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Abstract

DNA polymerase lambda contains template-dependent (DNA polymerase) and template-independent (terminal transferase) activities. In this study we enzymologically characterized the terminal transferase activity of polymerase lambda (pol lambda-tdt). Pol lambda-tdt activity was strongly influenced by the nature of the 3'-terminal sequence of the DNA substrate, and it required a single-stranded (ss) DNA 3'-overhang of about 9-12 nucleotides for optimal activity. The strong preference observed for pyrimidine versus purine nucleotide incorporation was found to be due, at least partially, to a steric block imposed by the residue Tyr-505 in the active site of pol lambda. Pol lambda-tdt was found to be able to elongate a 3'-ssDNA end by two alternative mechanisms: first, a template-independent one resulting in addition of 1 or 2 nucleotides, and second, a template-dependent one where a homopolymeric tract as short as 3 nucleotides at the 3'-end could be used as a template to direct DNA polymerization by a looping back mechanism. Furthermore repetitive cycles of DNA synthesis resulted in the expansion of such a short homopolymeric terminal sequence. Most importantly we found that the proliferating cell nuclear antigen was able to selectively block the looping back mechanism while stimulating the single terminal nucleotide addition. Finally replication protein A completely suppressed the transferase activity of pol lambda while stimulating the polymerase activity, suggesting that proliferating cell nuclear antigen and replication protein A can coordinate the polymerase and the terminal transferase activities of pol lambda.

DNA Elongation by the Human DNA Polymerase λ Polymerase and Terminal Transferase Activities Are Differentially Coordinated by Proliferating Cell Nuclear Antigen and Replication Protein A*

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DNA polymerase λ contains template-dependent (DNA polymerase) and template-independent (terminal transferase) activities. In this study we enzymologically characterized the terminal transferase activity of polymerase λ (pol λ -tdt). Pol λ -tdt activity was strongly influenced by the nature of the 3'-terminal sequence of the DNA substrate, and it required a single-stranded (ss) DNA 3'-overhang of about 9–12 nucleotides for optimal activity. The strong preference observed for pyrimidine *versus* purine nucleotide incorporation was found to be due, at least partially, to a steric block imposed by the residue Tyr-505 in the active site of pol λ . Pol λ -tdt was found to be able to elongate a 3'-ssDNA end by two alternative mechanisms: first, a template-independent one resulting in addition of 1 or 2 nucleotides, and second, a template-dependent one where a homopolymeric tract as short as 3 nucleotides at the 3'-end could be used as a template to direct DNA polymerization by a looping back mechanism. Furthermore repetitive cycles of DNA synthesis resulted in the expansion of such a short homopolymeric terminal sequence. Most importantly we found that the proliferating cell nuclear antigen was able to selectively block the looping back mechanism while stimulating the single terminal nucleotide addition. Finally replication protein A completely suppressed the transferase activity of pol λ while stimulating the polymerase activity, suggesting that proliferating cell nuclear antigen and replication protein A can coordinate the polymerase and the terminal transferase activities of pol λ .

DNA polymerase (pol)¹ λ is a recently described eukaryotic enzyme belonging to the pol X family, comprising other enzymes involved in DNA repair processes such as pol β , pol μ , and terminal deoxynucleotidyltransferase (TdT) (1). Human

pol λ (63.4 kDa) consists of a nuclear localization signal (residues 1–35), a BRCA1 C-terminal domain (residues 36–132), a proline-serine-rich region (residues 133–243), and a pol β -like core region (residues 244–575). The C-terminal part of pol λ (residues 244–575) is composed of a catalytic core that is similar to pol β (8-kDa domain and 31-kDa finger, palm, and thumb polymerization domain) and has 32% amino acid identity to pol β . Pol λ possesses multiple enzymatic activities, including DNA polymerase, terminal transferase, deoxyribose phosphate lyase, and polynucleotide synthetase, all localized in the C-terminal region containing the pol β -like core domain (2, 3). In addition, pol λ has been shown to interact with the replication protein proliferating cell nuclear antigen (PCNA) (4, 5) and with the DNA repair protein ligase IV/XRCC4 (6). On the basis of its biochemical properties, pol λ has been implicated in various DNA repair pathways such as abasic site translesion DNA synthesis, base excision repair, and the non-homologous end joining type of double strand break repair even though no direct demonstration of its role *in vivo* has been demonstrated so far (6–9). Of particular interest is the recent finding that pol λ is highly prone to generate –1 frameshifts due to its ability to promote microhomology base pairing between two DNA strands (10, 11) and that this ability is used by pol λ during abasic site bypass (12).

Pol λ shares with the highly homologous TdT and pol μ a template-independent (*i.e.* terminal transferase) DNA synthetic activity. The biochemical properties of TdT have been extensively characterized (13–15), and this enzyme is known to be able to add several nucleotides, in a strictly template-independent manner, to the 3'-ends of ss or dsDNA templates (16, 17). Conversely pol μ has been shown to require 3'-recessed DNA ends for optimal terminal transferase activity (18, 19). The fact that pol λ possesses both DNA polymerase and terminal transferase activity within the same active site raises the question of how these two activities are regulated. In the present work, we aimed to investigate in details the biochemical properties of pol λ -terminal transferase (pol λ -tdt) as well as the possible regulatory roles of the two auxiliary proteins, PCNA and replication protein A (RP-A).

MATERIALS AND METHODS

Chemicals

[³H]dTTP (30 Ci/mmol), [³H]dCTP (18 Ci/mmol), [³H]dATP (73 Ci/mmol), [³H]dGTP (73 Ci/mmol), and [γ -³²P]ATP (3000 Ci/mmol) were from Amersham Biosciences. Unlabeled dNTPs, poly(dA), and oligo(dT)_{12–18} were from Roche Applied Science. The oligonucleotides were from MWG Biotech (Florence, Italy). Whatman was the supplier of the GF/C filters. All other reagents were of analytical grade and purchased from Merck or Fluka.

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¹ The abbreviations used are: pol, polymerase; pol λ -tdt, terminal transferase activity of polymerase λ ; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; TdT, terminal deoxynucleotidyltransferase; ss, single-stranded; ds, double-stranded; SSB, single-stranded DNA-binding protein.

Enzymes and Proteins

Recombinant human DNA pol λ was expressed and purified as described previously (20). The DNA pol $\lambda\Delta N$ (amino acids 244–575) and the Y505A mutants were generated by PCR. The mutated sequences were then cloned and expressed as described previously (20, 21). After purification, the proteins were >90% homogenous as judged by SDS-PAGE and Coomassie staining (data not shown) and had specific activities of 200,000 units/mg for wild type pol λ and 180,000 units/mg for the pol $\lambda\Delta N$ mutant. 1 unit of pol activity corresponds to the incorporation of 1 pmol of total dTMP into acid-precipitable material in 60 min at 37 °C in a standard assay containing 0.5 μ g (as nucleotides) of poly(dA)/oligo(dT)_{10:1} and 10 μ M dTTP. The wild type PCNA and the three mutant proteins SHV43, QLGI125, and LAPK251 were generated, expressed in *Escherichia coli*, and purified as described previously (22). Recombinant human RP-A was isolated as described previously (23). Human pol β was from Trevigen.

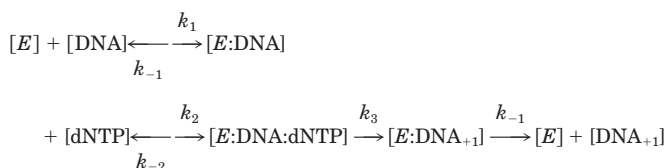
Enzymatic Assays

DNA Polymerase Assay—Pol λ activity on poly(dA)/oligo(dT)_{10:1} was assayed in a final volume of 25 μ l containing 50 mM Tris-HCl (pH 7.0), 0.25 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM MnCl₂, 0.2 μ M poly(dA)/oligo(dT)_{10:1} (3'-OH ends), 50 nM pol λ , and 5 μ M [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 was precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described previously (24). For denaturing gel analysis, the reaction mixture (10 μ l) included 50 mM Tris-HCl (pH 7.0), 0.25 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM MnCl₂. Enzymes, unlabeled dNTPs, and template were as indicated in the figure legends. For processivity measurements, the reactions were performed in the presence of a 100-fold molar excess of cold over labeled DNA substrate.

Terminal Transferase Assay—Pol λ terminal transferase activity on the different oligonucleotides was assayed in a final volume of 25 μ l containing 50 mM Tris-HCl (pH 7.0), 0.25 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM MnCl₂, and 0.2 μ M DNA (3'-OH ends) unless otherwise stated. Pol λ and [³H]dNTPs (10 Ci/mmol) were added as indicated in the figure legends. All reactions were incubated at 37 °C for 10 min unless otherwise indicated in the figures, and the DNA was precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting as described previously (24).

Kinetic Analysis

A minimal reaction pathway for the pol λ -tdt reaction is depicted below in Scheme 1.



SCHEME 1. Minimal kinetic pathway for single nucleotide addition by pol λ -tdt

Time course experiments were performed as described in the figure legends. Samples were taken at 0, 15, 30, 45, 60, 75, 90, 120, 240, 600, and 1800 s. Data were analyzed according to mixed exponential Equation 1 (25),

$$A(1 - e^{-k't}) + k_{ss}t \quad (\text{Eq. 1})$$

where A is the burst amplitude; k' is the apparent first-order rate constant for the single turnover association of the enzyme to its substrate (burst rate); k_{ss} is the rate limiting constant for the steady-state reaction, which was assumed to be equal to k_{-1} ; and t is time.

Active site concentration was derived from Equation 2.

$$A = [E]_0 \cdot \left(\frac{k'}{k' + k_3} \right)^2 \quad (\text{Eq. 2})$$

The catalytic rate k_3 and the affinity of the enzyme for its substrates (K_s) were calculated according to the relationship in Equation 3,

$$k' = \frac{k_3 \cdot [S]}{[S] + K_s} \quad (\text{Eq. 3})$$

where $[S]$ is the variable substrate concentration.

TABLE I
Activity of pol λ -tdt on different oligonucleotide substrates^a

ss DNA Substrate (3'-terminal sequence)	dTTP (pmol x min ⁻¹)	dATP (pmol x min ⁻¹)	dCTP (pmol x min ⁻¹)	dGTP (pmol x min ⁻¹)
POSITION -5 -4 -3 -2 -1 0				
1. - A T T C A T	0.001	0.001	0.001	n.d. ^c
2. - G G C A A G	0.014	0.001	0.001	n.d.
3. - T A C T T G	0.001	0.0011	0.001	n.d.
4. - C A T C C A	0.007	0.001	0.001	n.d.
5. - A G A T G G	0.011	0.002	0.001	n.d.
6. - G G C T C C	0.02	0.0012	0.007	n.d.
7. - A A C T A C	0.001	0.001	0.001	n.d.
8. - G A T A A C	0.064	0.03	0.045	n.d.
9. - T C C C A G	0.025	0.015	0.001	n.d.
10. - C A A T A G	0.01	0.005	0.001	n.d.
11. - C A T A A C	0.125	0.05	0.045	n.d.
12. - C A T A A G	0.01	0.001	0.001	0.001
13. - C A T A A T	0.03	0.012	0.03	0.001
14. - C A T A G G	0.01	0.001	0.001	0.001
15. - A A C A A C	0.05	0.06	0.03	0.001
16. - C A A A A C	0.2	0.055	0.08	0.001
17. - A G G G T T	0.01	0.06	0.001	0.001
18. - G G G T T A	0.01	0.001	0.001	0.001
19. - G G T T A G	0.005	0.001	0.001	0.001
20. - T T A G G G	0.005	0.001	0.001	0.001
21. - C C C T A A	0.005	0.001	0.001	0.001
22. - C C T A A C	0.005	0.001	0.001	0.001
23. - T A A C C C	0.07	0.001	0.001	0.15
Consensus	R R Y N C^b			
	R Y R N C			
	R R R N C			

^a Oligonucleotides 1–14 were 27-mers, oligonucleotides 15 and 16 were 30-mers, and oligonucleotides 17–23 were 24-mers. The first 18 nucleotides at the 5'-end of the sequence were identical for all substrates and were 5'-AGGATGTATGTTTATTAGTA-3'. Highest activities are in bold. Sequences matching the consensus are underlined.

^b R, purine; Y, pyrimidine; N, any.

^c n.d., not determined.

The actual rates k_1 , k_{-1} , k_2 , and k_{-2} were calculated according to the relationships in Equations 4–7.

$$k'_{\text{DNA}} = \frac{k_{+1} \cdot k_3}{k_{-1} + k_3} \quad (\text{Eq. 4})$$

$$k'_{\text{dNTP}} = \frac{k_{+2} \cdot k_3}{k_{-2} + k_3} \quad (\text{Eq. 5})$$

$$K_{\text{DNA}} = \frac{k_{-1}}{k_{+1}} \quad (\text{Eq. 6})$$

$$K_{\text{dNTP}} = \frac{k_{-2}}{k_{+2}} \quad (\text{Eq. 7})$$

Since the kinetics of the DNA polymerase reaction on poly(dA)/oligo(dT) as well as the dGTP addition on the ssDNA oligo 23 were linear, the kinetic parameters k_{cat} and K_{DNA} were derived by analyzing the initial velocities of the reaction according to the Michaelis-Menten equation. All the experiments were performed in triplicate. Error bars (\pm S.D.) are shown in the plots.

RESULTS

The Terminal Transferase Activity of Pol λ Is Influenced by the 3'-End Terminal Sequence of the ssDNA Substrate—The activity of both cellular and viral terminal transferases have been shown to be influenced by the 3'-terminal sequence of the ssDNA substrate (26). To verify whether this was true for pol λ , we analyzed 23 different oligonucleotides as substrates for pol λ -tdt activity. These oligonucleotides bear random sequences (Table I, oligos 1–10) or two particular subsets of DNA sequences such as triplet repeats AAC, AAG, AGG, AAT, (AAC)₂, or AAAAAC (Table I, oligos 11–16) or telomeric sequences present in the G-rich and in complementary strands of human telomeres (Table I, oligos 17–23). As described previously, pol λ -tdt showed a strong preference for pyrimidine nucleotide

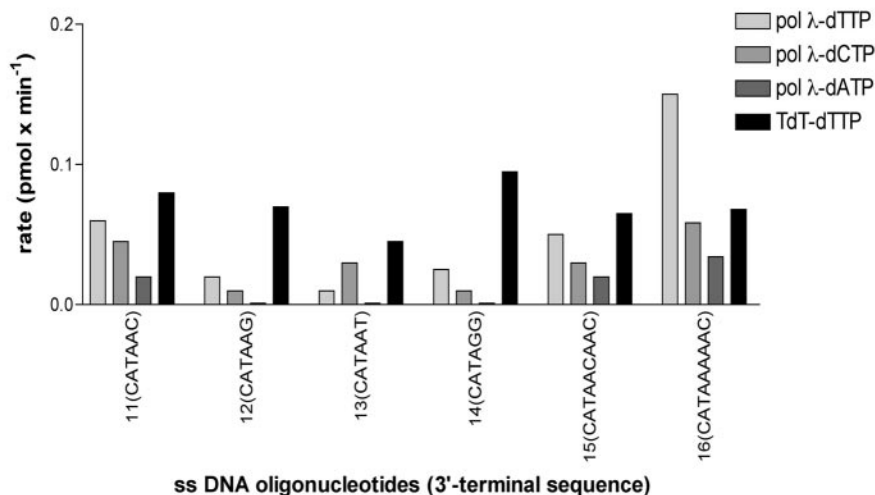
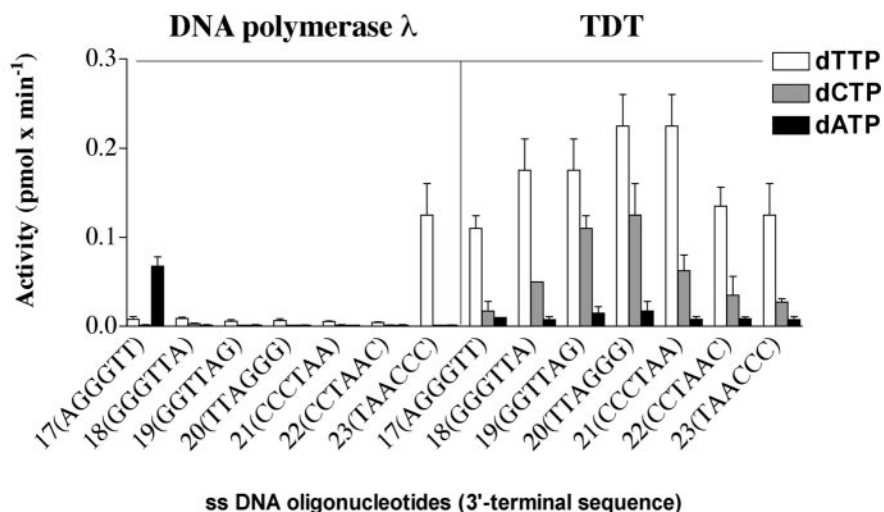
A

FIG. 1. Pol λ -tdt differs from calf thymus TdT in its nucleotide and DNA substrate specificity. *A*, terminal transferase reactions were performed as described under "Material and Methods" in the presence of 50 nM pol λ or 50 nM calf thymus TdT and a 4 μ M concentration of each of ss oligonucleotide DNA substrates 11 (-AAC), 12 (-AAG), 13 (-AAT), 14 (-AGG), 15 (-AACAAAC), and 16 (-AAAAAAC) and in the presence of 2 μ M labeled dTTP (light gray bars), dCTP (gray bars), and dATP (dark gray bars) for pol λ or 2 μ M labeled dTTP (black bars) for calf thymus TdT. *B*, terminal transferase reactions were performed as described under "Material and Methods" in the presence of 50 nM pol λ or 50 nM calf thymus TdT and in the presence of a 4 μ M concentration of each of the ss oligonucleotide telomeric DNA substrates indicated in the panel and in the presence of 2 μ M labeled dTTP (white bars), dCTP (gray bars), and dATP (black bars) for both pol λ -tdt and calf thymus TdT.

B

incorporation; however, we found that the activity was also strongly influenced by the 3'-terminal sequence (2). Pol λ -tdt activity was maximal on the AAC, (AAC)₂, and AAAAAAC repeats (Table I, oligos 11, 15, and 16, respectively). Inspection of the sequences of the last nucleotides at the 3'-end of all 23 substrates utilized allowed the identification of a minimal consensus for optimal activity, which was (from position 0 to -4) RRYNC, RRRNC, or RYRNC where C is cytosine, R is a purine, Y is a pyrimidine, and N is any nucleobase (Table I). In particular, a strong discrimination against two adjacent pyrimidines at the -4, -3 and/or -3, -2 positions was noted.

Pol λ -tdt Differs from Calf Thymus TdT in Nucleotide and DNA Substrate Specificities—Next we compared the activity of pol λ -tdt and calf thymus TdT on the triplet repeats (Table I, oligos 11–16) and the telomeric DNA sequences (Table I, oligos 17–23). As shown in Fig. 1A, the order of preference for terminal nucleotide addition on substrates 11–16 for pol λ -tdt was dT > dC > dA, but this activity was additionally influenced by

the 3'-terminal sequence. Calf thymus TdT added dTTP with almost the same efficiency to the ssDNA oligonucleotides tested, thus showing no sequence preferences. When the activity of pol λ -tdt was next tested on telomeric substrates 17–23, an even stronger nucleotide and sequence specificity was observed (Fig. 1B). Pol λ was able to add specifically dATP to the sequence -AGGGTT (oligo 17) and dTTP to the sequence -TAACCC (oligo 23), while it was almost inactive on all other substrates tested. Calf thymus TdT, on the other hand, showed higher incorporation of dT and dC with respect to dA but did not display a significant preference for any telomeric sequences analyzed. Together these results suggested that the substrate selectivity observed for pol λ -tdt is an intrinsic feature of this enzyme that is not shared by the canonical TdT.

The Pol λ -tdt Activity Requires a ssDNA Protruding 3'-End—The details of pol λ -tdt properties were further tested on the -AAC terminal sequence (substrate 11, Table I), which was used as a reference substrate for further characterization. The

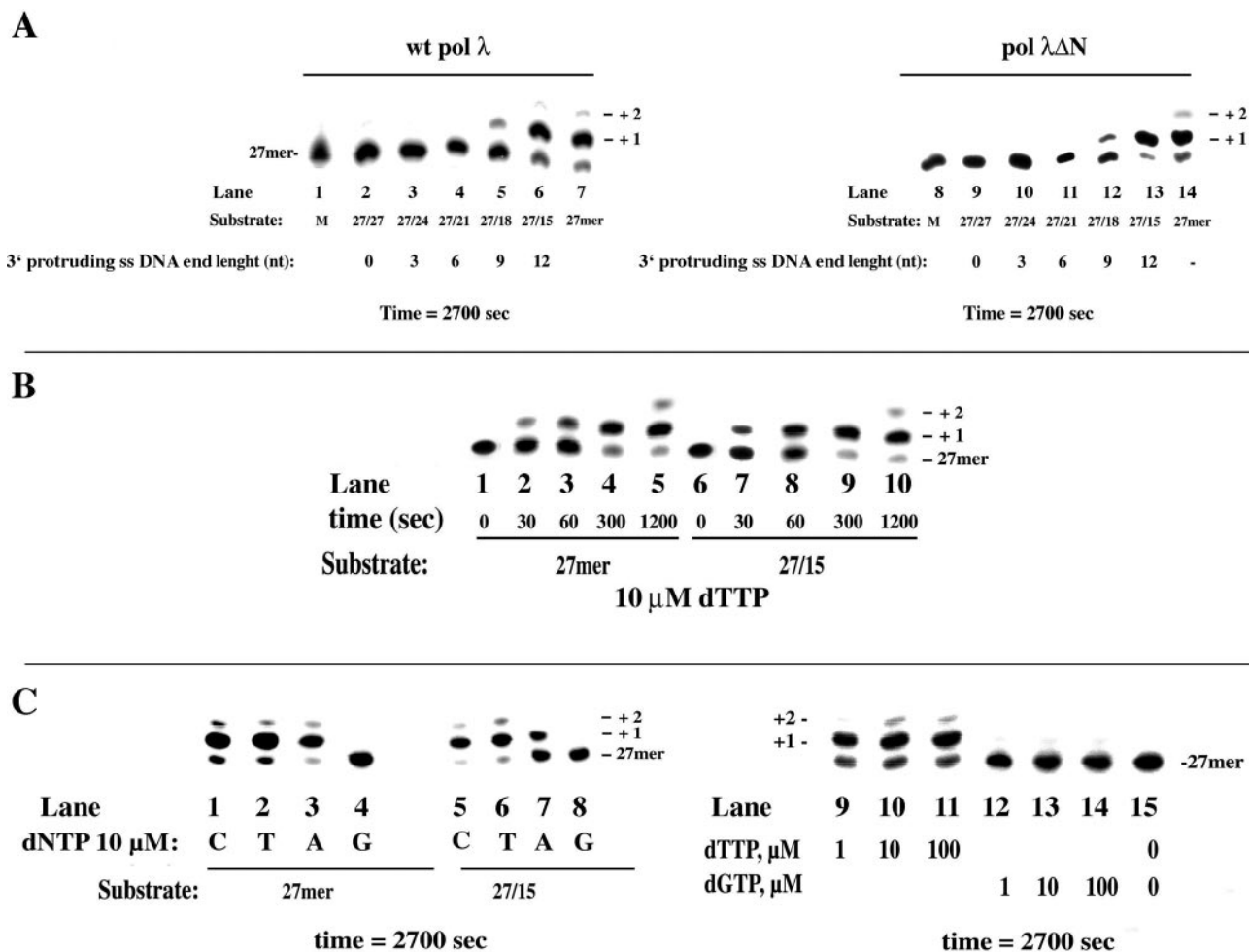


FIG. 2. The pol λ -tdt activity requires a ssDNA 3'-overhang of 9–12 nucleotides. *A*, terminal transferase reactions for pol λ were performed as described under "Material and Methods" in the presence of a 50 nM concentration of each of the different 5'-labeled oligonucleotide substrates (27-mer, 27/15-mer, 27/18-mer, 27/21-mer, 27/24-mer, and 27/27-mer) bearing 3'-ssDNA overhangs of different lengths as indicated at the bottom of the panel. Reactions were carried out for 45 min at 37 °C in the presence of 5 μ M dTTP with 50 nM full-length pol λ (lanes 2–7) or with a 50 nM concentration of the N-terminally truncated mutant pol $\lambda\Delta N$ (lanes 8–14). *M*, ss 27-mer oligonucleotide alone as marker. *B*, time course experiments for dTTP incorporation were performed in the presence of 50 nM pol λ and with 10 μ M dTTP and a 50 nM concentration of 5'-end-labeled ss 27-mer DNA (oligo 11, Table I) (lanes 1–5) or a 50 nM concentration of 5'-end-labeled ss 27-mer DNA (oligo 19, Table I) but annealed to a labeled complementary 15-mer to generate a partially ds 27/15-mer (lanes 6–10). *C*, terminal transferase assays for pol λ were performed for 45 min at 37 °C as described under "Material and Methods" in the presence of 50 nM pol λ and a 50 nM concentration of the ss 27-mer (oligo 11) (lanes 1–4) or the partially ds 27/15-mer (lanes 5–8) DNA substrates in the presence of single nucleotides at a fixed concentration (lanes 1–8) or in the presence of the ss 27-mer DNA only and increasing concentrations of dTTP (lanes 9–11) or dGTP (lanes 12–14). *wt*, wild type; *nt*, nucleotides.

first objective was to test whether pol λ -tdt requires an ssDNA or whether it is able to utilize dsDNA blunt ends as does TdT or even 3'-recessed ends as does pol μ . Different complementary oligonucleotides were annealed to substrate 11 to create partially ds oligonucleotides with ss 3'-overhangs of decreasing lengths. Pol λ -tdt required a 3'-protruding ssDNA tract between 9 and 12 nucleotides for terminal addition (Fig. 2A, lanes 1–7). The same results were obtained with a truncated pol λ -tdt mutant lacking the BRCA1 C-terminal and Pro-rich domains at its N-terminal part (Fig. 2A, lanes 8–14), suggesting that the interaction with the ssDNA tract involves mainly the catalytic domain of pol λ . Since the product distribution indicated a distributive pattern with the main product of a single nucleotide addition, we next investigated the kinetics of the reaction. On the 27-mer ssDNA substrate (oligo 11), only a +1 product was formed within the first 5 min of the reaction, whereas a +2 product appeared only at later times (Fig. 2B, lanes 1–5). The same pattern was observed when the 27/15-mer substrate with a 12-nucleotide ss 3'-overhang was tested (Fig. 2B, lanes 6–10). This was consistent with a mechanism of single nucleotide

addition following enzyme dissociation/reassociation until incorporation of the next nucleotide could occur.

Finally the nucleotide substrate specificity of pol λ -tdt on this template was investigated. As shown in Fig. 2C, only dTTP, dCTP, and dATP were incorporated on the ss 27-mer (lanes 1–3) or the partially ds 27/15-mer (lanes 5–7), whereas dGTP was not (Fig. 2C, lanes 4 and 8). Titration of increasing dGTP concentrations showed that discrimination on this DNA substrate against its incorporation was almost absolute (Fig. 2C, lanes 12–14). Also dGTP was not incorporated on any of the substrates analyzed with the exception of oligo 23 as will be discussed later (Table I and see also below).

The Nucleotide Substrate Selectivity of Pol λ -tdt Is Influenced by the Tyr-505 of the Polymerase Active Site—To investigate the molecular basis for the pyrimidine versus purine nucleotide discrimination by pol λ -tdt, the kinetic parameters for single nucleotide addition on the reference substrate (oligo 11, Table I) were tested. Under these conditions, the reaction followed a time-dependent biphasic curve with a fast rate (burst) corresponding to the first nucleotide addition followed by a slow

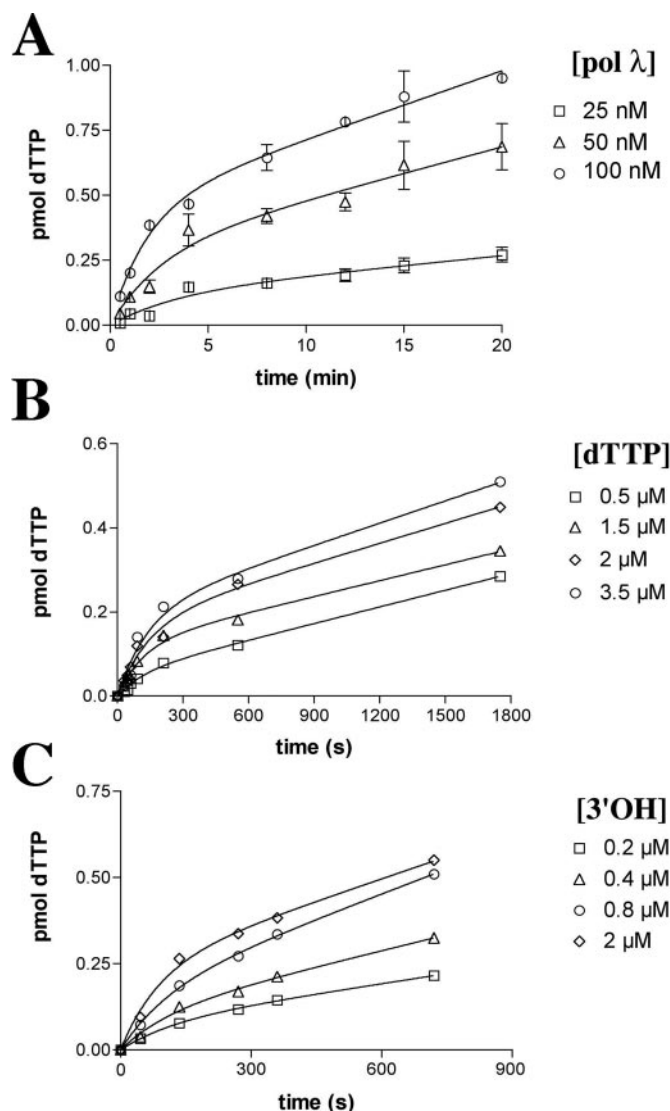


FIG. 3. Kinetic analysis of terminal nucleotide addition by pol λ -tdt. Time course experiments were performed under the conditions as described under "Material and Methods" in the presence of labeled dTTP and ss 27-mer DNA substrate (oligo 11). A, kinetics of terminal nucleotide addition with different enzyme concentrations (25 nM, squares; 50 nM, triangles; 100 nM, circles) in the presence of 2 μ M ssDNA substrate and 2 μ M dTTP. B, kinetics of terminal nucleotide addition with different dTTP concentrations (0.5 μ M, squares; 1.5 μ M, triangles; 2 μ M, diamonds; 3.5 μ M, circles) in the presence of 2 μ M ssDNA substrate and 50 nM pol λ . C, kinetics of terminal nucleotide addition with different DNA concentrations (0.2 μ M, squares; 0.4 μ M, triangles; 0.8 μ M, circles; 2 μ M, diamonds) in the presence of 2 μ M dTTP and 50 nM pol λ . Data were fitted to a mixed exponential equation as described under "Material and Methods." Points represent the means of three independent experiments with error bars representing S.D.

steady-state rate likely determined by the dissociation of the enzyme and subsequent binding and addition of the second nucleotide (Fig. 3). The burst amplitude increased as a function of enzyme (Fig. 3A), nucleotide (Fig. 3B), and DNA substrate (Fig. 3C) concentrations. By using a mixed exponential kinetic model (see "Materials and Methods" for details), the kinetic parameters for the whole reaction pathway of dTTP and dATP addition were derived. The kinetic pathway is depicted in Scheme 1, and the values are summarized in Table II. As can be seen, pol λ -tdt showed a nearly 6-fold higher efficiency ($k_3/K_{d\text{ATP}}$) for dTTP versus dATP addition. This resulted mainly from a combination of faster association (k_2) and catalytic (k_3) rates for dTTP, suggesting the presence of some steric

barrier for dATP within the active site of pol λ -tdt. We have previously shown that the Tyr-505 of pol λ is part of the nucleotide binding site, a finding that has been subsequently confirmed by the resolution of the crystal structure of pol λ (20). By exploiting the mutant Y505A, we were able to demonstrate that removal of the side chain of Tyr-505 increased dramatically both the association (k_2) and the catalytic (k_3) rates for dATP addition, suggesting that this residue was responsible for the A versus T discrimination (Table II). As a result, the Y505A mutant showed a 13-fold preference for dATP over dTTP terminal addition. When pol λ -tdt was tested on telomeric DNA sequences, both the sequence and the nucleotide specificities were extremely selective since only two 3'-terminal sequences were recognized as substrates by pol λ -tdt, -GGGTT (substrate 17) on the G-rich strand and AACCC (substrate 23) on the complementary strand (Table I and Fig. 1B). Moreover the nucleotide addition specificity was almost absolute with only dTTP being incorporated on the TAACCC sequence (giving the product TAACCC \underline{T}) and remarkably dATP on the GGGTT sequence (giving the product GGGTT \underline{A}). Therefore, we wanted to investigate the reasons for this absolute substrate specificity. For this, the kinetic parameters for single nucleotide addition on the telomeric substrates 17 and 23 were determined (Table III). It can be seen that the preference for dATP on substrate 17 and for dTTP on substrate 23 was due to faster association (k_2) and catalytic (k_3) rates in both cases for the preferred nucleotide. Remarkably substrate 17 was slightly different from the optimal consensus sequence in that it has a T instead of a C at position 0 (see also Table I). When the same substrates were tested in the presence of the Y505A mutant, again the binding (k_2) and the catalytic (k_3) rates for dATP addition were increased with respect to the wild type enzyme on both substrates. However, the effect was less pronounced than in the case of substrate 11 (see Table II for comparison), suggesting that for the telomeric sequences examined also the DNA structure/sequence might play a major role in determining the nucleotide specificity. Taken together these results suggested that the residue Tyr-505 could act as a steric barrier for the large purine ring, whereas it facilitated accommodation of the pyrimidine nucleotides in the active site of the enzyme.

Pol λ -tdt Shows Two Alternative Mechanisms for ssDNA Elongation—The minimal change in the sequence of oligos 11 and 15 to generate a homopurinic tract at the 3'-end (oligo 16) resulted in a 2–4-fold increase in dTTP incorporation by pol λ -tdt (Table I and Fig. 2). Determination of the kinetic parameters for nucleotide incorporation revealed that the catalytic rate (k_3) was specifically increased, whereas no difference on the affinity for DNA was observed (Table IV). Product analysis of the terminal nucleotide reaction catalyzed by pol λ -tdt on templates 11 and 16 showed that only limited synthesis occurred with generation of +1 or +2 products (Fig. 4A, lanes 1–3) when either dTTP, dATP, or dCTP were used as substrates for terminal addition in the presence of the 27-mer AAC substrate (oligo 11). However, when dTTP was tested in the presence of the 30-mer AAAAAC substrate (oligo 16), long products of +15 were synthesized, whereas no difference was evident in the presence of dATP (Fig. 4A, lanes 4 and 5). An identical result was observed when a partially ds 30/15-mer oligonucleotide was tested (Fig. 4A, lanes 6 and 7).

Since long products were synthesized in the presence of a homopolymeric tract at the 3'-end only with the complementary nucleotide (in the case with dTTP in the presence of an AAAA tract), one possibility could be that pol λ -tdt could "loop back" the 3'-end of the substrate and use the terminal sequence as a template for its DNA polymerase activity. This would also predict that an alternative elongation should be observed with

TABLE II
Kinetic parameters for dTTP or dATP incorporation by wild type and Y505A pol λ -tdt on DNA substrate 11

Substrate	Wild type								Y505A							
	k_1	k_{-1}	K_{DNA}	k_2	k_{-2}	K_{dNTP}	k_3	k_3/K_{dNTP}	k_1	k_{-1}	K_{DNA}	k_2	k_{-2}	K_{dNTP}	k_3	k_3/K_{dNTP}
	$s^{-1} M^{-1}$	s^{-1}	μM	$s^{-1} M^{-1}$	s^{-1}	μM	s^{-1}	$s^{-1} M^{-1}$	$s^{-1} M^{-1}$	s^{-1}	μM	$s^{-1} M^{-1}$	s^{-1}	μM	s^{-1}	$s^{-1} M^{-1}$
dTTP	8.4×10^5	0.008	0.11	4.3×10^4	0.015	0.36	1.6	4.44×10^{-6}	7.5×10^5	0.006	0.15	5.7×10^4	0.015	0.26	0.5	1.9×10^{-6}
dATP	ND	ND	ND	1.5×10^4	0.009	0.6	0.46	0.76×10^{-6}	ND	ND	ND	1×10^5	0.009	0.09	2.3	25.5×10^{-6}

^a ND, not determined.

TABLE III
Kinetic parameters for dTTP and dATP incorporation by wild type and Y505A pol λ -tdt activity on telomeric substrates 17 and 23

Substrate	Wild type						Y505A					
	K_{DNA}	k_2	k_{-2}	K_{dNTP}	k_3	k_3/K_{dNTP}	K_{DNA}	k_2	k_{-2}	K_{dNTP}	k_3	k_3/K_{dNTP}
	μM	$s^{-1} M^{-1}$	s^{-1}	μM	s^{-1}	$s^{-1} M^{-1}$	μM	$s^{-1} M^{-1}$	s^{-1}	μM	s^{-1}	$s^{-1} M^{-1}$
Telomeric substrate 17 (-AGGGTT)												
dTTP	0.33	1.2×10^5	0.025	0.2	0.1	0.45×10^{-6}	0.45	1×10^4	0.0075	0.7	0.3	0.85×10^{-6}
dATP	0.52	4×10^5	0.02	0.05	0.85	17×10^{-6}	0.8	1.1×10^6	0.01	0.09	2.6	28.8×10^{-6}
Telomeric substrate 23 (-TAACCC)												
dTTP	0.54	1.1×10^5	0.01	0.09	1.1	12.2×10^{-6}	0.85	8.1×10^4	0.009	0.11	0.6	5.4×10^{-6}
dATP	0.9	4.5×10^4	0.009	0.2	0.25	1.25×10^{-6}	1.2	8.1×10^4	0.009	0.11	0.32	2.9×10^{-6}

TABLE IV
The preference for the AAAAAC 3'-terminal sequence by pol λ -tdt is due to an increase in the rate of nucleotide incorporation

	K_{DNA}	dATP			dTTP		
		K_{dNTP}	k_3	k_3/K_{dNTP}	K_{dNTP}	k_3	k_3/K_{dNTP}
	μM	μM	s^{-1}	$s^{-1} M^{-1}$	μM	s^{-1}	$s^{-1} M^{-1}$
-AACAAAC	1.2	0.4	0.5	1.25×10^{-6}	0.26	0.72	2.76×10^{-6}
-AAAAAC	0.75	0.42	1.27	3.02×10^{-6}	0.3	2.5	8.33×10^{-6}

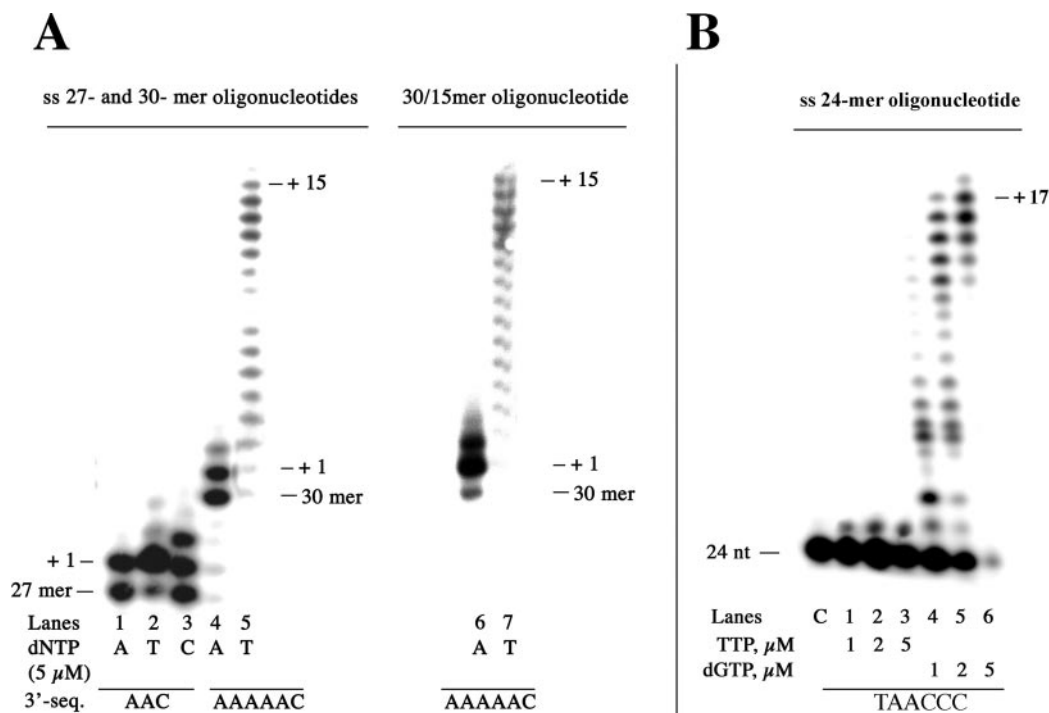


FIG. 4. Product analysis of the terminal nucleotide addition by pol λ -tdt on different ssDNA templates. A, terminal transferase reactions for pol λ were performed as described under "Material and Methods" in the presence of 50 nM pol λ and a 50 nM concentration of each of the 5-labeled ss 27-mer (oligo 11, Table I) (lanes 1–3), the ss 30-mer (oligo 16, Table I) (lanes 4 and 5), or the partially ds 30/15-mer oligonucleotide DNA substrates (lanes 6 and 7). B, terminal transferase reactions for pol λ were performed as described under "Material and Methods" in the presence of 50 nM pol λ and a 50 nM concentration of the oligo 23 (Table I) ssDNA substrate. Lanes 1–6, reactions were incubated for 8 min in the presence of increasing concentrations of dTTP or dGTP. The single nucleotide added in each reaction is indicated at the bottom of the panel along with its final concentration and the 3'-terminal sequence (3'-seq.) of the substrates.

other homopolymeric tracts. The substrate oligo 23 (Table I) bears a CCC triplet at its 3'-end and was a substrate for terminal dTTP addition (Table I and Fig. 1 B). Thus, we next asked whether on this substrate pol λ -tdt could incorporate the dGTP complementary to the terminal repeat. The results of the

product analysis of dTTP versus dGTP incorporation catalyzed by pol λ -tdt on substrate 23 showed that by adding increasing concentrations of dTTP, a +1 product was formed exclusively (Fig. 4B, lanes 1–3), while addition of the same concentrations of dGTP resulted in longer products (Fig. 4B, lanes 4–6).

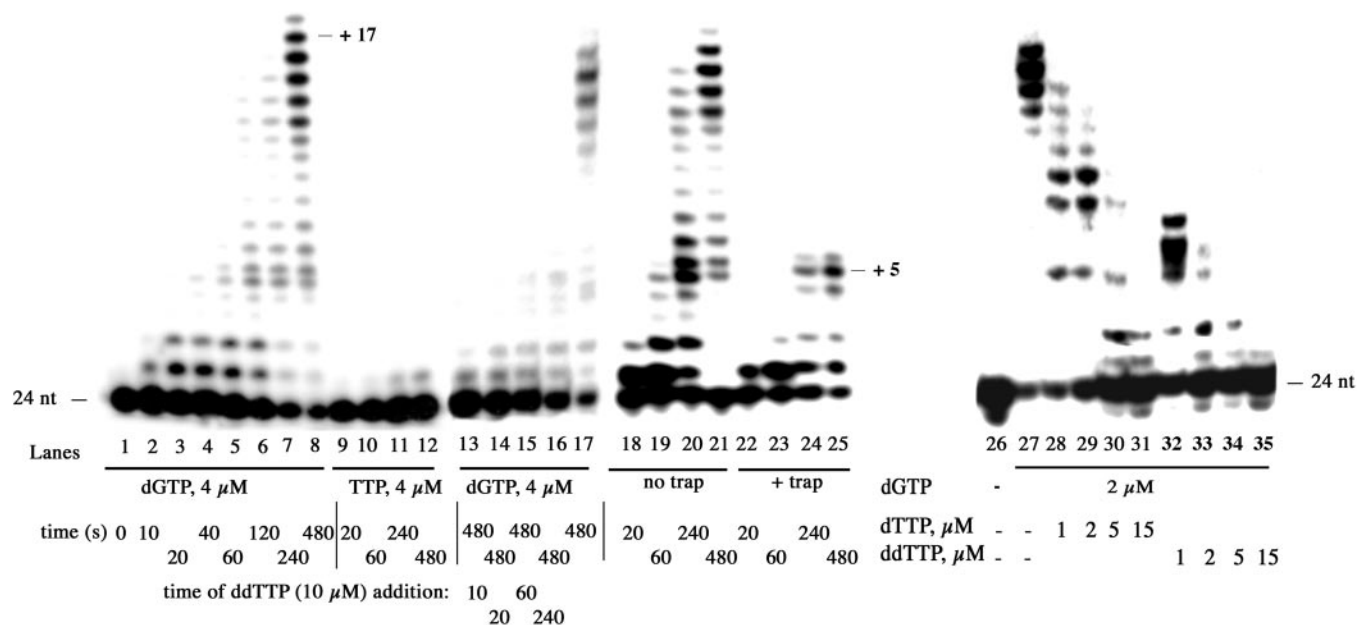


FIG. 5. **Characterization of the terminal nucleotide addition by pol λ -tdt on the -CCC ssDNA substrate.** Terminal transferase reactions for pol λ were performed as described under "Material and Methods" in the presence of 50 nM pol λ and a 50 nM concentration of the oligo 23 (Table I) ssDNA substrate. In lanes 1–8, the reactions were incubated for various time points in the presence of a fixed dGTP concentration. In lanes 9–12, the reactions were incubated in the presence of a fixed dTTP concentration for various times as indicated. Lanes 13–17, reactions were incubated for 8 min in the presence of a fixed dGTP concentration. 10 μ M ddTTP was added at different time points during the incubation. In lanes 18–25, preformed pol λ -DNA complexes were incubated for different time points in the presence of 4 μ M dGTP and in the absence (lanes 18–21) or in the presence (lanes 22–25) of a 100-fold molar excess of unlabeled ssDNA substrate (oligo 23, Table I). Lanes 26–35, terminal nucleotide addition reactions for pol λ -tdt were performed in the presence of 2 μ M dGTP and in the absence (lane 27) or in the presence of increasing concentrations of dTTP (lanes 28–31) or ddTTP (lanes 32–35). nt, nucleotides.

Pol λ -tdt Switches from Template-independent to Template-dependent 3'-End Elongation Depending on the Nature of the Incoming Nucleotide—A time course experiment with oligo 23 and dGTP showed that a +1 product appeared already after 10 s (Fig. 5, lane 2), which was then efficiently elongated up to +17–18 nucleotides (lanes 3–8). Addition of dTTP, on the other hand, resulted in the accumulation of +1 products only (lanes 9–12). By comparing the products synthesized in the presence of either dGTP or dTTP at early time points (Fig. 5, e.g. compare lane 5 with lane 10), it can be seen that the kinetics of dGTP addition was faster than the one of dTTP. Moreover the kinetics of dTTP addition was biphasic as already noted for the substrate 11, whereas addition of dGTP was linear with time (data not shown). These results suggested the involvement of two different pol λ mechanisms.

To further characterize this activity, pol λ -tdt was incubated in the presence of the DNA substrate and dGTP followed by the addition of the chain terminator ddTTP (10 μ M) at different time points. Addition of ddTTP at 10, 20, 60, and 240 s (Fig. 5, lanes 13–16) completely abolished further dGTP incorporation (compare lanes 13–16 to the uninhibited reaction in lane 17). Moreover by comparing the reactions terminated with ddTTP to the time course experiments in its absence, it can be seen that addition of ddTTP terminated the chains almost exactly at the length they were at the time of addition of the chain terminator (Fig. 5, compare lanes 13–16 with lanes 2–8).

Next the processivity of dGTP addition was investigated by analyzing the length of the products synthesized at different times either in the absence (Fig. 5, lanes 18–21) or in the presence (lanes 22–25) of a cold trap (100-fold molar excess of unlabeled oligo 23). In the presence of the trap, products of +5 and +6 nucleotides accumulated, suggesting that incorporation of dGTP into the +18 products was more processive than the terminal 1 nucleotide addition of dTTP (see also Fig. 2).

According to the above data, it can be hypothesized that pol

λ -tdt could switch from one elongation mode to the other depending on the presence of the nucleotide complementary to the homopolymeric tract. To directly show this, competition experiments were performed by testing different molar ratios of dTTP or ddTTP over dGTP. Both dTTP (Fig. 5, lanes 28–31) and ddTTP (lanes 32–35) were able to inhibit dGTP incorporation. However, ddTTP addition resulted in shorter products than dTTP (Fig. 5, e.g. compare lane 28 with lane 32), suggesting that after incorporation of a dTTP residue the chain could be further elongated by either dGTP or dTTP, whereas ddTTP was acting as the chain terminator. These results suggested that pol λ -tdt could elongate a ssDNA 3'-end by two alternative mechanisms: first, a template-independent one resulting in addition of 1 or 2 nucleotides, and second, a template-dependent one where a homopolymeric tract as short as 3 nucleotides at the end can be used as a template to direct polymerization via a looping back mechanism. Repetitive cycles of DNA synthesis resulted moreover in the expansion of the short homopolymeric terminal sequence as indicated by the generation of products longer than the homopolymeric tract. As controls, other oligonucleotides (Table I, oligos 11, 15, 17, and 21) were tested for their ability to support complementary nucleotide addition. None of them could trigger long products by pol λ -tdt (Fig. 4 and data not shown). In summary, these results clearly indicated that the homopolymeric tract should start at position 0 or –1 to be used by the looping back mechanism and that its minimal size is 3 nucleotides.

PCNA Differentially Affects Two Alternative Mechanisms for ssDNA Elongation by Pol λ -tdt—We have previously shown that pol λ interacts physically and functionally with PCNA (5). Thus, we next determined whether PCNA could influence the two ssDNA elongation activities of pol λ described above. Increasing amounts of human PCNA were titrated in the presence of either dTTP or dGTP with the -CCC terminal substrate (Table I, oligo 23) or in the presence of dTTP or dATP with the

-AAAAAC terminal substrate (Table I, oligo 16). As shown in Fig. 6, on the oligo 23, PCNA inhibited dGTP addition, whereas it stimulated dTTP incorporation. Similarly, on oligo 16, PCNA inhibited the incorporation of the complementary nucleotide dTTP, while it stimulated dATP addition. We have shown

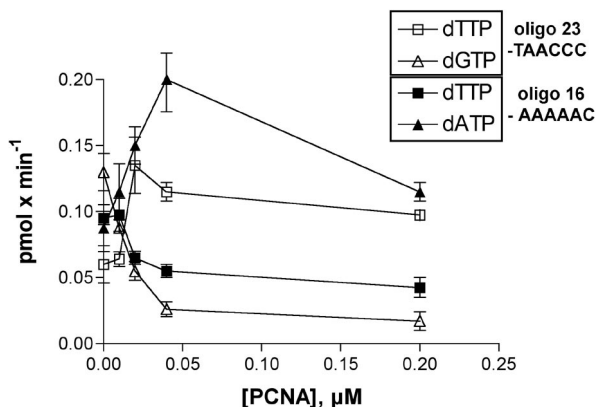


FIG. 6. Effect of PCNA on ssDNA elongation by pol λ -tdt. Increasing amounts of human recombinant PCNA were titrated in terminal nucleotide addition assays in the presence of 100 nM pol λ , 2 μ M ssDNA 24-mer (oligo 23, Table I) (empty symbols), and 2 μ M labeled dGTP (squares) or dTTP (triangles) or 2 μ M ssDNA 30-mer (oligo 16, Table I) (filled symbols) and 2 μ M labeled dTTP (circles) or dATP (triangles). PCNA concentrations used (as trimer) were 10, 20, 40, and 200 nM.

previously that interaction of PCNA with pol λ stabilizes the binding of the enzyme to a 3'-OH end (4). Thus, these results might suggest that PCNA induces a steric block, preventing looping back of the 3'-end by pol λ and thus inhibiting the template-directed incorporation of the complementary nucleotide, whereas it stimulated terminal nucleotide addition by preventing enzyme dissociation from the 3'-end.

PCNA Can Inhibit the Looping Back Mechanism by Pol λ , and This Requires Their Physical Interaction—The inhibitory mechanism by PCNA was next investigated by titrating increasing PCNA amounts in the presence of dGTP and different fixed concentrations of the -CCC substrate (oligo 23) (Fig. 7A), and variation of the K_{DNA} and k_3 parameters as a function of the PCNA concentrations were derived by computer fitting analysis (see "Material and Methods"). The calculated K_{DNA} and k_3 values were then plotted as a function of the PCNA concentration (Fig. 7B). Similarly increasing amounts of PCNA were titrated into the reaction in the presence of fixed concentrations of oligo 23, and the resulting inhibition curves are shown in Fig. 7C along with the calculated inhibition constant (K_D) of PCNA for pol λ . From these experiments, it was evident that both the K_{DNA} and k_3 parameters for the pol λ -tdt reaction were decreased by PCNA (Fig. 7B), whereas the inhibition by PCNA was not affected by the concentration of the 3'-OH ends (Fig. 7C). These results were consistent with an uncompetitive mechanism of inhibition by PCNA. The simplest molecular mechanism giving rise to uncompetitive inhibition is the pref-

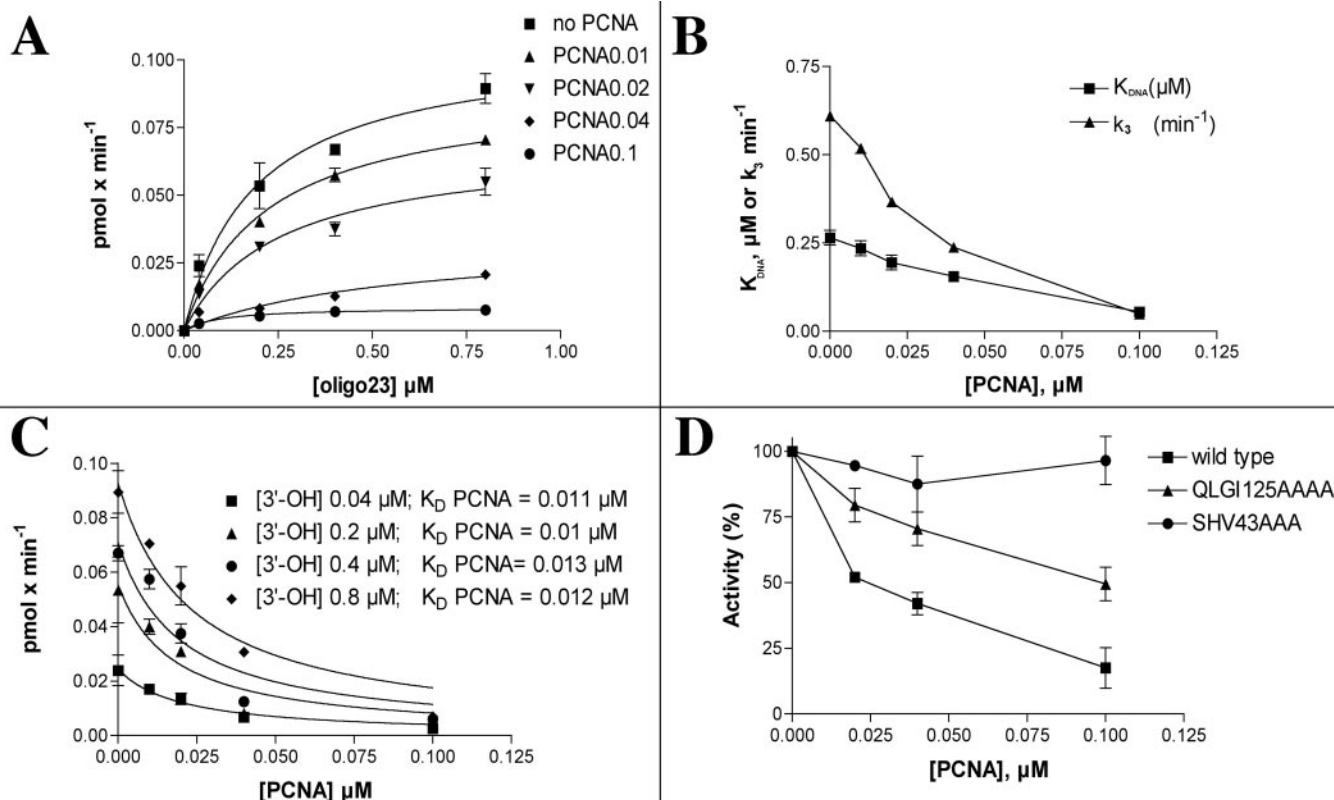


FIG. 7. Physical interaction between pol λ and PCNA is required for inhibition of the looping back mechanism of ssDNA elongation. A, different fixed PCNA concentrations were titrated in terminal nucleotide addition assays in the presence of 100 nM pol λ , 2 μ M labeled dGTP, and increasing amounts of ssDNA 24-mer (oligo 23, Table I). PCNA concentrations as trimers were as follows: none (squares), 10 nM (upward triangles), 20 nM (downward triangles), 40 nM (diamonds), and 100 nM (circles). DNA concentrations used were 0.04, 0.2, 0.8, and 2 μ M. B, the k_3 and K_{DNA} values were calculated by fitting the initial velocities of the reaction in the absence or presence of increasing PCNA concentrations to the hyperbolic equation $v = k_3[E]_0/(1 + K_{DNA}/[DNA])$, and their variation was plotted as a function of PCNA. C, increasing amounts of PCNA were titrated in the presence of 100 nM pol λ , 2 μ M labeled dGTP, and increasing amounts of ssDNA 24-mer (oligo 23, Table I). Dose-response curves were generated, and the inhibition constant for PCNA (K_D) was calculated at each DNA concentration. PCNA concentrations as trimers were 10, 20, 40, and 100 nM. D, increasing amounts of PCNA wild type (squares) or the QLGI125AAAA (QLGI125AAAA, triangles) and SHV43 (SHV43AAA, circles) mutants were titrated in terminal nucleotide addition assays in the presence of 100 nM pol λ , 2 μ M labeled dGTP, and 2 μ M ssDNA 24-mer (oligo 23, Table I). The PCNA concentrations as trimer were 20, 40, and 100 nM.

erential association of the inhibitor to the complex of the enzyme with its substrates (25). Thus, these results suggested that, to inhibit dGTP incorporation, PCNA had to associate to the pol λ -DNA complex.

To verify whether PCNA inhibition also required physical interaction with pol λ , we tested the two human PCNA mutants QLG125 (Q125A/L126A/G127A/I128A) and SHV43 (S43A/H44A/V45A). We have previously shown that these two PCNA mutants were impaired in physical interaction with pol λ but were correctly folded and formed homotrimers in solution (4). The mutant SHV43, which has been shown to be unable to interact with pol λ , failed to inhibit dGTP incorporation on the -CCC substrate, whereas the mutant QLG125, which has been shown to have a reduced affinity for pol λ , could still inhibit the reaction but to a lesser extent with respect to wild type PCNA (Fig. 7D). This almost perfect correspondence between the effects of the two PCNA mutants, in terms of interaction with pol λ , and their ability to suppress dGTP incorporation suggested that physical interaction between PCNA and pol λ was required for inhibition of the template-directed elongation of ssDNA.

RP-A Differentially Regulates the DNA Polymerase and the tdt Activities of Pol λ —Since pol λ contains both polymerase and tdt activities, one major question is how these two activities might be regulated. One possible mechanism might be suggested by the experiments shown in Fig. 2A, which indicated how different DNA structures, a 3'-protruding end for the tdt and a 3'-recessed end for the DNA polymerase, are required for these two activities. In a eukaryotic cell, the ssDNA is covered by the ssDNA-binding protein RP-A. When increasing concentrations of RP-A were titrated in the presence of dTTP and two fixed concentrations of the reference substrate -AAC (oligo 11) were used, RP-A inhibited pol λ -tdt activity in a dose-dependent manner (Fig. 8A). Moreover the inhibition was dependent on the DNA substrate concentrations, thus suggesting a competitive mechanism. This was confirmed by measuring the effects of increasing amounts of RP-A on the K_{DNA} and k_3 kinetic constants of pol λ . RP-A affected specifically the K_{DNA} of pol λ , suggesting that RP-A-covered ssDNA cannot be used for terminal nucleotide addition (Fig. 8B). Next the effects of RP-A on the DNA polymerase activity of pol λ were tested. RP-A stimulated nucleotide incorporation by pol λ in a standard DNA polymerase assay with the homopolymeric primer-template poly(dA)/oligo(dT) (Fig. 9, A and B). The amount of RP-A required for optimal stimulation was dependent on the concentration of the DNA substrate, suggesting that pol λ preferred RP-A-covered ssDNA as a template for the DNA polymerization reaction. The stimulatory effect was more pronounced when a long circular ss natural DNA (M13DNA containing a single primer) was tested as a template. While pol λ was almost inactive on uncovered singly primed M13DNA, RP-A greatly stimulated its activity. Moreover this stimulatory effect by RP-A was specific since comparable amounts of *E. coli* single-stranded DNA-binding protein SSB inhibited the reaction (Fig. 9C). Finally the kinetic parameters for DNA utilization by pol λ were determined in the absence or in the presence of RP-A either with the poly(dA)/oligo(dT) primer-template or with the -AAC ssDNA substrate (Table I, oligo 11). The variation of the efficiency parameter (k_{cat}/K_m) for DNA utilization of pol λ as a function of the RP-A concentration was plotted (Fig. 9D). RP-A specifically increased the DNA utilization efficiency of the pol λ DNA polymerase activity but inhibited the pol λ -tdt activity, suggesting that RP-A might be involved in regulating the balance between these two catalytic activities of pol λ .

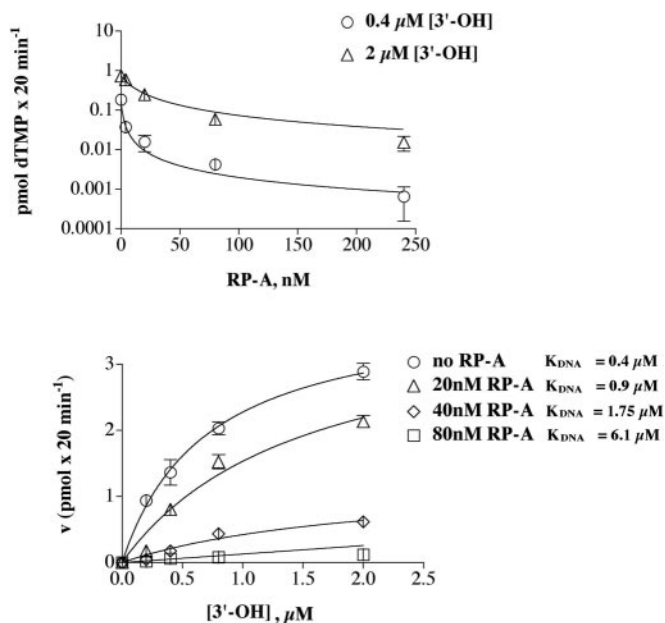


FIG. 8. RP-A inhibits the terminal transferase activity of pol λ . A, increasing amounts of RP-A were titrated in terminal nucleotide addition assays in the presence of 100 nM pol λ , 2 μM labeled dTTP, and 0.4 μM (circles) or 2 μM (triangles) ssDNA 27-mer (oligo 11, Table I). RP-A concentrations as heterotrimers were 10, 20, 80, and 240 nM. B, the variation of the terminal nucleotide addition velocity as a function of ssDNA concentrations was studied in the presence of 100 nM pol λ and 2 μM labeled dTTP and in the absence (circles) or in the presence of 20 nM (triangles), 40 nM (diamonds), or 80 nM (squares) RP-A. 24-mer ssDNA (oligo 11, Table I) concentrations used were 0.2, 0.4, 0.8, and 2 μM . Steady-state K_{DNA} values were calculated by fitting the initial velocities to the hyperbolic equation $v = k_{\text{cat}}[E]_0/(1 + K_{\text{DNA}}/[\text{DNA}])$.

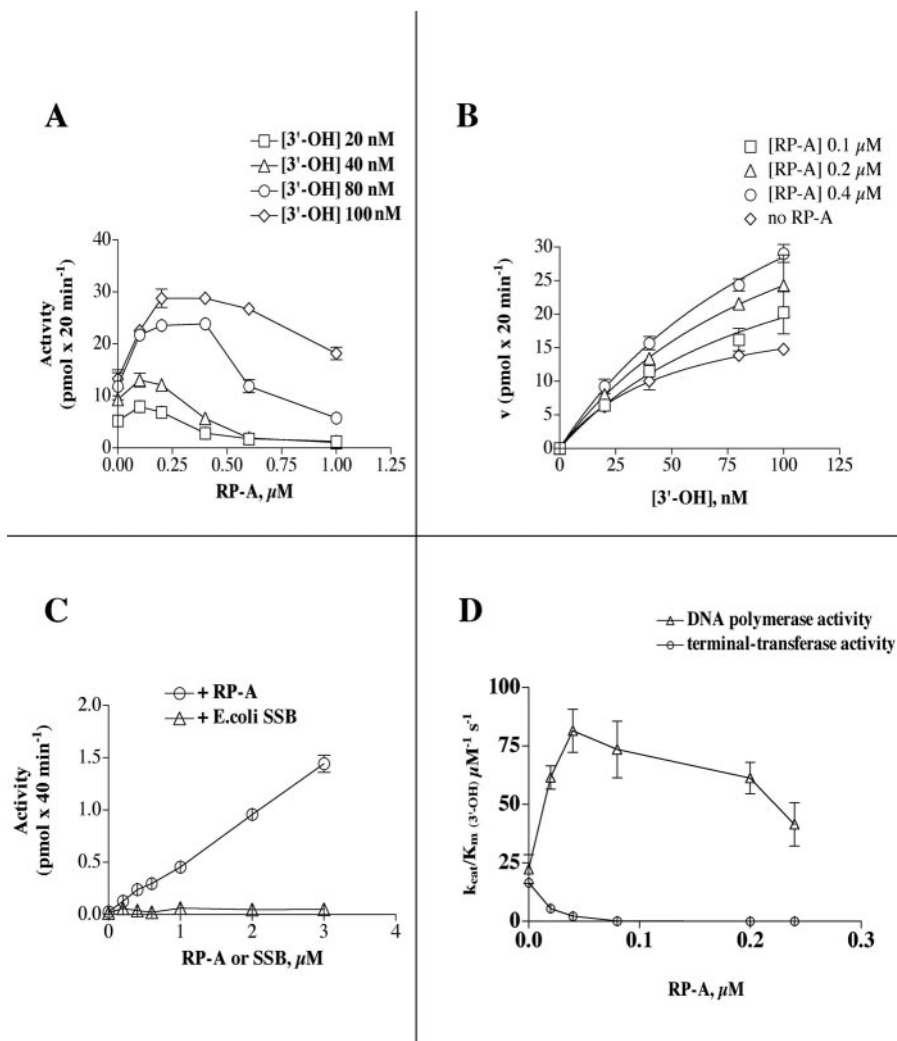
DISCUSSION

A fascinating feature of pol λ is the presence of two different, potentially conflicting activities within the same active site, namely a template-directed DNA polymerase and a template-independent terminal transferase activity. Given the unknown role(s) of this enzyme *in vivo*, understanding how these two activities are regulated is of great interest. Based on the results presented in this work, we propose that at least three basic mechanisms might coordinate these two functions of pol λ .

The Structure and Sequence of the DNA Substrate—In the presence of a 3'-recessed hydroxyl end, pol λ synthesizes DNA strictly in a template-directed manner (27). Even when challenged with a non-instructional lesion such as an abasic site, its bypass activity does not depend on terminal nucleotide addition (terminal transferase) but rather proceeds through a next nucleotide stabilized primer misalignment mechanism (12). In this work, on the other hand, we showed that pol λ absolutely requires a ssDNA tract of at least 9–12 nucleotides for terminal transferase activity. In addition, the sequence of the DNA substrate also plays an important role. We have shown that pol λ as a DNA polymerase can promote template slippage on repetitive DNA sequences (12). In the present study we showed that only certain sequences at the ssDNA 3'-hydroxyl end can be further elongated by its terminal transferase activity. Thus, the different structure and sequence of the DNA substrate encountered by pol λ might influence its properties as a DNA polymerase or as a terminal transferase.

The Interaction with PCNA—Previous work showed that pol λ interacts with PCNA, which, in turn, stabilizes the binding of the enzyme to the 3'-hydroxyl end and stimulates its processivity as well as its lesion bypass activity (4). Here we showed that pol λ , because of its remarkable ability to promote base pairing between short homologous DNA tracts, can elongate a

FIG. 9. RP-A stimulates the DNA polymerase activity of pol λ . *A*, DNA polymerase assays were performed as described under "Material and Methods" in the presence of 50 nM pol λ , 2 μ M labeled dTTP, and 20 nM (squares), 40 nM (triangles), 80 nM (circles), or 100 nM (diamonds) poly(dA)/oligo(dT) template-primer as 3'-OH ends. Increasing concentrations of RP-A were titrated in the assays at each DNA concentration. RP-A concentrations as heterotrimers were 0.1, 0.2, 0.4, and 1 μ M. *B*, the variation of the DNA polymerase reaction velocity as a function of poly(dA)/oligo(dT) concentration was studied in the presence of 50 nM pol λ and 2 μ M labeled dTTP and in the absence (diamonds) or in the presence of 0.1 μ M (squares), 0.2 μ M (triangles), or 0.4 μ M (circles) RP-A. Poly(dA)/oligo(dT) concentrations as 3'-OH ends were 20, 40, 80, and 100 nM. *C*, increasing amounts of RP-A (circles) or *E. coli* SSB (triangles) were titrated into nucleotide incorporation assays in the presence of 50 nM pol λ , 40 nM (as 3'-OH ends) singly primed M13DNA, and 10 μ M labeled dNTPs. RP-A or SSB concentrations used were 0.1, 0.2, 0.4, 1, 2, and 3 μ M. *D*, the kinetic parameters k_{cat} and K_m for DNA utilization by pol λ were calculated as described under "Material and Methods" under DNA polymerase (*i.e.* with poly(dA)/oligo(dT)) and terminal transferase (*i.e.* with oligo 11) assay conditions in the absence or presence of increasing amounts of RP-A. In the case of the terminal transferase reaction, $k_{cat} = k_3$ and $K_m = K_{DNA}$. The variation of the DNA utilization efficiency parameter (k_{cat}/K_m) for the DNA polymerase (triangles) or the terminal transferase (circles) activity was plotted as a function of different RP-A concentrations, which were 0.1, 0.2, 0.4, 1, and 1.2 μ M, respectively.



ssDNA 3'-end in two ways: first, a template-independent one resulting in addition of 1 or 2 nucleotides, and second, a template-dependent one where a homopolymeric tract as short as 3 nucleotides at the 3'-end of the substrate can be used as a template to direct DNA polymerization via a looping back mechanism. It has to be noted that not all the homopolymeric sequences served as a substrate. In fact, the oligonucleotide substrate 20, bearing a -GGG sequence at its 3'-end, did not support dCTP incorporation (Table I). This could indicate that a single nucleotide addition by the tdt activity of pol λ on a suitable sequence is required first to promote the looping back mechanism. PCNA can modulate these two different activities by preventing the looping back, likely through a steric block, while stimulating the terminal addition by binding to pol λ .

The Action of RP-A—Both template-dependent and template-independent activities of pol λ require ssDNA. However, in mammalian cells, ssDNA is readily covered by RP-A. Here we showed that RP-A-covered ssDNA is a good substrate for the DNA polymerase activity of pol λ , whereas it is no longer a substrate for its terminal transferase activity. Thus, RP-A might be another regulator of the activity of pol λ in the cell.

Can these properties be extrapolated to the *in vivo* roles of pol λ ? Pol λ has been proposed to act in the nonhomologous end joining mechanism of double strand break repair (6, 9). Indeed one of the intermediates of double strand break repair is 3'-ssDNA overhangs, which are an optimal substrate for pol λ terminal transferase activity. Thus, it is possible to envision a scenario where pol λ can either terminally add

nucleotides, extending the ssDNA overhangs and promoting microhomology base pairing, or can loop back the 3'-end, creating a closed, partial dsDNA end. The balance between these two activities might be determined by the presence of RP-A and PCNA. It is possible that a looped dsDNA end could be a "signal" to trigger other types of cellular response, for example resembling an intermediate of VDJ recombination (28), or it could be melted and used to perform strand invasion on the complementary DNA strand, starting homologous recombination. The fact that this looping back mechanism is triggered by homopolymeric DNA tracts places it at the heart of a possible double strand break repair mechanism in genomic regions containing highly repetitive DNA sequences, which were found to be hotspots for recombination.

Another possible scenario could be telomeric end healing. The remarkable nucleotide and sequence selectivity shown by pol λ terminal transferase on telomeric sequences of opposite strands might suggest that this enzyme could participate in the repair of double strand breaks at chromosome ends (29). Its ability to generate long ss terminal tracts as well as to promote homology pairing would fit well with the occurrence of unusual DNA structures at the telomeres such as t-loops.

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