5'-Deoxyribose Phosphate Lyase Activity of Human DNA Polymerase ? in Vitro

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5′-Deoxyribose Phosphate Lyase Activity of Human DNA Polymerase ι in Vitro

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DNA polymerase iota (pol ι) is one of several recently discovered DNA polymerases in mammalian cells whose function is unknown. We report here that human pol ι has an intrinsic 5′-deoxyribose phosphate (dRP) lyase activity. In reactions reconstituted with uracil-DNA glycosylase (UDG), apurinic/apyrimidinic (AP) endonuclease and DNA ligase I, pol ι can use its dRP lyase and polymerase activities to repair G•U and A•U pairs in DNA. These data and three distinct catalytic properties of pol ι implicate it in specialized forms of base excision repair (BER).

Pol ι (*1*–*3*) is a member of the RAD30 family and the larger UmuC/DinB superfamily of DNA polymerases (*4*–*6*) whose function is unknown. Pol ι performs primer extension reactions with low processivity (*1*), a property shared by DNA polymerase β, whose primary function is in single-nucleotide BER (*7*). The human *POLI* gene encoding the 80-kD pol ι contains a helix-hairpin-helix motif (*8*, *9*) (Fig. 1A) similar to that found in the NH_2-terminal domain of pol β, which catalyzes excision of a 5′-dRP group from DNA during BER (*10*–*12*).

To investigate possible functions of pol ι, we tested whether glutathione *S*-transferase (GST)–tagged human pol ι (*1*) could remove a dRP group from an AP endonuclease–incised AP site (Fig. 1B). dRPase activity was detected (Fig. 1C), and an initial time course indicated that the dRP group was removed by pol ι at 30 to 50% of the rate catalyzed by pol β (*13*, *14*). The dRPase reaction of pol β proceeds by β elimination through formation of a Schiff base intermediate with Lys^{72} that can be trapped by reduction with sodium borohydride (*11*, *12*). To determine whether the dRPase activity in the pol ι preparation was intrinsic to the protein and whether the reaction proceeds by a lyase mechanism, we performed the dRPase reaction in the presence of sodium borohydride. We then examined the products for a Schiff base intermediate of the appropriate mobility in an SDS–polyacrylamide gel. A major band (Fig. 1D) was detected with the mobility expected for a covalent intermediate of the GST–pol ι fusion protein and the DNA substrate. This indicates that the dRP activity in the protein preparation is intrinsic to pol ι and is not due to a contaminant. Two less intense bands of greater mobility were also observed, corresponding to two lower molecular weight species (*1*). Immunoblot analysis confirmed that these bands contained the GST–pol ι fusion protein lacking COOH-terminal residues (*15*). In contrast, we failed to detect a covalent protein-DNA intermediate (*16*) with human pol κ (*17*). We conclude that human pol ι has an intrinsic dRP lyase activity and that the reaction proceeds via β elimination involving an active site residue containing a primary amine.

The association of dRP lyase enzymatic activity with a distributive DNA polymerase and the requirement for both polymerization and dRP removal in BER suggest that pol ι participates in BER. To test this in vitro, we prepared two 34-nucleotide (nt) oligomer DNA substrates to monitor repair of uracil at position 16 located opposite either A or G in the template strand (Fig. 2A). These sub-

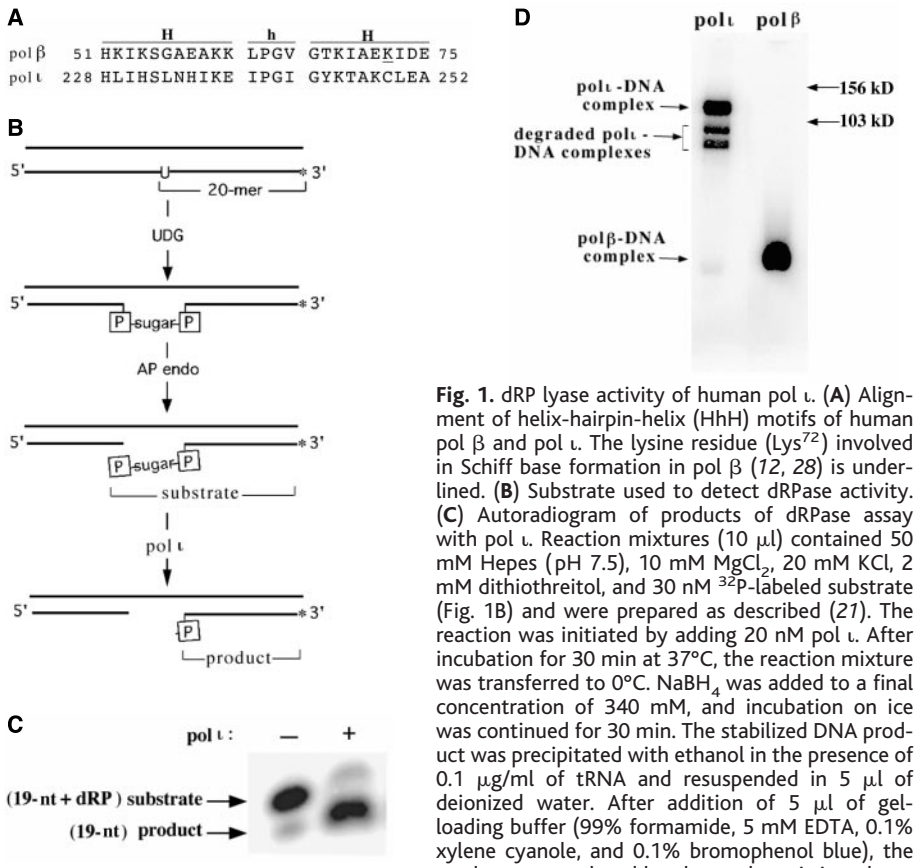

Fig. 1. dRP lyase activity of human pol ι. (**A**) Alignment of helix-hairpin-helix (HhH) motifs of human pol β and pol ι. The lysine residue (Lys^{72}) involved in Schiff base formation in pol β (*12*, *28*) is underlined. (**B**) Substrate used to detect dRPase activity. (**C**) Autoradiogram of products of dRPase assay with pol ι. Reaction mixtures (10 μl) contained 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl, 2 mM dithiothreitol, and 30 nM ^{32}P-labeled substrate (Fig. 1B) and were prepared as described (*21*). The reaction was initiated by adding 20 nM pol ι. After incubation for 30 min at 37°C, the reaction mixture was transferred to 0°C. $NaBH_4$ was added to a final concentration of 340 mM, and incubation on ice was continued for 30 min. The stabilized DNA product was precipitated with ethanol in the presence of 0.1 μg/ml of tRNA and resuspended in 5 μl of deionized water. After addition of 5 μl of gel-loading buffer (99% formamide, 5 mM EDTA, 0.1% xylene cyanole, and 0.1% bromophenol blue), the product was analyzed by electrophoresis in a denaturing 16% polyacrylamide gel and visualized by phosphor screen autoradiography. (**D**) Schiff base intermediates formed with GST–pol ι fusion protein and pol β. Arrows on the right indicate the position of molecular size markers. Trapping of polymerase-DNA covalent complex with $NaBH_4$ was performed as described (*11*). Mixtures (10 μl) contained 100 nM ^{32}P-labeled DNA previously incised with AP endonuclease suspended in 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl, and 2 mM dithiothreitol. Reactions were initiated by adding pol ι (16 nM) or pol β (50 nM), and 20 mM $NaBH_4$. After 30 min on ice, reactions were terminated by addition of an equal volume of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were resolved in a 10% SDS-PAGE gel, and products were visualized by phosphor screen autoradiography.

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strates were incubated with four human enzymes: uracil DNA glycosylase (UDG), AP endonuclease, DNA ligase I, and either pol β or pol ι. Conditions were chosen for excision repair of uracil in the G•U substrate by pol β (*18*). The product of complete BER (uracil removal → DNA backbone incision → gap-filling synthesis → dRP removal → ligation) is the full-length 34-nt oligomer (Fig. 2B) resulting from replacement of the uracil with the correct base (either C or T). The lower band (16-nt oligomer) represents incorporation of C (or T) without ligation.

As expected from previous studies (*18*), pol β participates in repair of the mispaired G•U substrate (Fig. 2B, lanes 4 to 6), with complete repair requiring all four proteins and all five enzymatic activities. Pol β also participates in repair of the correctly paired A•U substrate (Fig. 2B, lanes 10 to 12). When pol ι was used in place of pol β, robust repair was again observed with both the G•U (lanes 1 to 3) and A•U substrates (lanes 7 to 9). These data indicate that, at least in vitro, pol ι can participate with three known BER enzymes in the excision repair of uracil.

We next investigated which types of BER might be performed by pol ι. As anticipated for a BER polymerase, pol ι efficiently fills 1- to 3-base gaps that are typical BER intermediates (Table 1, expt. 1 to 3). Remarkably, pol ι inserts T opposite A with an efficiency that is 35 to 110 times that for formation of the other three canonical Watson-Crick base pairs [expt. 1, also see (*1–3*)]. This strong preference for forming one particular correct base pair has not been reported for any other DNA polymerase. It suggests that after removal of uracil resulting from incorporation of dUTP opposite A during DNA replication (*19*), pol ι may efficiently replace it with T. If so, pol ι should accurately insert T opposite A during gap-filling synthesis.

To determine whether this is the case, we performed fidelity assays with a gapped M13mp2 DNA substrate that contained a single template A. The template encodes a colorless M13 plaque phenotype because of the presence of a TGA nonsense codon in the *lacZ* α-complementation gene. A•dCMP, A•dGMP, and A•dAMP errors are scored as blue plaque revertants among the total copied products (*20*). The results (Table 2) indicate that pol ι has an average error rate at this template A of $\leq 2.2 \times 10^{-4}$. This is lower than or equal to the error rate for human pol β in this same assay (Table 2) and is similar to pol ι misinsertion rates for these mismatches during primer extension (*1–3*). Thus, were pol ι to participate in repair of the estimated 2000 uracils incorporated during replication

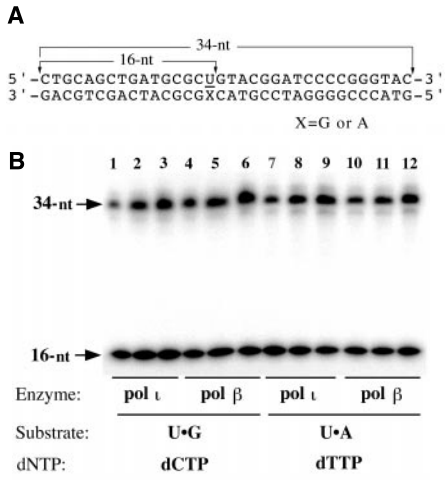

Fig. 2. BER of G•U and A•U substrates with pol β or pol ι. (**A**) DNA substrates. The uracil that is removed and replaced with either C or T is underlined. (**B**) Analysis of products of BER reactions. Uracil-containing DNA (Fig. 2A) was treated with UDG for 20 min at 37°C in a reaction mixture (20 μl) that contained 50 mM Hepes (pH 7.5), 20 mM KCl, 2 mM dithiothreitol, 10 nM UDG, and 760 nM DNA. Substrates (250 nM final concentration) were added to a reaction mixture (total volume, 30 μl) containing 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl, 2 mM dithiothreitol, 4 mM ATP, 2 μM dCTP or dTTP, 10 μCi [α-^{32}P]dCTP or [α-^{32}P]dTTP, 10 nM AP endonuclease, 200 nM DNA ligase I, and 11 nM pol ι or 5 nM pol β. Incubation was at 37°C, and 10-μl aliquots were removed at 5, 15, and 30 min. Reactions were terminated by adding EDTA to 15 mM. DNA products were recovered by ethanol precipitation with 0.1 μg/ml of tRNA and resuspended in 5 μl of water. An equal volume of gel-loading buffer (99% formamide, 5 mM EDTA, 0.1% xylene cyanole, and 0.1% bromophenol blue) was added, and the products were resolved by electrophoresis in a denaturing 10% polyacrylamide gel and visualized by phosphor screen autoradiography. Lanes 1, 4, 7, and 10 for a 5-min incubation; lanes 2, 5, 8, and 11 for a 15-min incubation; lanes 3, 6, 9, and 12 for a 30-min incubation.

Table 1. Kinetic analysis of nucleotide insertion by human pol ι with substrates containing matched and mismatched template-primer termini in short gaps. The assays for nucleotide insertion and mismatch extension kinetics were performed as described in (*1*) and (*29*). Oligonucleotides (Loftstrand Laboratories, Gaithersburg, Maryland) were gel-purified before use. The templates for experiment 1 used a 40-nt oligomer with the sequence 5′-AGCGTCTTAATCTAAGCXXTCGCTATGTTTTCAAGGATTC-3′, where XX is TG, TA, AT, or TC (expt. 1). The 16-nt oligomer primer for both experiments was 5′-CTTGAAAACATAGCGA-3′ and was 5′-labeled with [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq, Amersham Pharmacia Biotech, Piscataway, New Jersey) by using T4 polynucleotide kinase (Life Technologies, Gaithersburg, Maryland). For experiment 1, a second oligonucleotide, 5′-AGCTTAGATTAAGACGCT-3′ (or 5′-TGCTTAGATTAAGACGCT-3′ for the A•T template) was annealed to the template to generate a 1-base gap at the target nucleotide. For experiments 2 and 3, a template oligonucleotide was synthesized with the *lacZ* target sequence used in the gap-filling assay (Table 2). Radiolabeled oligonucleotide primers were then hybridized such that the first nucleotide incorporated was opposite the template T at either position 87 (underlined in expt. 2) or position 86 (underlined in expt. 3). Insertion opposite T at position 86 in experiment 3 was examined by using substrates containing either a terminal T•A pair or a G•T mismatch. For experiments 2 and 3, a second unlabeled oligonucleotide was annealed to yield a 3-base or 2-base gap, respectively.

Oligonucleotide	dNTP	K_m (μM)	V_{max} (%/min)	$(V_{max}/K_m)_{app}$	
Expt. 1: Insertion from matched termini in a 1-nt gap					
5′-GCGA AGCTT-3′ 3′-CGCTATCGAA-5′	dTTP	0.99 ± 0.1	45 ± 2.5	45	
5′-GCGA TGCTT-3′ 3′-CGCTTACGAA-5′	dATP	19 ± 6.9	7.8 ± 2.0	0.41	
5′-GCGA TGCTT-3′ 3′-CGCTTACGAA-5′	dGTP	8.7 ± 0.9	12 ± 0.7	1.4	
5′-GCGA AGCTT-3′ 3′-CGCTGTCGAA-5′	dCTP	12 ± 1.9	16 ± 0.1	1.3	
5′-GCGA AGCTT-3′ 3′-CGCTCTCGAA-5′	dGTP	4.2 ± 0.72	5.2 ± 0.36	1.2	
Expt. 2: Insertion opposite T from matched termini in a 3-nt gap					
					f_{ins}*
5′-AGTC AATT-3′ 3′-TCAGTTGTTAA-5′	dATP	5.1 ± 0.81	14 ± 1.3	2.7	1.0
5′-AGTC AATT-3′ 3′-TCAGTTGTTAA-5′	dGTP	2.5 ± 0.31	31 ± 2.9	12	4.4
Expt. 3: Insertion from matched and mismatched termini in a 2-nt gap					
					f^0_{ext}†
5′-AGTCA AATT-3′ 3′-TCAGTTGTTAA-5′	dATP	9.2 ± 1.6	4.3 ± 4.6	0.47	1.0
5′-AGTCG AATT-3′ 3′-TCAGTTGTTAA-5′	dATP	57 ± 9.5	3.0 ± 0.3	0.053	0.11
5′-AGTCA AATT-3′ 3′-TCAGTTGTTAA-5′	dGTP	7.2 ± 1.2	4.9 ± 0.42	0.68	1.0
5′-AGTCG AATT-3′ 3′-TCAGTTGTTAA-5′	dGTP	440 ± 37	1.4 ± 0.05	0.0032	0.005

*The relative discrimination at insertion is $f_{ins} = (V_{max}/K_m)_{incorrect}/(V_{max}/K_m)_{correct}$. †The relative discrimination at excision is $f^0_{ext} = (V_{max}/K_m)_{mismatched}/(V_{max}/K_m)_{matched}$.

of the mammalian genome (*19*), few errors would be introduced.

Previous studies have also shown that, when copying single-stranded templates (*1–3*), pol ι preferentially inserts G rather than A opposite T (*1–3*). Kinetic analysis performed here demonstrates a similar preference with short gapped substrates (Table 1, expt. 1 and 2). To determine whether this preference also holds for stable misincorporation during gap-filling in the presence of all four dNTPs, we performed fidelity assays with a second M13mp2 DNA substrate that contained a 5′-GTTGA template sequence. In contrast to results with a gap containing a single template A, synthesis by pol ι to fill the 5-nt gap generated products with a *lacZ* reversion frequency of 61% (Table 2, bottom). Sequence analysis of 119 independent *lacZ* revertants generated by pol ι revealed that all but two contained a single T to C base substitution. Thus, pol ι incorporated G opposite the first template T encountered (T at position 87) with 72% efficiency (Table 2). This is similar to the preference for insertion of G opposite T observed by kinetic analysis using this same template sequence (Table 1, expt. 2, T at position 87 is underlined), but is different from results seen with the human pol β (Table 2).

Only one of 119 *lacZ* revertants contained a substitution (a TT to CC tandem double change) that was consistent with G misincorporation opposite the second template T encountered during gap-filling, located at position 86. Thus, G incorporation opposite T at position 87 was followed by preferential incorporation of A opposite T at position 86. This suggests a remarkable change in nucleotide insertion specificity opposite template T by pol ι, depending on whether the terminus is matched (G insertion preferred) or mismatched (A insertion preferred). This switch in specificity was confirmed by kinetic analysis. With a 2-nt gapped DNA substrate containing a correct terminal T•A base pair (Table 1, expt. 3, lines 1 and 3), pol ι inserted A opposite T slightly less efficiently than it inserted G opposite T (V_{max}/K_m values of 0.47 and 0.68). However, when the gapped DNA substrate contained a terminal T•G pair (expt. 3, lines 2 and 4), pol ι exhibited a 22-fold preference for insertion of A rather than G opposite T (f^0_{ext} values of 0.11 and 0.005). In both cases, extension of the T•G mispair was less efficient than extension of the terminus with the correctly matched T•A base pair.

Pol ι's intrinsic dRP lyase activity and its capability for filling short gaps are consistent with a role for this polymerase in BER of a variety of base lesions. Pol ι is one member of the recently discovered UmuC/DinB superfamily DNA polymerases, several of which are reported to have low fidelity (*6*, *21*). Conservation of such polymerases in organisms containing large, stable genomes indicates they are carefully regulated. Regulation may be particularly important for pol ι, given the preferential insertion of G opposite T. Two possible functions for pol ι were considered in earlier studies (*1–3*, *21–23*): translesion DNA synthesis and somatic hypermutation of immunoglobulin genes. In the latter hypothesis, the preferential insertion of G opposite T is viewed as an error that ultimately leads to a mutation. However, an alternative point of view is that, under certain circumstances, insertion of G opposite T is a "correct" event that stabilizes mammalian genomes encoding this unusual yet conserved DNA polymerase. As one hypothetical example, the parental guanine of a G•T mismatch generated by deamination of 5-methylcytosine may occasionally be removed by a glycosylase that could use this mismatched substrate. For example, 3-methyladenine DNA glycosylases can excise undamaged guanine from normal DNA at a biologically significant rate (*24*), and bacterial (*25*) and human (*26*) MutY DNA glycosylases can excise undamaged adenine when mispaired opposite G. These data are consistent with removal of undamaged guanine from a G•T mismatch by a glycosylase. If the G were inadvertently excised, pol ι could replace the guanine opposite the T in a BER reaction that requires its dRP lyase activity. This would maintain the parental genotype until the T is excised and replaced with C. To prevent mutations at adjacent template T, pol ι should perform this unusual guanine insertion reaction only once, with any subsequent events preferentially placing A opposite T (as in Table 1, expt. 3). In this scenario, the formation of a T-dGMP pair by pol ι at a site of 5-methylcytosine deamination would not be an error, but would rather be a correct incorporation that stabilizes the genome.

Whatever the roles of pol ι are in BER, the preference for incorporating G rather than A opposite T suggests that pol ι is excluded from participating in certain DNA transactions involving template T, such as repair of the common alkylation lesion 3-methyladenine. Exclusion of pol ι from BER of some lesions is consistent with the fact that its dRP lyase does not prevent cytotoxicity induced by methylating agents in cells lacking the dRP lyase activity of pol β (*27*). Thus, it will be critical to understand how pol ι is targeted to use its intrinsic polymerase and dRP lyase activities only at the appropriate time and place and is otherwise prevented from conducting widespread, inaccurate DNA synthesis in a cell.

Table 2. Fidelity of pol ι during short gap–filling synthesis. Gap-filling reaction mixtures (20 μl) contained 40 mM tris-HCl (pH 8.0 at 22°C), 10 mM $MgCl_2$, 10 mM dithiothreitol, 6.25 μg bovine serum albumin, 60 mM KCl, 2.5% glycerol 0.5 mM each of dATP, dTTP, dGTP, and dCTP, 1.6 nM gapped M13mp2 DNA, 6.5 nM or 20 nM pol ι, and 400 units of T4 DNA ligase. After incubation at 37°C for 60 min, EDTA was added to 15 mM, and the reaction products were resolved by electrophoresis in a neutral 0.8% agarose gel (*20*). Covalently closed, circular DNA products were electroeluted from gel slices, recovered by ethanol precipitation, introduced into *Escherichia coli* MC1061 by electroporation, and plated (*20*). After scoring revertant and total plaques, we sequenced the DNA of revertants to define the sequence change responsible for the change of phenotype. M13mp DNA not subjected to DNA synthesis in vitro yielded *lacZ* mutant frequencies of 0.00051% and 0.00056%, respectively, for the substrates with 1- and 5-nt gaps. The results for Klenow fragment pol and pol β are from (*30*) and (*20*), respectively. The misincorporation rates listed for the 1-nt gap are for the most frequent of three possible errors made by that polymerase. The *lacZ* mutant frequency values for pol ι are the average of two independent determinations.

DNA polymerase	*lacZ* mutant frequency (%)	Misincorporation rate (%)
	Single template A gap	
Exo⁻ Klenow pol	0.05	0.068
Human pol β	0.03	0.022
Human pol ι	0.013	≤0.022
	Five-base template 5′-GTTGA gap	
		T•dGMP mismatch
Human pol β	0.26	0.29
Human pol ι	61	72*

*Covalently closed M13mp2 DNA containing a T•G mismatch at the T of the TGA codon in the 5-nt gap yields 85% blue plaques. This indicates that 85% of pol ι errors made in vitro are expressed in *E. coli*. Thus, pol ι incorporated G opposite the first template T encountered at a frequency of 72% (0.61 divided by 0.85).

References and Notes

1. A. Tissier, J. P. McDonald, E. G. Frank, R. Woodgate, *Genes Dev*. **14**, 1642 (2000).
2. R. E. Johnson, M. T. Washington, L. Haracska, S. Prakash, L. Prakash, *Nature* **406**, 1015 (2000).
3. Y. Zhang, F. Yuan, X. Wu, Z. Wang, *Mol. Cell. Biol*. **20**, 7099 (2000).
4. R. Woodgate, *Genes Dev*. **13**, 2191 (1999).
5. R. E. Johnson, M. T. Washington, S. Prakash, L. Prakash, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12224 (1999).
6. E. C. Friedberg, W. J. Feaver, V. L. Gerlach, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5681 (2000).
7. S. H. Wilson *et al*., in *Cold Spring Harbor Symp. Quant. Biol*. **65**, 143 (2001).
8. J. P. McDonald *et al*., *Genomics* **60**, 20 (1999).
9. G. P. Mullen, S. H. Wilson, *Biochemistry* **36**, 4713 (1997).
10. Y. Matsumoto, K. Kim, *Science* **269**, 699 (1995).

11. C. E. Piersen, R. Prasad, S. H. Wilson, R. S. Lloyd, *J. Biol. Chem.* **271**, 17811 (1996).
12. L. J. Deterding, R. Prasad, G. P. Mullen, S. H. Wilson, K. B. Tomer, *J. Biol. Chem.* **275**, 10463 (2000).
13. The apparent specific activity of pol ι dRP lyase was determined from the intensity of the product band observed after 5 min of incubation and corrected for background. This activity of 68×10^{-3} per minute is one-half to one-third that observed for pol β at the same DNA concentration (20 nM).
14. R. Prasad, W. A. Beard, P. R. Strauss, S. H. Wilson, *J. Biol. Chem.* **273**, 15263 (1998).
15. E. G. Frank, R. Woodgate, data not shown.
16. K. Bebenek, V. L. Gerlach, W. J. Feaver, E. C. Friedberg, T. A. Kunkel, data not shown.
17. V. L. Gerlach, W. J. Feaver, P. J. Fischhaber, E. C. Friedberg, *J. Biol. Chem.* **276**, 92 (2001).
18. D. Srivastava *et al.*, *J. Biol. Chem.* **273**, 21203 (1998).
19. H. Nilsen *et al.*, *Mol. Cell* **5**, 1059 (2000).
20. W. P. Osheroff, H. K. Jung, W. A. Beard, S. H. Wilson, T. A. Kunkel, *J. Biol. Chem.* **274**, 3642 (1999).
21. M. F. Goodman, B. Tippin, *Nature Rev. Mol. Cell Biol.* **1**, 101 (2000).
22. V. Poltoratsky, M. F. Goodman, M. D. Scharff, *J. Exp. Med.* **192**, F27 (2000).
23. A. Tissier *et al.*, *EMBO J.* **19**, 5259 (2000).
24. K. G. Berdal, R. F. Johansen, E. Seeberg, *EMBO J.* **17**, 363 (1998).
25. A. L. Lu, D. Y.Chang, *Cell* **54**, 805 (1988).
26. T. Ohtsubo *et al.*, *Nucleic Acids Res.* **28**, 1355 (2000).
27. R. W. Sobol *et al.*, *Nature* **405**, 807 (2000).
28. R. Prasad *et al.*, *J. Biol. Chem.* **273**, 11121 (1998).
29. L. V. Mendelman, J. Petruska, M. F. Goodman, *J. Biol. Chem.* **265**, 2338 (1990).
30. D. T. Minnick *et al.*, *J. Biol. Chem.* **274**, 3067 (1999).
31. We thank E. de Rosny for assistance in calculating the kinetic constants, D. Nguyen for technical assistance, and W. A. Beard and M. J. Longley for helpful comments.

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An RNA Ligase Essential for RNA Editing and Survival of the Bloodstream Form of *Trypanosoma brucei*

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RNA editing in trypanosomes occurs by a series of enzymatic steps that are catalyzed by a macromolecular complex. The TbMP52 protein is shown to be a component of this complex, to have RNA ligase activity, and to be one of two adenylatable proteins in the complex. Regulated repression of *TbMP52* blocks editing, which shows that it is a functional component of the editing complex. This repression is lethal in bloodforms of the parasite, indicating that editing is essential in the mammalian stage of the life cycle. The editing complex, which is present in all kinetoplastid parasites, may thus be a chemotherapeutic target.

RNA editing in kinetoplastid protozoa produces mature mitochondrial (mt) mRNAs by a series of catalytic steps that insert and delete the number of uridylates (Us) specified by guide RNAs (gRNAs). The pre-mRNA undergoes endonucleolytic cleavage, U addition or removal, and ligation. The gRNAs duplex with the pre-mRNAs, and these RNAs associate with the multiprotein complex that catalyzes the editing (*1*–*3*). We recently purified the editing complex of *Trypanosoma brucei* and identified several candidate genes for its components (*4*). Monoclonal antibody (mAb) P3C1-G2, which is specific for recombinant and native protein from one of these genes, *TbMP52*, immunoprecipitates in vitro editing activity, indicating that the TbMP52 protein is a component of the editing complex (*4*). We report here that *TbMP52* encodes an RNA ligase and that down-regulation of its expression in vivo stops the production of edited mRNA and results in the death of the bloodforms of the parasite.

The predicted *T. brucei* TbMP52 protein sequence has high homology to a putative ortholog in *Leishmania major* and to TbMP48, a putative paralog of TbMP52 in *T. brucei* that is also present in the purified editing complex (*4*). Although no homologs with known function could be identified, motif and domain searches (*5*) identified ligase signature boxes (*6*) in both TbMP52 and TbMP48 (Fig. 1). This includes the KXXG (*7*) box (region I, Fig. 1), the lysine of which becomes adenylated in ligases during ligation (*8*). These findings suggested that *TbMP52* and *TbMP48* encode the larger and the smaller adenylatable protein, respectively, that are present in purified RNA editing complexes and have been suggested to represent editing ligases (*9*–*11*). In vitro–transcribed and –translated recombinant TbMP52 protein (rTbMP52) ligated 71% of a synthetic RNA substrate (Fig. 2, A and B). rTbMP52 also autoadenylated upon incubation with [^{32}P]α–adenosine triphosphate (α-ATP) (Fig. 2C). mAb P3C1-G2, which is specific for TbMP52, immunoprecipitated the larger of the two adenylatable proteins from native complexes after dissociation into individual proteins (Fig. 2D). TbMP48 also contains the KXXG box, and its predicted size matches that of the smaller adenylatable protein (*9*, *11*). Thus, *TbMP48* is likely to encode this second adenylatable component of the complex, which may also be an RNA ligase.

The in vivo function of *TbMP52* was investigated by targeted gene replacement of both endogenous alleles and insertion of a *TbMP52* allele that was under the control of a tetracycline (tet) inducible promoter, leading to mutant clone 1B3 (*12*–*14*). Western analysis showed that *TbMP52* is conditionally expressed in clone 1B3 (Fig. 3A). The amount of TbMP52 protein was substantially reduced in non-induced (no tet) cells as compared to induced cells. Loss of TbMP52 also led to a substantial reduction in complexes containing adenylatable proteins that can be immunoprecipitated (Fig. 3B). mAb P1H3-D7, which is specific for a 69-kD component of the editing complex, immunoprecipitates active editing complexes (*15*) and complexes that contained both adenylatable proteins from wild-type and induced 1B3 cells. TbMP52 and TbMP48 were reduced by 91.3 and 42.8% in the precipitates of non-induced 1B3 cells, respectively. Thus, there appear to be fewer editing complexes in cells with reduced TbMP52, which indicates that this protein is an integral component of the editing complex.

TbMP52 expression was found to be essential for proliferation of *T. brucei*. Clone 1B3 grew continuously in vitro in the presence of tet, with a generation time indistinguishable from that of the wild type (Fig. 3C, solid squares). However, repression of *TbMP52* expression in the absence of tet resulted in cessation of growth after about 70 hours (Fig. 3C, open squares). Over 95% of the population died within 48 hours, and many lysed cells were evident. Reintroduction of tet 21 hours after cessation of growth (91 total hours, arrow in Fig. 3C) prevented the population decline, and growth resumed at the normal rate approximately 90 hours later (181 total hours), indicating that the cell death and population

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