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A coproofreading Zn^{2+}-dependent exonuclease within a bacterial replicase

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The proofreading exonucleases of all DNA replicases contain acidic residues that chelate two Mg^{2+} ions that participate in catalysis. DNA polymerase III holoenzymes contain their proofreading activity in a separate subunit, ε, which binds the polymerase subunit, α, through α's N-terminal php domain. Here we demonstrate that the α php domain contains a novel Zn^{2+}-dependent 3′→5′ exonuclease that preferentially removes mispaired nucleotides, providing the first example of a coediting nuclease.

During DNA replication, a high level of fidelity is attained by the action of a proofreading exonuclease that removes nucleotides misincorporated by an associated polymerase. The proofreading exonucleases of all eukaryotic, bacterial and viral DNA replicases contain acidic residues that chelate two Mg^{2+} ions that participate directly in catalysis[1]. In DnaE-based bacterial replicases, the proofreading exonuclease exists as a separate polypeptide chain, ε (ref. 2), which binds to the α-polymerization subunit through α's N-terminal php domain[3]. This domain was first identified by its sequence similarity to histidinol phosphatase, and the proposal was made that it might have a phosphatase-like activity, perhaps that of a pyrophosphatase[4]. The structure of YcdX, a protein more closely related to the Pol III php domain and whose function is unknown, revealed a Zn^{2+} trinuclear center with characteristics similar to several phosphoesterases[5]. This information prompted us to search for intrinsic hydrolytic activity in α in the absence of the ε subunit.

Because trace levels of the tightly binding ε subunit would be expected to contaminate even overproduced *Escherichia coli* α expressed in *E. coli*, and perhaps foreign α subunits as well, initially we chose to investigate the α subunit from a thermophile, *Thermus thermophilus*. The expected thermostability of any intrinsic activity detected should be distinguishable from endogenous *E. coli* activities. We first examined the ability of purified *T. thermophilus* α to extend a series of four primers differing in their terminal nucleotide composition. As previously observed for *E. coli* α (ref. 6), a primer containing a terminal C that forms a proper base pair with a template G was elongated rapidly. Also as observed with *E. coli* α, the next best substrate was terminated with T, followed by, in decreasing order of preference, the A•G mismatch and the poorly elongated G•G mismatch.

Notably, in parallel reactions lacking dNTPs, we observed time-dependent removal of mismatched primer termini that paralleled the rate of primer elongation (**Fig. 1**). With properly paired primer termini, only a trace of degradation was detected at times when the primer was elongated completely. In the absence of dNTPs and with extensive incubation times, the removal of properly base-paired termini was observed. Among the primers tested, the T•G mismatch was the best substrate for the *T. thermophilus* α exonuclease activity, which is consistent with the mismatch being the most efficiently processed and elongated. This suggests a mechanism where the mismatched nucleotide is removed before elongation. The degradation pattern and rate are consistent with a 3′→5′ exonuclease that removes one nucleotide at a time from the 3′ end of 5′ end–labeled primers.

All assays were performed at 55 °C in mixtures that had been preheated before the addition of enzyme, a condition expected to inactivate *E. coli* enzymes. To distinguish the observed activity from the most likely artifact—contamination by endogenous *E. coli* ε bound to a thermally stable scaffold provided by *T. thermophilus* α—we monitored the rate of inactivation of polymerase and exonuclease activities at an elevated temperature. We observed that the *T. thermophilus* polymerase activity and 3′→5′ exonuclease activities decayed

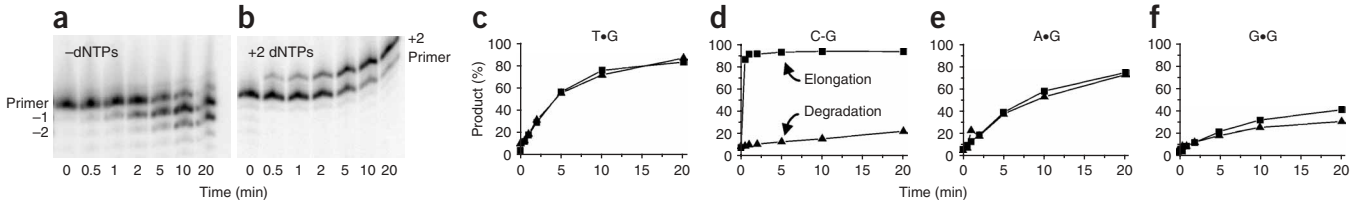

Figure 1 The α subunit of *T. thermophilus* Pol III has an intrinsic 3′→5′ exonuclease that removes mispaired primer termini, permitting their elongation. (**a**,**b**) Activity of *T. thermophilus* Pol III on a duplex DNA consisting of a 24-nucleotide primer annealed to a 60-nucleotide template containing a 3′ T•G mismatch in the primer, in the absence (**a**) and presence (**b**) of dCTP and dTTP. (**c**) Percentage of products in **a** that were shorter than the primer (triangles) or products in **b** that were longer than the primer (squares). (**d**–**f**) Products resulting from a perfectly matched primer-template duplex (**d**) or mismatched A•G (**e**) or G•G (**f**) primer-template duplex.

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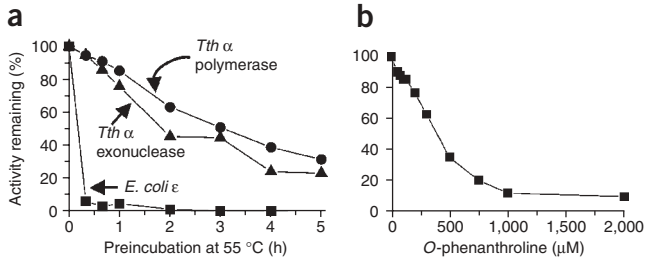

Figure 2 The intrinsic exonuclease activity of *T. thermophilus* α is thermally stable and susceptible to inhibition by the Zn^{2+} chelator *O*-phenanthroline. (**a**) *T. thermophilus* (*Tth*) α preincubated and assayed at 55 °C shows a parallel decrease in polymerase activity (circles) and exonuclease activity (triangles). *E. coli* ε (in the presence of equimolar *T. thermophilus* α; squares) was inactivated in <30 min at 55 °C. (**b**) *T. thermophilus* α was preincubated in the presence of *O*-phenanthroline in buffer A (**Supplementary Methods** online) for 1 h at room temperature before 1.5-fold dilution in the exonuclease assay as described in **Supplementary Methods**. *O*-phenanthroline concentration shown is the final concentration in the assay.

in parallel with a half-life of about 2.5 h at 55 °C (**Fig. 2a**). In contrast, *E. coli* ε exonuclease activity decayed rapidly, even if present in a 1:1 mixture with *T. thermophilus* α. These results are consistent with cooperative unfolding of a proofreading exonuclease and polymerase activity that are part of the same polypeptide and distinguish the activity observed from thermally labile contaminants. To meet the classical criterion for association of activities, we demonstrated that 3′→5′ exonuclease and DNA polymerase activities are coincident during gel filtration (**Supplementary Fig. 1** online).

To test our hypothesis, based on the YcdX structure, that the exonuclease activity of *T. thermophilus* α is attributable to a Zn^{2+}-binding php domain, we examined the sensitivity of the 3′→5′ exonuclease activity to a Zn^{2+} chelator. We observed a high level of sensitivity, with 80% of the activity being destroyed upon addition of 750 μM *O*-phenanthroline in the presence of 10 mM Mg^{2+} (**Fig. 2b**). Thus, we have demonstrated an intrinsic 3′→5′ exonuclease activity present in the php domain that, in *E. coli*, binds the ε proofreading subunit. This novel Zn^{2+}-dependent activity shows the classic hallmarks of a proofreading exonuclease. It preferentially removes mispaired primer termini and shares the primer terminus with a polymerase that cannot readily extend a mismatch[7]; as a result, the rate of mispaired-nucleotide removal is rate limiting for extension. Furthermore, it is distinct from the proofreading activities found to date in all other replicases.

What might be the function of a second editing activity? It is possible that the Zn^{2+}-dependent activity was the ancestral proofreading exonuclease and that it has been functionally replaced in most organisms by a more recently evolved Mg^{2+}-dependent enzyme. However, the genomes of *T. thermophilus* and other organisms whose php domains have the essential Zn^{2+} ligands found in YcdX also contain obvious homologs of ε. Thus, the presence of a functional php exonuclease and the existence of a functional ε subunit do not seem to be mutually exclusive. Perhaps the advantageous ability to partition a mismatched primer terminus between the polymerase and php active sites within a single polypeptide was then expanded to permit efficient and regulated shuttling of the primer terminus between the polymerase and an exogenous ε subunit. Such a situation may exist in eukaryotic replicases. These enzymes contain sequences homologous to calcineurin-type phosphoesterases, but the essential Mg^{2+}-chelating residues have been lost in evolution[4,8]. An active calcineurin-like exonuclease domain has been revealed in the eukaryotic double-strand–break repair protein, MreII, and in the Pol D class of DNA polymerases found in the Euryarchaeota subdomain of archaea[8,9]. It would be interesting to learn whether the calcineurin-like domain of the eukaryotic replicases interacts with the exonuclease domain of the polymerase and serves a coordinating role in proofreading.

The php and ε exonucleases are not redundant. The mutator phenotype of ε mutations (*mutD*) is quite profound[10], indicating that its activity is important. The strong sequence conservation of the php domain and the insertion of ε-like exonuclease sequences within it in Pol C–like Pol IIIs[3] support the argument that the php domain is not just a tether, but rather it contributes functionally. Perhaps the functions of these two activities are complementary. The php enzyme might be more active on mismatches not preferred by ε, or the relevant substrate for the php enzyme might not be the substrates examined in this work. Other possibilities include 3′ phosphatase activity or the ability to process phosphodiesterase substrates inherited from incomplete repair processes, such as 3′-phosphoglycolates or 3′ α,β-unsaturated aldehydes. An understanding of these processes must include a survey of possible substrates and identification and mutagenesis of essential php active site residues that do not perturb polymerase function or ε activity, along with an evaluation of the *in vivo* phenotype.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Beese, L.S. & Steitz, T.A. *EMBO J.* **10**, 25–34 (1991).
2. Scheuermann, R., Tam, S., Burgers, P.M., Lu, C. & Echols, H. *Proc. Natl. Acad. Sci. USA* **80**, 7085–7089 (1983).
3. Wieczorek, A. & McHenry, C.S. *J. Biol. Chem.*, published online 3 March 2006 (doi:10.1074/jbc.M513844200).
4. Aravind, L. & Koonin, E.V. *Nucleic Acids Res.* **26**, 3746–3752 (1998).
5. Teplyakov, A. *et al. Proteins* **51**, 315–318 (2003).
6. Kim, D.R. & McHenry, C.S. *J. Biol. Chem.* **271**, 20681–20689 (1996).
7. Brutlag, D. & Kornberg, A. *J. Biol. Chem.* **247**, 241–248 (1972).
8. Gueguen, Y. *et al. Eur. J. Biochem.* **268**, 5961–5969 (2001).
9. Hopfner, K.P. *et al. Cell* **105**, 473–485 (2001).
10. Cox, E.C. & Horner, D.L. *Genetics* **100**, 7–18 (1982).