (2011) 1845-1849.

[21] Y. Shimada, W. Fukuda, Y. Akada, M. Ishida, J. Nakayama, T. Imanaka, S.

Fujiwara, Property of cold inducible DEAD-box RNA helicase in hyperthermophilic archaea, Biochem. Biophys. Res. Commun., 389 (2009) 622–627.

Figure legends

Fig. 1. Creation of reverse transcriptase activity in K4pol by the mutation of Leu329→Ala. (A) Domain structure of bacterial DNA polymerases. (B-D) Modeled structure of K4pol-template RNA complex. The structure is constructed by Swiss model using *E. coli* Klenow fragment (1kfs) as a template. Carbon, nitrogen, oxygen, and phosphorus atoms of RNA are shown in green, blue, red, and orange, respectively. (B) Whole structure of $K4pol_{WT}$-template RNA complex. The amino acid residues predicted to be located around an RNA template (Tyr326, Leu329, Gln384, Lys387, Phe388, Met408, Asn422, Tyr438, and Phe451) are shown in green. (C) Close-up view of Leu329 of $K4pol_{WT}$-template RNA complex. (D) Close-up view of Ala329 of $K4pol_{L329A}$-template RNA complex. Electron densities of the side chain of Leu329 (C) and Ala329 (D) are shown in red.

Fig. 2. DNA- and RNA-dependent DNA polymerase activities of $K4pol_{L329A}$. (A) SDS-PAGE under reducing condition. Lane M, molecular-mass marker. (B) Agarose gel electrophoresis of reaction products in the DNA-dependent DNA polymerase assay. Lane M, molecular-mass marker. The DNA bands a, b, c, and d correspond to the gap-filled DNA, the gapped DNA (the annealing product of the 6,789-base single-strand DNA fragment and the 7,196-base single-strand circular DNA), the 6,789-bp double-strand DNA fragment, and the 7,196-base single-strand circular DNA, respectively. The reaction was carried out with 670 nM $K4pol_{L329A}$ at 50ºC for 5 min. (C) Incorporation of radioactivity of reaction products in the reverse transcriptase assay. The reaction was carried out with $K4pol_{WT}$ (50 nM at 50ºC), $K4pol_{L329A}$ (50 nM at

50ºC), $M1pol_{WT}$ (50 nM at 50ºC) or MMLV RT (2 nM at 37ºC). The initial concentrations of dTTP and T/P were 200 and 10 μM, respectively.

Fig. 3. Substrate dependence of the reaction rates (v_o) in the reverse transcriptase reaction. The reaction was carried out with $K4pol_{L329A}$ (50 nM at 50ºC), $M1pol_{WT}$ (50 nM at 50ºC) or MMLV RT (2 nM at 37ºC). Solid line represents the best fit of the Michaelis-Menten equation. (A) Dependence of v_o on the dTTP concentration. The initial T/P concentration was 10 μM. (B) Dependence of v_o on the T/P concentration. The initial concentration of dTTP was 200 μM.

Fig. 4. Temperature dependence of the reaction rate (v_o) of $K4pol_{L329A}$ in the reverse transcriptase reaction. The reaction was carried out with 50 nM $K4pol_{L329A}$. The initial concentrations of dTTP and T/P were 200 and 25 μM, respectively. Inset shows Arrhenius plot.

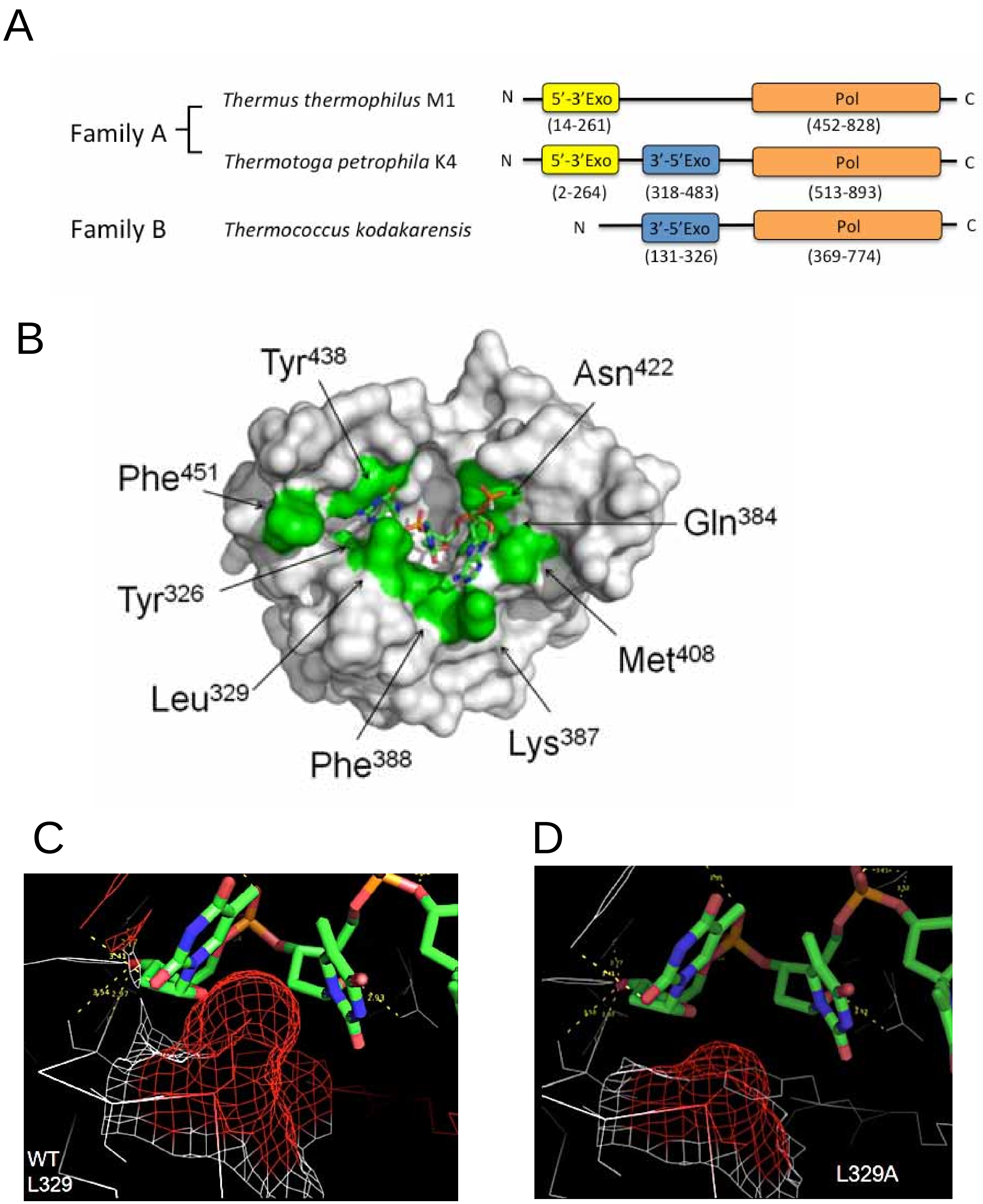

Fig. 1, Yasukawa *et al.*

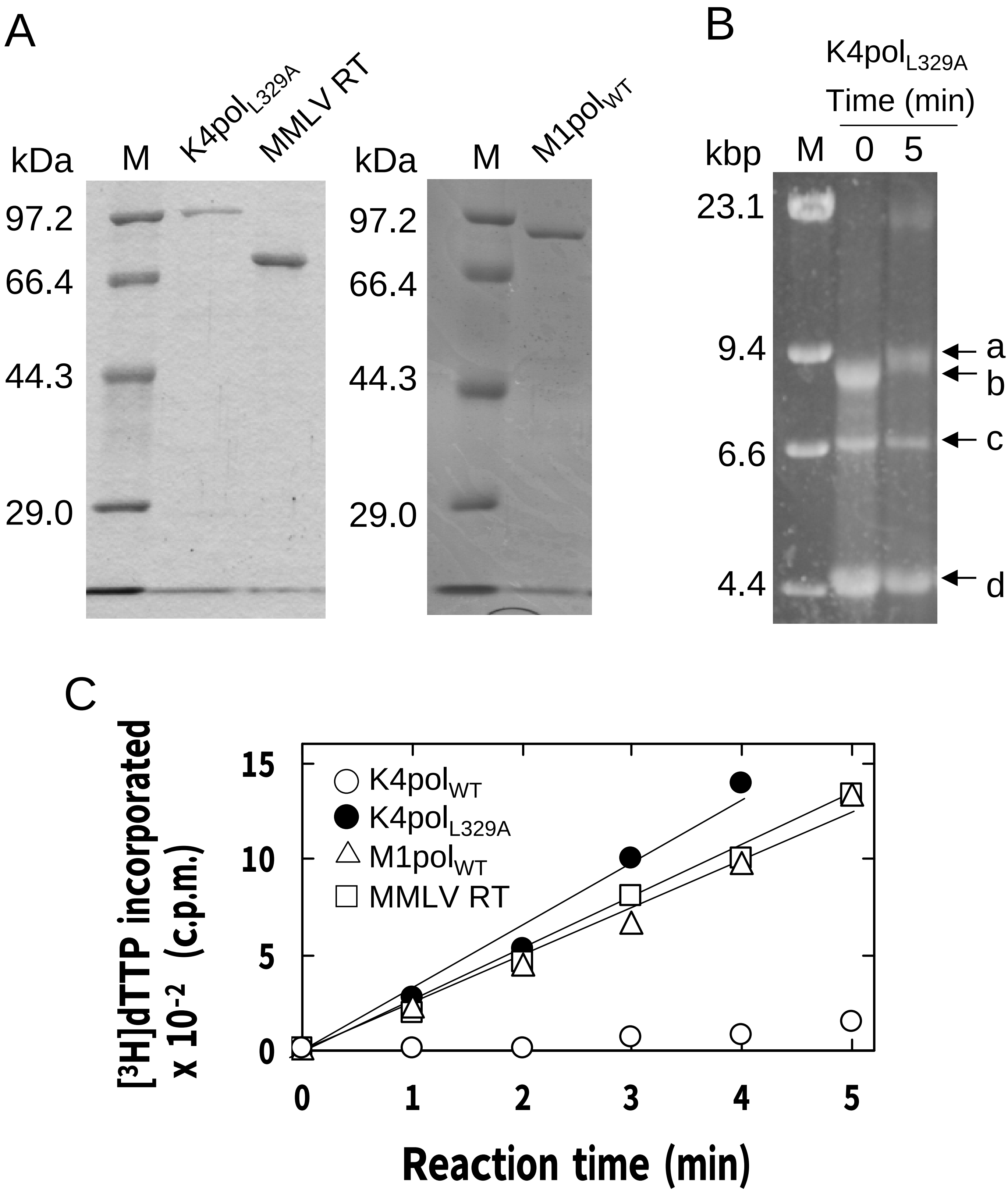

Fig. 2, Yasukawa *et al.*

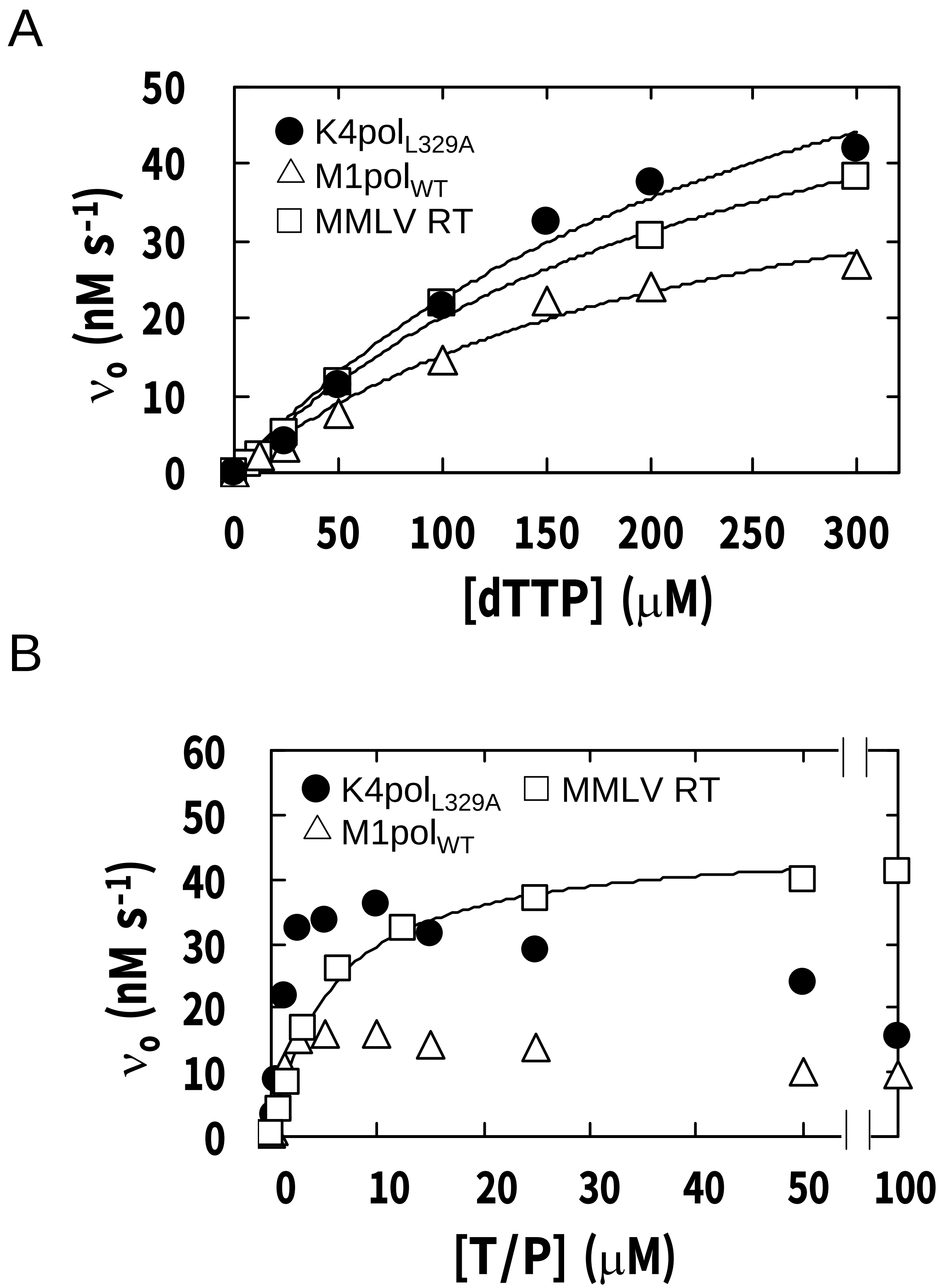

Fig. 3, Yasukawa *et al.*

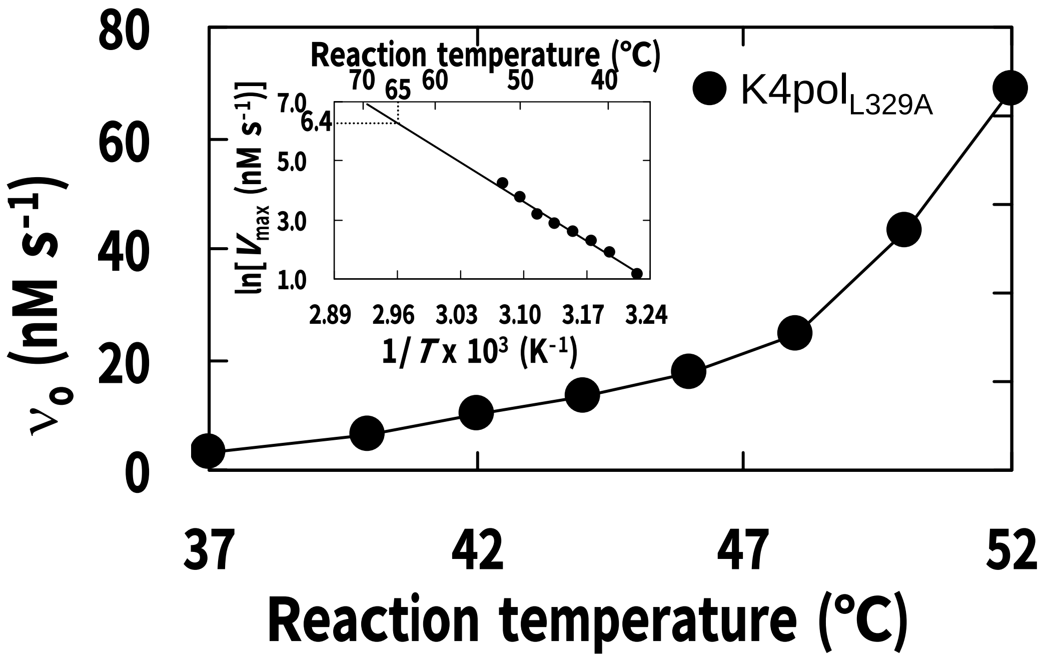

Fig. 4, Yasukawa *et al.*