The*f*cientificWorld
www.thescientificworld.com

The TREX2 3′→5′ Exonuclease Physically Interacts with DNA Polymerase δ and Increases Its Accuracy

Igor V. Shevelev[1,2], Kristijan Ramadan[1], and Ulrich Hübscher[1,*]

[1]*Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057, Zürich (Switzerland);* [2]*Department of Molecular and Radiation Biophysics, Petersburg Nuclear Physics Institute, Leningrad district, Gatchina, 188300 (Russia)*

Proofreading function by the 3′→5′ exonuclease of DNA polymerase δ (pol δ) is consistent with the observation that deficiency of the associated exonuclease can lead to a strong mutation phenotype, high error rates during DNA replication, and ultimately cancer. We have isolated pol δ from isotonic (pol δ_i) and detergent (pol δ_d) calf thymus extracts. Pol δ_d had a 20-fold higher ratio of exonuclease to DNA polymerase than pol δ_i. This was due to the physical association of the TREX2 3′→5′ exonuclease to pol δ_d, which was missing from pol δ_i. Pol δ_d was fivefold more accurate than pol δ_i under error-prone conditions (1 mM dGTP and 20 μM dATP, dCTP, and dTTP) in a M13mp2 DNA forward mutation assay, and fourfold more accurate in an M13mp2T90 reversion assay. Under error-free conditions (20 μM each of the four dNTPs), however, both polymerases showed equal fidelity. Our data suggested that autonomous 3′→5′ exonucleases, such as TREX2, through its association with pol δ can guarantee high fidelity under difficult conditions in the cell (e.g., imbalance of dNTPs) and can add to the accuracy of the DNA replication machinery, thus preventing mutagenesis.

KEY WORDS: isotonic and detergent extracted pol δ, proofreading, TREX2 3′→5′ exonuclease

DOMAINS: enzymology, protein-protein interaction, biochemistry

INTRODUCTION

Eukaryotic DNA polymerases (pol) δ and ε have the ability to proofread replication errors by using their 3′→5′ exonuclease activities. Genetic analysis has shown that fidelity is essential for DNA replication, since mutations affecting the exonuclease activity pol δ and pol ε result in high mutation rates *in vivo*[1]. Moreover, transgenic mice with exo⁻ mutated pol δ alleles showed a

striking increase in cancer susceptibility within 12 months of age[2]. Nevertheless, several experimental results point to the possibility of additional proofreading by autonomous (not an inherent exonuclease of pol δ or pol ε) 3′→5′ exonucleases during DNA replication or repair. The base substitution error rate of pol δ has been shown to be 27-fold higher in the presence of proliferating cell nuclear antigen (PCNA)[3]. The inefficient excision of nucleotide analogues has been demonstrated for pol δ and ε[4]. The nucleotide substitution error rate for *Schizosaccaromyces pombe* pol δ holoenzyme (pol δ, replication factor C, and PCNA) was 5×10^{-4} for T:G mispairs, two orders of magnitude lower than for *Escherichia coli* pol III holoenzyme at the same position[5]. Several mammalian 3′→5′ exonucleases such as Werner protein[6], p53[7], calf thymus[8], and rat liver autonomous 3′→5′ exonucleases[9] have been found to be associated with eukaryotic pol δ or other components of the replication fork. Finally, fidelity data of a human cell DNA replication complex suggested that additional fidelity factors are likely present during chromosomal replication *in vivo*[10,11]. In this paper we show that detergent extracted pol δ_d from calf thymus has a 20-times-higher exonuclease to DNA polymerase ratio than isotonic extracted pol δ_i (pol δ from isotonic extract). This higher ratio is due to the association of an additional 3′→5′ exonuclease, and the exonuclease was identified as TREX2[12].

As a consequence of this association pol δ_d (pol δ from detergent extract) was four to five times more accurate at error-prone conditions as determined in an *in vitro* forward mutation assay, suggesting that autonomous 3′→5′ exonuclease can proofread for pol δ.

MATERIAL AND METHODS

Poly(dA)$_{1000-1500}$ and oligo(dT)$_{12-18}$ were purchased from Pharmacia and Sigma. Poly(dA)/oligo(dT) template (base ratio 10:1) was prepared as described by Weiser et al.[13]. PCNA was overexpressed in *E. coli* strain BL21 (DE3) harboring the expression plasmid pT7/PCNA and purified as described by Stucki et al.[14]. PCNA-dependent and -independent pol assays were carried out according to Weiser et al.[13]. 3′→5′ exonuclease was measured according to Belyakova et al.[15]. One unit of DNA polymerase activity corresponds to the incorporation of 1 nM total dNTPs into acid precipitate material in 60 min at 37ºC in a standard assay for pol δ[13]. One unit of exonuclease activity is defined as the amount of the enzyme causing the conversion of 10% 3′–end labeled ss DNA (0.2 μg, 10,000 cpm/μg) to acid soluble products in 60 min at 37ºC in 50 mM Tris-HCl pH 7.5, 1 mM DTT, and 5 mM MgCl$_2$. 3′→5′ exo:pol is the ratio of excised nucleotides (pmol) vs. those incorporated (pmol), when concentrations of limiting substrates being 0.1 Km include optimal conditions for each enzyme in separate parallel experiments.

Preparation of isotonic and detergent cell extracts: all isolation steps were performed at or near 0°C. The isotonic extract — (I): 300 ml buffer A (50 mM Tris pH 7.0, 10% (v/v) glycerol, 0.5 mM EDTA, 50 mM NaCl, 0.25 mM sucrose, 5 μg/ml pepstatin, 1 μg/ml leupeptin, 0.8 mM PMSF, and 1 mM DTT) — was added to 100 g calf thymus. The tissue was thawed, homogenized in a Sorvall Omnimixer, stirred gently for 2 h, and centrifuged at 25,000 × g for 45 min. The supernatant (supernatant I) was achieved. The pellet was again extracted as described above, and the supernatant (supernatant II) was mixed with supernatant I. From the mixed supernatants I and II, isotonic extract was obtained. The detergent extract — (D): the resulting pellet (40 g) (after supernatant II extraction) was resuspended in 120 ml of buffer B (50 mM Tris pH 7.0, 10% [v/v] glycerol, 0.5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 5 μg/ml pepstatin, 1 μg/ml leupeptin, 0.8 mM PMSF, and 1 mM DTT), homogenized by 20 strokes in Dounce homogenizer, stirred for 2 h, and centrifuged at 12,000 × g for 45 min. The supernatant III was achieved. The pellet was again extracted on the same manner, and supernatant IV was achieved. Detergent extract was obtained from the mixed supernatants III and IV.

TABLE 1
Characteristics of Calf Thymus pol δ_i and pol δ_d[a]

Pol	(mg/ml)	Pol +PCNA (U/ml)	Pol -PCNA (U/ml)	Specific Activity (U/mg)	3′→5′ Exo (U/ml)	Exo/Pol Ratio
pol δ_i	0.1	64	1.8	640	204	3
pol δ_d	0.08	18	1.2	225	1100	61

[a]For details see Materials and Methods.

The subsequent purification steps for both pol δ_i and pol δ_d were described earlier[13], and some enzyme characteristics are shown in Table 1.

Pol δ from calf thymus isotonic extract was also purified with modified protocol that we used earlier. The isotonic extract was prepared on the same manner as before described. Fraction I (isotonic extract) was adsorbed to 60 ml phosphocellulose column (Whatman), and pol activity was eluted with linear gradient of 50–600 mM NaCl. Fractions eluted between 80–100 mM NaCl had pol activity with PCNA dependency (2-fold). These fractions were pooled to yield fraction II. Fraction II was loaded to 1 ml HiTrap heparin column (Amersham Pharmacia), and pol activity eluted with linear gradient of 50–1000 mM NaCl. Fractions eluted between 100–150 mM NaCl had PCNA dependency (5-fold). These fractions were pooled to yield fraction III. Fraction III was adsorbed on HiTrap Q column (Amersham Pharmacia), and pol activity eluted with linear gradient of 50–1000 mM NaCl. Fractions with 20-fold PCNA dependency (fraction IV) were eluted between 100–150 mM NaCl, containing mainly pol δ. Fraction IV was adsorbed on monoQ column (Amersham Pharmacia), and pol δ was eluted between 200–250 mM NaCl. The final fraction had 50-fold PCNA dependency.

Bacteriophage M13mp2 and *E. coli* strains CSH50, NR9099, and MC1061 were described by Kunkel[16]. Nonsense mutant M13 mp2T90 (point mutation G:T in the position +90 of *LacZ* gene) was selected after sequencing analysis of colorless mutants as mutation hot point in DNA M13mp2 forward mutation assay. The sequence of the 26 mer oligonucleotide was: 5′-cga tta agt tgg gta acg cca ggg tt-3′. Immunoprecipitation and immunobloting experiments were made according to Koundrioukoff et al.[17]. Chicken nonimmune IgY was used as a control for nonspecific binding in the immunoprecipitation experiment. The fusion human TREX2-MBP protein was expressed and purified as described by Mazur et al.[18] and used for producing chicken IgY according to Gassmann et al.[19]. Rabbit antisera against a peptide in N-terminal region of human pol δ (p125) was the gift of P. Fisher. Monoclonal antibody against human B-subunit of pol δ (p50) was kindly provided by H.-P. Nasheuer (Jena). Recombinant His-tagged p50 protein (B-subunit of pol δ) was overexpressed in *E. coli* and used as a positive control for the coimmunoprecipitation experiments.

RESULTS AND DISCUSSION

In eukaryotes, spontaneous mutation rates have been estimated to be 10^{-10}–10^{-12} errors per base pair per generation. On the other hand, the principal elongation enzyme pol δ with its own 3′→5′ exonuclease activity site makes replication errors with probability of 10^{-5}[20]. Our explanation for such an inaccuracy can be the loss of subunits of the eukaryotic pols that function during replication to improve the fidelity. Presumably, part of these errors could be eliminated by autonomous 3′→5′ exonucleases. Indeed, there is the immunochemical evidence for the complex formation between pol δ and some potent autonomous 3′→5′ exonucleases from calf thymus[21].
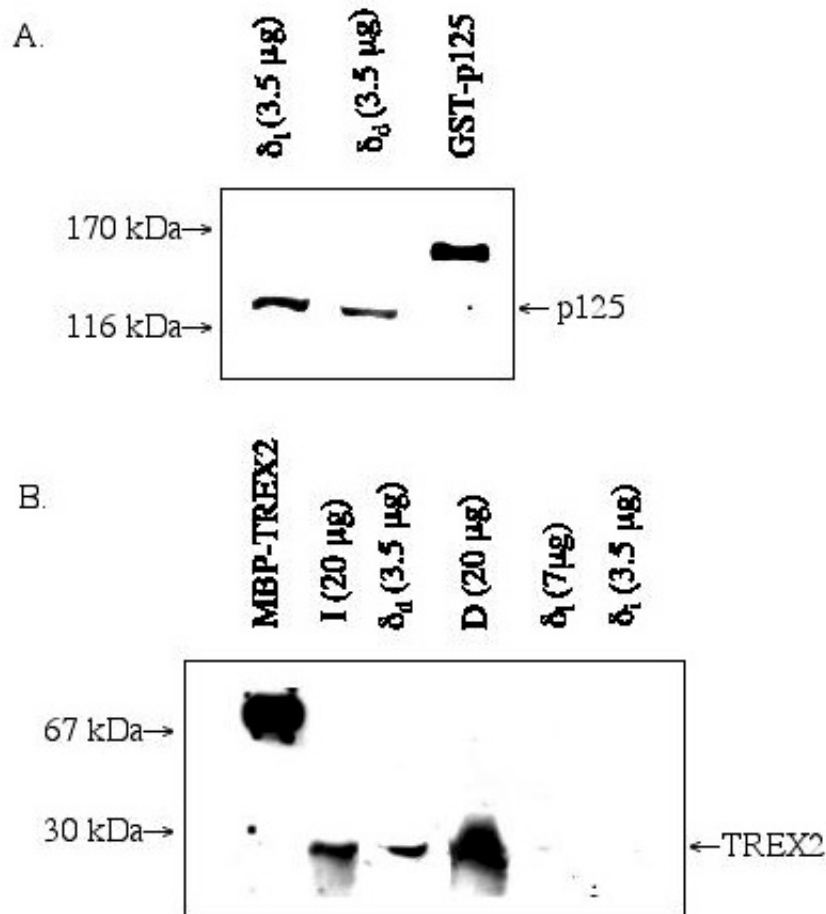
FIGURE 1. The TREX2 3′→5′ exonuclease copurifies with pol δ_d. A: Western blot analysis of calf thymus pol δ_i and δ_d with polyclonal antibody against C-terminal part of catalytical subunit of pol δ (p125). B: Isotonic (I), detergent (D) calf thymus extracts pol δ_i and pol δ_d were loaded on 8% SDS-PAGE and transferred on nitrocellulose membrane. Polyclonal chicken antibody against TREX2 recognizes TREX2 exonuclease in both extracts and pol δ_d.

Moreover, autonomous 3′→5′ exonucleases are responsible for 80% of the total 3′→5′ exonuclease activity in rat liver cells[9]. We showed, in our unpublished data, that additional 3′→5′ exonuclease such as DnaQ from *E. coli*, TREX1, TREX2, and Werner protein can enhance in 2- to 10-fold the accuracy of pol I of *E. coli*, pol δ, and ε from calf thymus under error-prone conditions in a M13mp2 forward mutation assay.

To approach this problem, we have monitored the purification of pol δ from isotonic and detergent calf thymus extracts. We obtained two pol δ preparations designated as pol δ_i and pol δ_d (Table1). Both pol δs were confirmed by immunobloting with antibodies against p125 (Fig. 1A) and p50 subunits (data not shown). The main difference between these two pol δs was a 20-times-higher ratio of 3′→5′ exonuclease to DNA polymerase. Both pol δs were free from endonuclease and 5′→3′ exonuclease activities (data not shown). Hoping to find a form of pol δ from isotonic extract with a more comparable exonuclease:DNA polymerase ratio, we have changed the purification protocol. However, we were able to purify pol δ by the modified protocol (see Material and Methods) than by Weiser et al.[13]. The other pol δ forms from isotonic extract had the same exonuclease:DNA polymerase ratio as pol δ_i (data not shown). These data suggested to us that pol δ_d might interact with an additional 3′→5′ exonuclease. The 3′→5′ exonuclease was identified in pol δ_d as TREX2 protein (Fig. 1B). The immunoprecipitation experiment also showed
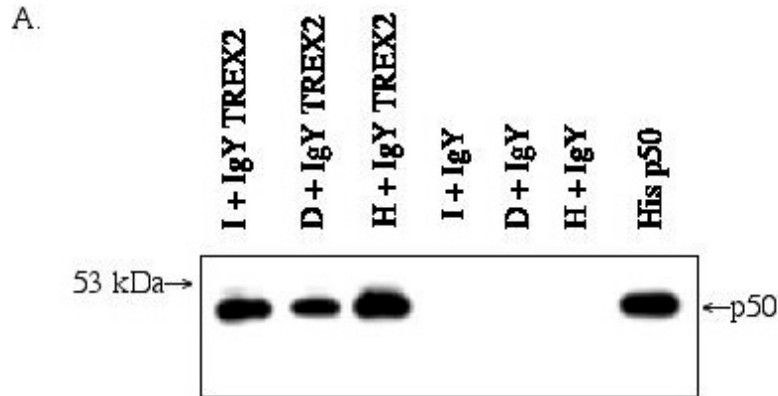
FIGURE 2. The TREX2 3′→5′ exonuclease physically interacts with pol δ. I, D (see Fig. 1B) and human (cell line Mg63, osteosarcoma) total cell extract (H) were immunoprecipitated with anti-TREX2 polyclonal antibody (IgY TREX2), or with chicken preimmune IgY as a control, and were analyzed by Western blot with rat monoclonal antibody against the small subunit of pol δ (p50). For this, a 12% SDS-PAGE and nitrocellulose membrane was used.

that TREX2 protein and pol δ form a complex in different prepared extracts (isotonic, detergent) (Fig. 2), indicating that TREX2 and pol δ interact. TREX2 also interacted with pol δ in human total cell extract suggesting that our finding is likely valid to any mammalian cells.

In order to understand a possible proofreading role of TREX2, we next used a forward mutation assay (Table 2). Pol δ_d showed the same mutation frequency as pol δ_i under error-free conditions (20 μM dNTPs). However, the TREX2 containing pol δ_d was about fivefold more accurate than pol δ_i under error-prone conditions (50-fold biased dGTP); 170 to 200 nucleotides were incorporated in each 250 target gap DNA for reactions of 2 h (data not shown). Next, the mutation frequency of base substitution was estimated for pol δ_i and pol δ_d (Table 3). To verify that in every case the nucleotides were incorporated in the nonsense codon (TAA, LacZ 90), an additional 26 mer oligonucleotide was hybridized to the gap molecules (the 3′-end of primer being located as three nucleotides from the point substitution T in the nonsense mutant instead of G in the M13mp2 at position +90 of LacZ gene). A fourfold higher fidelity for pol δ_d in comparison with pol δ_i was evident for base substitution under error-prone conditions.

TABLE 2
Mutation Frequency of DNA Copied by pol δ_i and pol δ_d in a Forward Mutation Assay[b]

Pol	dNTPs	Total Plaques	Mutants Plaques	Mutation Frequency ($\times 10^{-4}$)
None		7,000	4	5.7
pol δ_I	Equimolar	7,000	8	11.4
	Biased dGTP	4,000	45	112.5
pol δ_d	Equimolar	9,000	10	11.1
	Biased dGTP	6,000	14	23.2

[b]Replication reactions were carried out for 2 h at 37ºC in 10 μl containing: 50 mM Tris-HCl pH 7.5, 1 mM DTT, 100 μg/ml BSA, 6 mM MgCl₂, 50 ng of gapped circular M13mp2 DNA, 20 μM each of dATP, dCTP, dGTP, and dTTP (equimolar) or additional 1 mM dGTP (biased dGTP), and pol δ_i (0.064 units) or pol δ_d (0.054 units).

TABLE 3
Mutation Frequency of DNA Copied by pol δ_i and pol δ_d in a Reversion Mutation Assay[c]

Pol	dNTPs	Oligo[2]	Plaques Total	Mutant Plaques	Revertant Frequency ($\times 10^{-5}$)
None	-	-	400, 000	6	1.5
		+	520, 000	7	1.3
pol δ_I	Equimolar	-	320, 000	10	3.1
		+	460, 000	14	3.0
	Biased dGTP	-	150, 000	48	32.0
		+	220, 000	53	27.5
pol δ_d	Equimolar	-	280, 000	8	2.9
		+	540, 000	17	3.1
	Biased dGTP	-	210, 000	18	8.6
		+	290, 000	22	7.6

[c]Replication reactions were carried out for 2 h at 37ºC by using 50 ng gapped circular M13mp2T90 (sign – in oligo column), or for 10 min at 37ºC when a 26 mer oligonucleotide was hybridized in addition to the gapped circular M13mp2T90 DNA (sign + in oligo column). Other conditions were as in Table 2.

Our experiments for the proofreading function of TREX2 for pol δ have been demonstrated for the distributive synthesis of pol δ only. Next, it would be of interest to understand the proofreading of TREX2 for the processive synthesis of pol δ holoenzyme (pol δ, RF-C, RP-A, and PCNA).

CONCLUSION

In conclusion we have identified from a detergent extract 3′→5′ exonuclease TREX2 associated with pol δ. The fact that this editing enzyme can enhance the fidelity of pol δ under difficult conditions strongly suggests that an autonomous exonuclease can proofread for pol δ. This might be important when pol δ has to dissociate from the DNA under genotoxic stress. As a consequence, this proofreading mechanism might help to minimize mutagenesis and consequently cancer propagation.

ACKNOWLEDGEMENTS

REFERENCES

1. Tran, H.T., Gordenin, D.A., and Resnick, M.A. (1999) The 3′→5′ exonucleases of DNA polymerases delta and epsilon and the 5′→3′ Exo I have major role in postreplication mutation avoidance in *Sacchyromyces cerevisiae*. *Mol. Cell. Biol.* **19**, 2000–2007.
2. Goldsby, R.E., Lawrence, N.A., Hays, L.E., Olmsted, E.A., Chen, X., Singh, M., and Preston, B.D. (2001) Defective DNA polymerase delta proofreading causes cancer susceptibility in mice. *Nat. Med.* **7**, 638–639.

3. Mozzherin, D.J., McConnell, M., Jasko, M.V., Krayevsky, A.A., Tan, C.K., Downey, K.M., and Fisher, P.A. (1996) Proliferating cell nuclear antigen promotes misincorporation catalyzed by calf thymus DNA polymerase delta. *J. Biol. Chem.* **271**, 31711–31717.

4. Perrino, W.F., Mazur, D.J., Ward, H., and Harvey, S. (1999) Exonucleases and the incorporation of aranucleotides into DNA. *Cell Biochem. Biophys.* **30**, 331–352.

5. Chen, X., Zuo, S., Kelman, Z., O`Donnell, M., Hurwitz, J., and Goodman, M.F. (2000) Fidelity of eukaryotic DNA polymerase delta holoenzyme from *Schizosaccharomyces pombe*. *J. Biol. Chem.* **275**, 17677–17682.

6. Kamath-Loeb, A.S., Johansson, E., Burgers, P.M.J., and Loeb, L.A. (2000) Functional interaction between the Werner syndrome protein and DNA polymerase delta. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4603–4608.

7. Huang, P. (1998) Excision of mismatched nucleotides from DNA: a potential mechanism for enhancing DNA replication fidelity by the wild-type p53 protein. *Oncogene* **17**, 261–270.

8. Jiang, Y., Zhang, S.J., Wu, S.M., and Lee, M.Y.W.T. (1995) Immunoaffinity purification of DNA polymerase delta. *Arch. Biochem. Biophys.* **320**, 297–304.

9. Shevelev, I.V., Kravetskaya, T.P., Legina, O.K., and Krutyakov, V.M. (1996) "External" proofreading of DNA replication errors and mammalian autonomous 3′→5′ exonucleases. *Mutat. Res.* **352**, 51–55.

10. Roberts, J.D. and Kunkel T.A. (1988) Fidelity of a human cell DNA replication complex. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7064–7068.

11. Bebenek, K., Matsuda, T., Masutoni, C., Hanaoka, F., and Kunkel, T.A. (2001) Proofreading of DNA polymerase eta-dependent replication errors. *J. Biol. Chem.* **276**, 2317–2320.

12. Mazur, D.J. and Perrino, F.W. (1999) Identification and expression of the TREX1 and TREX2 cDNA sequences encoding mammalian 3′→5′ exonucleases. *J. Biol. Chem.* **274**, 19655–19660.

13. Weiser, T., Gassman, M., Thömmes, P., Ferrari, E., Hafkemeyer, P., and Hübscher U. (1991) Biochemical and functional comparison of DNA polymerases alpha, delta and epsilon from calf thymus. *J. Biol. Chem.* **266**, 10420–10428.

14. Stucki, M., Pascucci, B., Parlanti, E., Fortini, P., Wilson, S.H., Hübscher, U., and Dogliotti, E. (1998) Mammalian base excision repair by DNA polymerases delta and epsilon. *Oncogene* **17**, 835–843.

15. Belyakova, N.V., Kleiner, N.E., Kravetskaya, T.P., Legina, O.K., Naryzhny, S.N., Perrino, F.W., Shevelev, I.V., and Krutyakov, V.M. (1993) Proofreading 3′→5′ exonucleases isolated from rat liver nuclei. *Eur. J. Biochem.* **217**, 493–500.

16. Kunkel, T.A. (1985) The mutational specificity of DNA polymerase beta during in vitro DNA synthesis. *J. Biol. Chem.* **260**, 5787–5796.

17. Koundrioukoff, S., Jonsson, Z.O., Hasan, S., de Jong, R.N., van der Vliet, P.C., Hottiger, M.O., and Hübscher, U. (2000) A direct interaction between PCNA and Cdk2 targets PCNA interacting proteins for phosphorylation. *J. Biol. Chem.* **275**, 22882–22887.

18. Mazur, D. J. and Perrino, F.W. (2001) Excision of 3′ termini by the TREX1 and TREX2 3′→5′ exonucleases. *J. Biol. Chem* **276**, 17022–17029.

19. Gassmann, M., Thömmes, P., Weiser, T., and Hübscher, U. (1990) Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *FASEB J.* **4**, 2528–2532.

20. Kunkel, T.A. and Bebenek, K. (2000) DNA replication fidelity. *Annu. Rev. Biochem.* **69**, 497–529.

21. Li, L., Mo, J., Rodrigez-Belmonte, E.M., and Lee, M.Y.W.T. (2000) Identification of a Fourth Subunit of Mammalian DNA polymerase delta. *J. Biol. Chem.* **275**, 18739–18744.