SUPPLEMENTARY ONLINE MATERIAL

For manuscript:

Single Molecule Studies Reveal the Function of a Third Polymerase in the Replisome

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This supplement contains:

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Materials and Methods

Reagents and proteins. Pol III subunits (α, ε, θ, τ, γ, δ, δ', χ, ψ, β), primase, SSB, and DnaB helicase were purified as described[1-3]. DiPol III* (Pol III-core$_2\tau_2\gamma_1\delta\delta\ni\chi\psi$) and TriPol III* (Pol III-core$_3\tau_3\delta\delta'\chi\psi$) were reconstituted and purified as described[4]. Gel purified DNA oligonucleotides were synthesized by IDT. Glucose oxidase and Catalase were from Sigma. Yo-Pro1 was from Invitrogen (Molecular Probes). Photo-clear silicone-based elastomer (Sylgard 184) was from Dow-Corning, MI. Lipids were from Avanti Polar Lipids Inc.

Single-molecule imaging of DNA during Total Internal Reflection Fluorescence (TIRF) microscopy. Flow cells for single-molecule TIRF microscopy were prepared using photo-clear silicone-based elastomer poured into a negative lithography mold, as previously described[5]. A lipid bilayer was formed on the glass surface inside the flow cell using a mixture of freshly sonicated liposomes, as described[5,6]. Microscopy was performed using an Olympus IX70 inverted microscope equipped with a 60X TIRF objective, a Prior motorized stage, and a motorized shutter. Replication assays were performed as described below, and buffers to support replication were driven through the flow cell by a programmable syringe pump (KD Scientific). Yo-Pro1, included in the buffer flow during replication experiments, fluoresces upon intercalating into dsDNA products. Yo-Pro1 fluorescence was excited using a solid state 488 nm laser (Coherent) at 1.5 mW. The images were captured with a 512 X 512 back thinned EM CCD camera (Hamamatsu) through a 60x TIRF objective. Image collection and data work-up were facilitated using the Slidebook (Intelligent Imaging Inc.) and Matlab (Mathworks Inc.) software suites.

Replication assays during TIRF microscopy. A 100mer synthetic DNA minicircle substrate containing a 40 dT 5' biotinylated tail was prepared as described[4]. DnaB helicase (18.2 pmol, 365 nM) was assembled onto the minicircle DNA (655 fmol, 13.1 nM) in 50 μl Buffer A (20 mM Tris-HCl, pH 7.5, 5 mM DTT, 40 μg/ml BSA, 4% glycerol) containing 8 mM $Mg(OAc)_2$ and 0.25 mM ATP, followed by incubation for 30s at 37°C. To this was added a 25 μl reaction containing Pol III* (675 fmol, 27 nM), β_2 (1.85 pmol, 74 nM as dimer), 60 μM each dCTP and dGTP and 8 mM $Mg(OAc)_2$ in Buffer A. After 2 min at 37 °C, 1 μl of the reaction was diluted 1000-fold into 1 ml of Buffer B (8 mM $MgOAc_2$, 60 μM each of dCTP and dGTP, and 50 nM Yo-Pro1 in Buffer A). The reaction was passed through the flow cell at 500 μl/min for 30s, then at 10 μl/min for 1 min. Following this step, the flow cell was washed for 4 minutes with Buffer A containing no proteins, to remove any unbound proteins. DNA replication was initiated upon flowing (100 μl/min) Buffer A

containing 60 μM of each dNTP, 250 μM each of CTP, TTP, and UTP, 1 mM ATP, 462 nM SSB_4, DnaG primase at various concentrations (3 nM, 6 nM, 25 nM and 250 nM), 50 nM β_2, 50 nM Yo-Pro1, 0.8% glucose, 0.01% β-mercaptoethanol, 0.57U glucose oxidase, and 2.1U catalase. The force exerted on lambda DNA at this flow rate was determined to be 1.45pN as explained in our earlier study[5]. Replication is observed for 30 minutes (or longer) during which time the flow buffer lacks Pol III* (unless otherwise indicated) and DnaB, thus acting as a further wash step to remove any Pol III* (and DnaB) that is not bound to DNA. Unbound Pol III* is the more worrisome, considering that DnaB can not reload onto DNA in the presence of SSB, unlike Pol III*. As an empirical control to ensure that unbound Pol III* is eliminated during the usual 4 minute wash step of the replication reaction protocol, a buffer containing Pol III* and β was passed over the flow cell, then the cell was washed for 2 minutes before immobilizing minicircle DNA to which DnaB was attached. Replication was then initiated as described above. No products were observed, demonstrating that Pol III* is effectively removed in the wash step. It should also be noted that since ssDNA has a different contour length than dsDNA, we specifically chose DNA molecules containing no gaps for measurements of processivity in the experiments of Fig 1. These experiments are performed using 250nM primase and therefore this selection process makes little difference in the processivity number as under this condition the gaps are small, and most molecules have no observable gaps. For example, DiPol replisomes produce DNA molecules with fewer than 10% gaps, while TriPol replisomes produce less than 2% of molecules with gaps.

Bead-based replication assays. Reactions containing 100 fmol of the 100mer DNA minicircle substrate, 60 μM dCTP, 60 μM dGTP, 50 μM ATP-γ-S, 4 pmol $DnaB_6$, 2.5 pmol β_2, 0.5 pmol Pol III* were incubated in replication buffer in a total volume of 20 μl for 5 min at 37ºC prior to immobilization on streptavidin-coupled magnetic beads (Invitrogen) for 10 min. Beads were washed three times in replication buffer containing 50 nM β_2 and 20 μM ATP-γ-S, prior to re-suspending in replication buffer containing 60 μM dCTP, 60 μM dGTP and 2.5 pmol β_2. Replication was initiated by addition of 0.5 mM ATP, the indicated concentrations of DnaG primase (200nM or 320 nM), 480 nM SSB_4 and 60 μM of dATP, 10 μM dTTP, 3 μCi α^{32}P-dTTP for leading, or 60 μM of dTTP, 10 μM dATP and 3 μCi α^{32}P-dATP for lagging strand synthesis. After 15s, 20nM of a TRAP DNA was added where indicated. The TRAP DNA was M13mp18 ssDNA annealed to a 3' dideoxynucleotide terminated 30mer DNA (5'-GTTAAAGGCCGCTTTTGCGGGATCGTCACddC-3') and pre-incubated with 4.8 pmol SSB, 2.5

pmol β_2, and 1 pmol γ-complex in order to assemble the β clamp onto the TRAP DNA. Reactions were allowed to continue for 20 s. Total reaction volumes were 25 µl. Reactions were quenched by adding 25 µl of 40 mM HEPES (pH 7.5), 40 mM EDTA and 6% SDS. DNA was released from the beads by boiling at 95ºC for 3 min and resolving on 0.8 % alkaline agarose gels. Gels were analyzed using a Typhoon 9400 phosphorimager and ImageQuant software.

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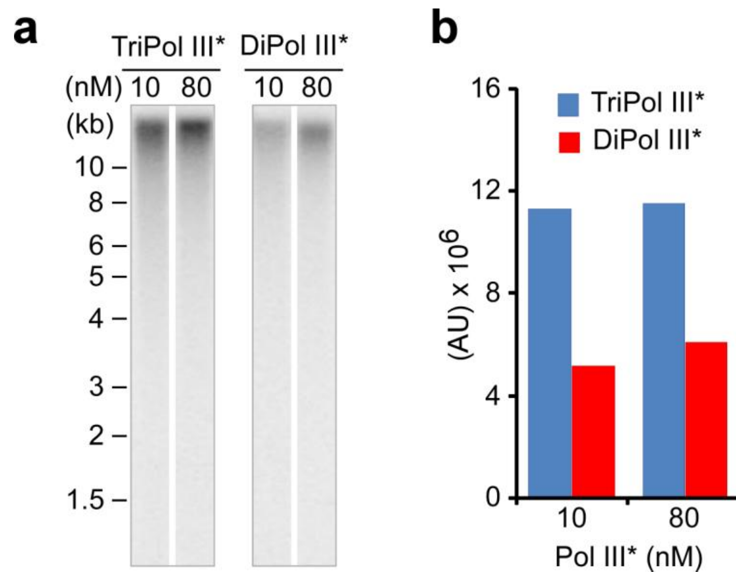
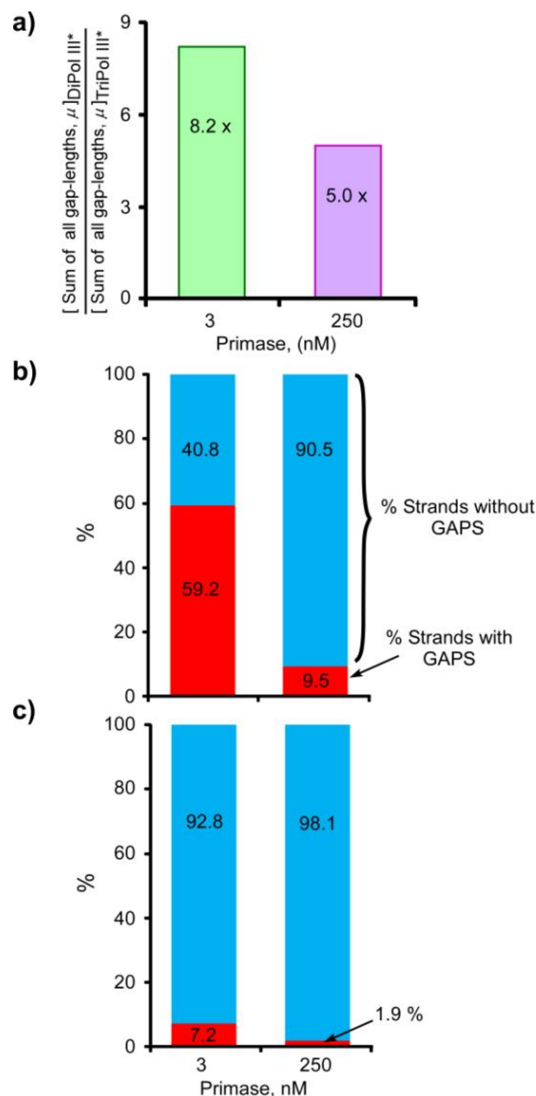
Supplementary Figure 1



Supplementary Figure 1. TriPol replisomes are more efficient than DiPol replisomes in total DNA synthesis. (a) Bead-based replication reaction products obtained using either *E. coli* TriPol replisomes or DiPol replisomes were resolved on a 0.8% alkaline gel. The replication reactions were performed at the specified replisome concentrations using 200 nM DnaG primase. (b) Quantitation of leading strand synthesis reactions. This assay is a measure of stability because both replisomes show equally processive DNA synthesis during the reaction time (35 s).

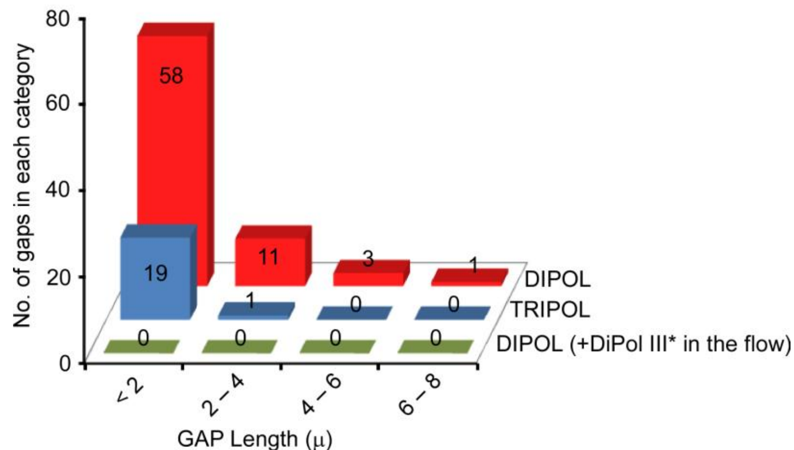
Supplementary Figure 2



Supplementary Figure 2. Variation in gaps observed at different primase concentration.

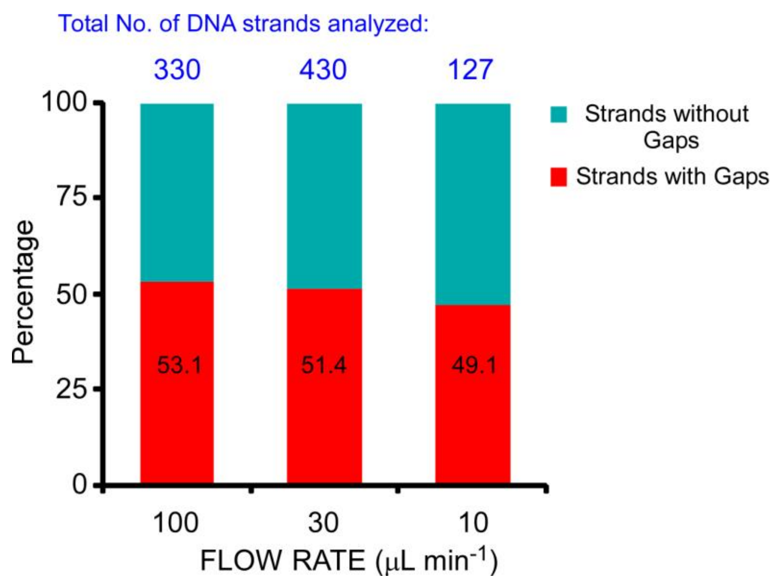
(a) Histogram depicting the ratio of the number of DNA strands containing gaps produced by Dipol replisomes versus the number of DNA strands containing gaps produced by Tripol replisomes, using either 3 nM or 250 nM primase. (b) Histogram depicting the percentage variation of the number of DNA strands containing gaps (red) and the strands without gaps (blue), in a series of experiments performed by Dipol replisomes at different primase concentrations of 3 nM and 250 nM. (c) Histogram showing a similar analysis as above, but in reactions performed using the Tripol replisome.

Supplementary Figure 3



Supplementary Figure 3. Dipol replisomes yield more gaps than Tripol replisomes. Overlay of three histograms showing the distribution of gap lengths (in μ) obtained in replication reactions performed by Dipol replisomes [no DiPol III* present in the flow] (red), Tripol replisomes [no TriPol III* present in the flow] (blue) and Dipol replisomes [with DiPol III* present in the buffer flow] (green) at high DnaG primase concentration (250 nM); a total of 540 DNA strands were counted in each experiment. The numbers of gaps in each length category are displayed on each column bar. Dipol replisomes present in the buffer flow eliminate the presence of ssDNA gaps in the replication products.

Supplementary Figure 4



Supplementary Figure 4. The hydrodynamic force imposed by the flow rate does not alter the frequency of ssDNA gaps in the DNA products. Histogram depicting the percentage of DNA products containing gaps (red) and products without gaps (blue), in a series of replication reactions performed by Dipol replisomes at buffer flow rates of 10, 30 and 100 μL/min, which correspond to forces of 0.1, ~0.4 and 1.45 pN, respectively.

Supplementary Video Material

Supplementary Video 1

Replication performed by Dipol replisomes. An example of a movie depicting real-time observation of coupled leading/lagging strand replication of a mini-rolling circle substrate by *E. coli* Dipol replisomes. The force of the hydrodynamic flow pushes the DNA-lipid complex to a diffusion barrier etched in the glass surface and concentrates numerous DNA molecules in the visual field shown here. The width of the visible area in the direction of the flow is 73 µm (equivalent to 220 kb) and the flow direction is from top to the bottom. Individual DNA molecules visualized with the fluorescent dye Yo-Pro1 are stretched by the buffer flow (100 µl/min) and imaged through Total Internal Reflection Fluorescence (TIRF) microscopy. Toward the end of the movie, the buffer-flow is stopped, letting the strands recoil, then the buffer flow is started again. Movie contains circa 7' 30" of experimental data rendered at 20 frames per second (original data acquisition is 1 frame/s at 100 ms exposure per frame).

Supplementary Video 2

Replication performed by Tripol replisomes. The video depicts the recording of a replication reaction performed by Tripol replisomes. The movie contains circa 5' 30" of experimental data rendered at 20 frames per second (original data acquisition is 1 frame/s at 100 ms exposure for each frame).

Supplementary Video 3

DNA molecules that harbor duplex regions contain gaps on the same molecule. The video depicts a recording at the end of a replication reaction using a DiPol replisome. The flow of the buffer solution is stopped then restarted, allowing the DNA strands to stretch and then recoil to their point of origin.

Supplementary Video 4

Use of fluorescent SSB to identify ssDNA in DNA products. The video depicts three successive recordings of different DNA products of DiPol replisomes, in which reactions contained fluorescently labeled SSB. The three successive recordings are easy to identify since they have different dimensions. The videos show that DNA products contain fluorescently labeled *E. coli* SSB

(with Oregon Green488 Maleimide). The duplex DNA is not visualized because Yo-Pro1 is omitted from the buffer flow for these experiments. To distinguish SSB bound to DNA from SSB that binds non-specifically to the surface of the flow cell, the buffer-flow is alternatively stopped and restarted in order to observe the recoiling of the DNA strands. Fluorescent SSB bound to DNA recoils and re-extends in synchrony with the changes in buffer flow (while non-specifically bound SSB does not change position).