Watching DNA polymerase η make a phosphodiester bond

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DNA synthesis has been extensively studied, but the chemical reaction itself has not been visualized. Here we follow the course of phosphodiester bond formation using time–resolved X–ray crystallography. Native human DNA polymerase η, DNA and dATP were co–crystallized at pH 6.0 without Mg2+. The polymerization reaction was initiated by exposing crystals to 1 mM Mg2+ at pH 7.0, and stopped by freezing at desired time points for structural analysis. The substrates and two Mg2+ ions are aligned within 40 s, but the bond formation is not evident until 80 s. From 80 to 300 s structures show a mixture of decreasing substrate and increasing product of the nucleotidyl–transfer reaction. Transient electron densities indicate that deprotonation and an accompanying C2′–endo to C3′–endo conversion of the nucleophile 3′–OH are rate limiting. A third Mg2+ ion, which arrives with the new bond and stabilizes the intermediate state, may be an unappreciated feature of the two–metal–ion mechanism.

Formation and breakage of chemical bonds underlie all life processes. DNA replication, which is essential for cell proliferation, is but one example1–2. In each reaction cycle, a dNTP complementary to the templating base is incorporated into DNA by a polymerase, during which a new bond is formed between the 3′–OH of the primer strand and the α–phosphate of the dNTP, and the phosphodiester bond between the α– and β–phosphates of dNTP is broken (Fig. 1a). As a result the primer strand is extended by one nucleotide and a pyrophosphate is released. This reaction has been shown to require two Mg2+ ions (A and B) and is inferred to be S2,2–type, forming a pentacovalent phosphate intermediate 3,4. A similar two–metal–ion–dependent mechanism is believed to be shared by all DNA and RNA polymerases and many nucleases5.

DNA synthesis has been analysed by kinetic measurements, dynamic simulations and structural studies6–7. Crystal structures of a number of DNA polymerases in substrate-bound forms have been determined using non–reactive substrate analogues or with Ca2+ instead of Mg2+ to prevent the nucleotidyl–transfer reaction8–18. These structures show active–site configurations in an array of pre–reaction ground states. Pre–steady–state kinetic studies of several DNA polymerases indicate that, after large conformational changes induced by substrate binding, unspecified subtle changes in the active site are the rate–limiting step19–27. The actual process of DNA synthesis has, to our knowledge, never been visualized.

Human DNA polymerase η (Pol η) is specialized in lesion bypass and has a preformed catalytic centre that undergoes limited conformational change during DNA synthesis28. Crystal structures of the catalytic domain of Pol η complexed with DNA and a non–reactive dNTP analogue poised for catalysis have been determined at 1.8 Å resolution29,30. Taking advantage of the slow reaction rate and hindered post–reaction DNA translocation in crystallo, we follow the course of DNA synthesis by X–ray crystallography and report here the structural changes and transient elements that are associated with the reaction.

The ground state of Pol η

The Pol η activity in a reaction buffer containing 0.9 mM Mg2+ and 0.1 mM Ca2+ was only one–third of that with 1 mM Mg2+ (Fig. 1b), indicating that both active–site metal ions have to be Mg2+ for the catalysis. In accordance with the bell–shaped pH–dependence curve characteristic for the acid–base catalysis17,21, the catalytic rate of Pol η is extremely low at pH 6.0 and rises with increasing pH from 6.0 to 8.0 (Fig. 1c). To prevent the nucleotidyl–transfer reaction, crystals of Pol η–substrate complexes were grown at pH 6.0 with only one Ca2+ per protein–DNA–dATP complex (Methods). After soaking in MES (pH 6.0–7.2) or HEPES buffer (pH 7.0–7.5) with Na+ or K+ but no divalent cation for 5 to 30 min, these crystals maintained excellent diffracting power up to pH 7.0, but decayed at pH 7.5 with reduced resolution and increased diffuse scattering.

The structure of Pol η crystal equilibrated at pH 6.8 was refined at 1.50 Å. A Ca2+ ion clearly occupies the B metal–ion–binding site and is coordinated in the octahedral geometry by oxygen atoms of the dATP molecule (Protein Data Bank 2X0W) and the active-site residues (Fig. 1d). The A metal–ion–binding site has low occupancy of a monovalent cation. The occupancy of Na+ or K+ in the A site increases with the pH (Supplementary Fig. 1a, b), which is probably correlated with deprotonation of the active–site carboxylates. Pol η binds Na+ more readily than K+, particularly below pH 7.0, probably because Na+ is similar to Mg2+ in size and is smaller than K+. As expected, there is no nucleotidyl–transfer reaction, and the structure is termed the ground–state ternary complex.

The ground state of Pol η is similar to the Pol η ternary complex with a non–reactive dATP analogue dAMPNP (2′–deoxyadenosine–5′–[(α, β)–imido]triphosphate) and two Mg2+ ions (Protein Data Bank ID 3MR2)29. However, without an A–site divalent cation, the 3′–OH of the primer strand shifts away from the dATP and forms hydrogen bonds with the side chains of the S113 and D115 residues, and the active–site carboxylates D13 and E116 adopt different rotamer conformations (Fig. 1d). Replacement of dAMPNP with dATP leads to stabilization of R61, which forms bidentate hydrogen bonds with the α–phosphate.

Catalysis in crystallo

The nucleotidyl transfer was initiated by transferring Pol η crystals to a pH 6.8 or 7.0 reaction buffer containing 1 mM Mg2+ but no dATP (Methods). After incubation at 293 K for 40 to 300 s, the reaction was terminated in ~40 s intervals by freezing crystals in liquid nitrogen at

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77 K. Diffraction data were collected to 1.50–1.95 Å Bragg spacings (Supplementary Table 1). The reaction process was monitored by the electron density corresponding to the new chemical bond in the $F_o - F_c$ map compared with the refined ground-state structure (Fig. 2a and Supplementary Fig. 2). The reaction time courses at pH 6.8 and 7.0 in crystalllo (Fig. 2b) are approximately 20–100-fold slower than in solution (Fig. 1c), probably owing to the reduced thermal motion. By 40 s, the A site was fully occupied with Mg$^{2+}$ (Fig. 2a and Supplementary Fig. 1c, d). About 50% of Ca$^{2+}$ in the B site was replaced by Mg$^{2+}$, and exchange of the remaining Ca$^{2+}$ took place slowly (Supplementary Fig. 3). Binding of two Mg$^{2+}$ ions leads to the alignment of the 3'-OH and dATP.$^{10,32}$ The refined structure is nearly identical to that with dAMPNPP, except for tighter dATP coordination by R61 and closer proximity of the Mg$^{2+}$ ions (3.4 Å apart versus 3.6 Å). As there is no sign of bond formation, the structure is termed the reactant state.

Electron density corresponding to a new bond between the 3'-OH and α-phosphorus of dATP begun to emerge at 80 s, increased quickly over the next 60 s and reached a maximum after 200 s, when the reaction was 60–70% complete (Fig. 2b). The seemingly reduced rate after 140 s is probably due to the reverse reaction in crystalllo. The slight decline of product after 250 s (Fig. 2b) is due to a sideway product translocation, which is clear with longer incubation (Supplementary Fig. 4a). To alleviate an impediment to proper DNA translocation by the crystal lattice, we replaced an AT base pair with a mismatch at the DNA end that formed lattice contacts (Methods and Supplementary Table 2). The DNA with a TG mismatch led to isomorphous crystals and proper translocation of the DNA product (Supplementary Fig. 4b). Interestingly, the time courses of nucleotidyl transfer in the AT and TG crystals are nearly identical and are unaffected by the lattice contacts (Fig. 2b).

At the peak of chemical-bond formation between 200 and 250 s, the scissile phosphate can be refined in a pentacovalent transition state structure. The active site is superimposed with the $2F_o - F_c$ electron density contoured at 1.2σ (top panel) or with the non-reactive dAMPNPP ternary complex (3MR2, silver, bottom panel). Hydrogen bonds and metal-ion coordination are shown as dashed lines.

Between the reactant and product state, the protein, DNA and dATP are superimposable except for atoms in the reaction centre. Most notably, the α-phosphorus of dATP moves 1.4 Å along a straight line between the attacking and leaving oxygen atoms (separated by 4.6 Å; Fig. 2d). The shift of the α-phosphate is accompanied by alteration of residue R61 of Pol η, which flips away from the scissile phosphate and is replaced by a new metal ion and water molecules (Fig. 2a, d; see details below). On the primer strand, changes are confined to the 3' nucleotide. The 3'-OH, together with the deoxyribose, moves towards the α-phosphate by 0.5 Å, and the sugar pucker changes from C2'-endo in the reactant state to C3'-endo in the product state (Fig. 2d). With the loss of the nucleophile and α-phosphate as ligands, the A-site Mg$^{2+}$ dissociates in the product state, as evidenced by the declining occupancy (Supplementary Fig. 1c, d). Concomitantly, D13 assumes a second conformation and forms a hydrogen bond with K224.

The C3'-endo (A form) conformation at the 3' primer end was observed in ternary complexes with the A-, B- and X-family DNA polymerases and is thought to be important for forming a shallow minor groove for dNTP selection.$^{9,11,12,14,15,17,18,32}$ Among the Y-family
polymerases, the primer end has always been observed as C2'-endo. In the Pol \( \eta \) ground-state and reactant-state complexes, the dATP and the nucleotide 5'- to the primer end have the A-form conformation, but only in the product-state structure does the primer end adopt the C3'-endo conformation to avoid clashes between its C2' atom and the non-bridging oxygen of dATP during and immediately after the nucleophilic attack (Figs 2d and 3a). Because the electron density is weak for the sugar moiety at the 3'-primer end, we tested the effect of the A-form conformation by using a primer with a ribonucleotide at its 3'-end. The catalytic efficiency \( (k_{\text{cat}}/K_m) \) of Pol \( \eta \) is comparable whether a ribonucleotide or deoxyribonucleotide is at the primer end (Fig. 3b), as observed for DNA pol \( \beta \) (ref. 36), indicating that the A-form conformation is probably necessary for DNA synthesis in general.

**A transient water molecule**

Two unexpected spheres of electron density appear in the 40-second reactant-state structure, the electron density is superimposable with a water molecule observed in the Pol \( \eta \)-dAMPNPP ternary complex. This water molecule is hydrogen-bonded with the 3'-OH, the O4' of dATP and another water molecule in the 80-second reactant-state structure, but it is incompatible with the C3'-endo conformation in the product state (Fig. 3a). The 3'-OH is also hydrogen-bonded to a water molecule in an X-family DNA polymerase crystallized with dUMPNNP (2'-deoxyuridine-5'-[(\( \alpha, \beta \)-imido)triphosphosphate]). A water-mediated and substrate-assisted catalytic mechanism has previously been proposed. In addition to the water molecule, S113 is hydrogen-bonded with the 3'-OH in the ground state and may pick up the proton and pass it to E116 in the reactant state. (Fig. 1d). However, the S113A-mutant Pol \( \eta \) retains \( 95\% \) of \( k_{\text{cat}} \) with a threefold increase of \( K_m \) (Fig. 3b and Supplementary Table 3). Interestingly, mutations of the S113 equivalent in the A- and B-family DNA polymerases (highly conserved histidine and threonine, respectively) also have limited impact on catalytic efficiency. We therefore propose that 3'-OH is prone to deprotonation when coordinated by Mg\(^{2+}\) and aligned with the incoming nucleotide. The proton can be passed on to the transient water molecule and then to bulk solvent. When the 3'-end of the primer is a ribonucleotide, the O2' may relay the proton out instead. An incoming dNTP most probably participates in deprotonation of the nucleophile because, with the nitrogen substitution in dAMPNNP, the water molecule is bound stably to the 3'-OH.

**The third metal ion**

The second emerging sphere of electron density is very close to the \( \alpha \)-phosphate on the opposite side of the A- and B-site Mg\(^{2+}\) ions (Fig. 2a). It starts to appear at 140 s and intensifies with the reaction time. By 230 s with the structure refined at 1.52 Å resolution, the octahedral geometry and the short coordination distances indicate that

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**Figure 2 | Reaction time course.** a, Two views (top and bottom diagrams) of \( F_{(40-230 \text{s})} - F_{(40 \text{s})} \) omit maps (4.0σ) superimposed onto the 40-s structure (pH 7.0). The emerging densities are shown by arrows or circled. b, A plot of the absolute peak height of new bond density versus reaction time at pH 6.8 and 7.0 in AT and TG crystals (Supplementary Table 1a, b). The noise level (1σ) is also hydrogen-bonded to a water molecule in an X-family DNA polymerase crystallized with dUMPNNP (2'-deoxyuridine-5'-[(\( \alpha, \beta \)-imido)triphosphosphate]). A water-mediated and substrate-assisted catalytic mechanism has previously been proposed. In addition to the water molecule, S113 is hydrogen-bonded with the 3'-OH in the ground state and may pick up the proton and pass it to E116 in the reactant state. (Fig. 1d). However, the S113A-mutant Pol \( \eta \) retains \( 95\% \) of \( k_{\text{cat}} \) with a threefold increase of \( K_m \) (Fig. 3b and Supplementary Table 3). Interestingly, mutations of the S113 equivalent in the A- and B-family DNA polymerases (highly conserved histidine and threonine, respectively) also have limited impact on catalytic efficiency. We therefore propose that 3'-OH is prone to deprotonation when coordinated by Mg\(^{2+}\) and aligned with the incoming nucleotide. The proton can be passed on to the transient water molecule and then to bulk solvent. When the 3'-end of the primer is a ribonucleotide, the O2' may relay the proton out instead. An incoming dNTP most probably participates in deprotonation of the nucleophile because, with the nitrogen substitution in dAMPNNP, the water molecule is bound stably to the 3'-OH.

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**A transient water molecule**

Two unexpected spheres of electron density appear in the \( F_{(40-230 \text{s})} - F_{(40 \text{s})} \) maps in the course of new bond formation (Fig. 2a). The first is within hydrogen-bonding distance of the 3'-OH. Its electron density peaks at 80 s and declines considerably after 140 s when the product state becomes prominent. Although absent in the ground state and the 40-second reactant-state structure, the electron density is superimposable with a water molecule observed in the Pol \( \eta \)-dAMPNPP ternary complex. This water molecule is hydrogen-bonded with the 3'-OH, the O4' of dATP and another water molecule in the 80-second reactant-state structure, but it is incompatible with the C3'-endo conformation in the product state (Fig. 3a). The 3'-OH is also hydrogen-bonded to a water molecule in an X-family DNA polymerase crystallized with dUMPNNP (2'-deoxyuridine-5'-[(\( \alpha, \beta \)-imido)triphosphosphate]). A water-mediated and substrate-assisted catalytic mechanism has previously been proposed. In addition to the water molecule, S113 is hydrogen-bonded with the 3'-OH in the ground state and may pick up the proton and pass it to E116 in the reactant state. (Fig. 1d). However, the S113A-mutant Pol \( \eta \) retains \( 95\% \) of \( k_{\text{cat}} \) with a threefold increase of \( K_m \) (Fig. 3b and Supplementary Table 3). Interestingly, mutations of the S113 equivalent in the A- and B-family DNA polymerases (highly conserved histidine and threonine, respectively) also have limited impact on catalytic efficiency. We therefore propose that 3'-OH is prone to deprotonation when coordinated by Mg\(^{2+}\) and aligned with the incoming nucleotide. The proton can be passed on to the transient water molecule and then to bulk solvent. When the 3'-end of the primer is a ribonucleotide, the O2' may relay the proton out instead. An incoming dNTP most probably participates in deprotonation of the nucleophile because, with the nitrogen substitution in dAMPNNP, the water molecule is bound stably to the 3'-OH.
endonuclease IV, the three Zn$^{2+}$ ions are also essential for catalysis. They bridge the two reaction products destined to separate and prevent product release.

Figure 3. Deprotonation of the 3′-OH. a, Superposition of the refined reactant state (80 s, yellow) and product state (300 s, blue). The transient water molecules, probably deprotonating the 3′-OH, are circled. The sugar pucker at the primer 3′ end changes from C2′-endo in the reactant state to C3′-endo in the product state. The clashes between α-phosphate and the C2′ if it is C2′-endo, this is a divalent cation and most probably Mg$^{2+}$ (Fig. 4). This third metal ion is liganded by four water molecules, two of which occupy the space of the departed R61 (Fig. 2d). Two additional ligands are the leaving oxygen (bridging between the α- and β-phosphate) and the non-bridging oxygen of the α-phosphate (Fig. 4). This Mg$^{2+}$ thus bridges the two reaction products destined to separate and prevents DNA translocation. In the TG crystals with 1 mM Mg$^{2+}$ addition, which consists of a mixture of reactant-state (80 s, yellow) and product-state (300 s, blue). The transient water molecule if the water molecule does not depart, are indicated by red dashed double arrowheads. Hydrogen bonds and metal-ion coordination are shown as dashed lines. b, Wild-type (WT) and S113A-mutant Pol η can both extend primer with a ribonucleotide at the 3′ end (lighter colour) as with pure DNA (darker colour).

Concluding remarks

Previously, flash-freeze was used to trap covalent intermediates and conformational changes associated with a chemical reaction. Here, we extend the technology to record DNA synthesis in real time and at atomic resolution (Fig. 5 and Supplementary Movie 1). The arrival of a water molecule to deprotonate the 3′-OH occurs only after two-Mg$^{2+}$-ion-induced substrate alignment and in the presence of a correct dNTP but not a non-reactive dAMPNPP. Mg$^{2+}$-dependent and water-mediated deprotonation may occur in all DNA polymerases. The third metal ion that replaces the side chain of R61 during the catalysis and between the C2′ and the transient water molecule if the water molecule does not depart, are indicated by red dashed double arrowheads. Hydrogen bonds and metal-ion coordination are shown as dashed lines. In the presence of a correct dNTP but not a non-reactive dAMPNPP.

Figure 4. The third Mg$^{2+}$ ion in Pol η catalysis. A stereo view of the three metal ions (Mg$^{2+}$, A, C) observed in the intermediate state of 230 s after Mg$^{2+}$ addition, which consists of a mixture of product state (blue) and reactant state (yellow). The third Mg$^{2+}$ (Mg$^{2+}$, C) has six ligands, four of which are water molecules. The 2F$_{o}$ − F$_{c}$ map contoured at 1.5σ (grey) and F$_{o}$ − F$_{c}$ map with the third Mg$^{2+}$ omitted contoured at 4.0σ (green) are overlaid with the ball-and-stick model. Hydrogen bonds and metal-ion coordination are shown as dashed lines.
A proposed mechanism for metal-ion-dependent polymerase reaction. The B-site metal ion is stably associated with the incoming dNTP and the enzyme. Binding of Mg$^{2+}$ at the A site aligns the reactants, in particular the 3'-OH, and promotes its deprotonation, which is assisted by dNTP and the position equivalent to R61 in Pol η (ref. 50). The involvement of an additional transient metal ion may be a general feature of both the one- and two-metal-ion mechanism.

**METHODS SUMMARY**

Pol η, DNA and dATP complexes mixed with Ca$^{2+}$ in a 1:1 molar ratio were concentrated to 3 mg ml$^{-1}$ Pol η and crystallized by the hanging-drop vapour-diffusion method$^{39}$. For in crystallo reactions, crystals were first stabilized in 0.1 M MES (pH 6.8–7.2), 5 μM dATP, 20% (w/v) polyethylene glycol 2000 monomethyl ether (PEG2K-MME) and 1 mM DTT for ~30 min, transferred in a nylon loop to the reaction buffer containing 0.1 M MES (pH 6.8–7.2), 1 mM MgCl$_2$, 20% (w/v) PEG2K-MME and 1 mM DTT for the desired amount of time, followed by a quick dipping in a cryo-solution supplemented with 20% (w/v) glycerol and flash-cooled in liquid nitrogen. The reaction time courses were determined based on the $F_k - F_0$ maps with all data scaled against and compared to the refined ground-state structures. The inter-state maps were refined as a mixture of reactant state and product state, and their ratio at each time point was determined according to the reaction course (Fig. 2b) and difference ($F_k - F_0$) electron-density maps. Catalytic rates ($k_{cat}$), $k_{cat}/k_{m}$, and $k_{m}$ were measured using the template/primer listed in Supplementary Table 3.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions T.N. and Y.Z. carried out crystallography. Y.Z. did the mutagenesis and kinetic analyses. T.N. handled crystals, refined structures and analysed reaction time courses. W.Y. designed the project. T.N., Y.Z. and W.Y. prepared the manuscript. Y.Y. supervised T.N. and Y.-J.H. advised Y.Z. All authors discussed the results and commented on the manuscript.

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Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank with accession codes from 4ECQ to 4ECZ, 4ED0 to 4ED3, and 4ED6 to 4ED8. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to W.Y. (wei.yang@nih.gov).
METHODS

Protein and crystal preparation. S113A-mutant Pol η was made using QuikChange (Stratagene). Wild-type Pol η (residues 1–432) and S113A-mutant proteins were expressed and purified as described29. The ternary complexes of Pol η, DNA and dATP were mixed with Ca²⁺ at 1:1 molar ratio and concentrated to a protein concentration of ~3 mg ml⁻¹. Oligonucleotides used for original (AT pair at the DNA end) and three ‘loosely packed’ crystals (TG or GT mispaired and an unpaired A) are shown in Supplementary Table 2. An unpaired nucleotide at the 5' end was disordered in the original Pol η structures29, and was therefore removed to improve the diffraction quality. All crystals were obtained using the hanging-drop vapour-diffusion method against a reservoir solution containing 0.1 M MES (pH 6.0) and 9–17% (w/v) PEG2K-MME and streak seeding.

Chemical reaction in crystallo. Based on diffraction qualities, the complexes containing the original AT and TG-end DNA were selected for chemical reaction in crystallo. These crystals were first transferred and incubated in a pre-reaction buffer containing 0.1 M MES (pH 6.8–7.2), 5 μM dATP, 20% (w/v) PEG2K-MME and 1 mM diithiothreitol (DTT) for ~30 min. For the pH 6.8 time course (Fig. 2b, Supplementary Table 1b) and translocation in the TG crystal, the pre-reaction buffer also contained 5 μM MgCl₂. The different pre-reaction buffer might cause the large variations of the pH 6.8 reactions. Chemical reaction was initiated by transferring the crystals with a nylon loop into a reaction buffer containing 0.1 M MES (pH 6.0) and 9–17% (w/v) PEG2K-MME and streak seeding. After incubation for a desired time point (40 s and intervals of ~40 s), the crystals were quickly dipped in a cryo-solution supplemented with 20% (w/v) glycerol and flash-cooled in liquid nitrogen. For in crystallo reactions, MES buffer was titrated by KOH and not NaOH to avoid binding of Na⁺ ion at the A site. To observe product release, TG crystals were soaked in 5 μM dATP and 5 μM MgCl₂ for 30 min and chased with addition of 1 mM MgCl₂ for 1–8 min.

Data collection and structure determination. Diffraction data were collected at 100 K on beam lines 22ID and 22BM of the advanced photon source. Data were processed with HKL200051 or XDS52, and converted to structure factors by CCP453. Collaborative Computational Project, Number 4. The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D 50, 760–763 (1994).

To determine the reaction time courses (Fig. 2b and Supplementary Figs 1–3), all data were scaled against and compared to the refined ground-state structure, and electron densities for the new bond (between the 3'-OH of the primer strand and the α-phosphorus of dATP) and the metal ions were measured in the F₀ – F₁ map (with the B-site metal ion omitted) at each time point using programs in CCP453. After refinement of the ground-state structure, the intermediate structures were refined as a mixture of reactant state and product state using PHENIX54 and Coot55. The ratio of reactant state and product state at each time point was determined according to the time course of the new bond formation (Fig. 2b) and difference (F₀ – F₁) in electron-density maps. For high-resolution refinement (~1.5 Å resolution), the translation–libration–screw refinement was applied. Data collection and refinement statistics are summarized in Supplementary Table 1.

Kinetic measurements. Kinetic measurements including Kₘ and kₗ were done using the template/primer shown in Supplementary Table 3. The basic reaction mixture contained 2.5 nM Pol η, 5 μM 5'-fluorescein-labelled primer and template, and 0–80 μM dNTP in 40 mM Tris–HCl (pH 7.5), 5 mM MgCl₂, 10 mM diithiothreitol, 100 mM KCl, 0.1 mg ml⁻¹ bovine serum albumin and 5% glycerol. For the metal-ion-competition assay, Mg²⁺ was increased from 0 to 1.0 mM in 0.1-mM steps and Ca²⁺ was decreased to keep the combined concentration of 1.0 mM. The effects of pH were screened from pH 6.0 to pH 8.0. All reactions took place at room temperature for 4 or 8 min and were stopped by addition of 10× formamide loading buffer to the final concentrations of 8% formamide, 1 mM EDTA (pH 8.0) and 0.1 mg ml⁻¹ xylene cyanol. After heating to 90 °C for 3 min and immediately placing on ice, products were resolved on 20% polyacrylamide sequencing gels containing 5.5 M urea. Quantification and curve fitting were carried out as described29. Kₘ, kₗ and (kₗ/Kₘ) of wild-type and S113A-mutant Pol η catalysing single-nucleotide incorporation using deoxyribonucleotide versus ribonucleotide primer are summarized in Supplementary Table 3 and plotted in Fig. 3b.

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