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Coordinating DNA replication by means of priming loop and differential synthesis rate

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Abstract

Genomic DNA is replicated by two DNA polymerase molecules: one works in close association with the helicase to copy the leading-strand template in a continuous manner while the second copies the already unwound lagging-strand template in a discontinuous manner through synthesis of Okazaki fragments^{1, 2}. Considering the lagging-strand polymerase has to recycle after every Okazaki fragment through the slow steps of primer synthesis and hand-off to the polymerase^{3–5}, it is not understood how the two strands are synthesized with the same *net* rate^{6–9}. Here, we show with the T7 replication proteins^{10, 11} that RNA primers are made on the fly during ongoing DNA synthesis, and the leading-strand T7 replisome does not pause during primer synthesis contrary to previous reports^{12, 13}. Instead, the leading-strand polymerase remains limited by the speed of the helicase¹⁴, so synthesizes DNA at a slower rate than the lagging-strand polymerase. We show that the primase-helicase T7 gp4 maintains contact with the priming sequence during ongoing DNA synthesis; hence, the nascent lagging-strand template organizes into a *priming loop* that keeps the primer in physical proximity to the replication complex. Our findings provides three synergistic mechanisms of coordination: 1) Primers are made concomitantly with DNA synthesis; 2) the priming-loop assures efficient primer utilization and hand-off to the polymerase; 3) the lagging-strand polymerase copies DNA faster, which allows it to keep up with leading-strand DNA synthesis overall.

To investigate the functional cooperativity between the enzymatic activities of the T7 replication complex, we measured the kinetics of DNA unwinding, DNA synthesis, and primer synthesis on synthetic replication fork substrates with and without the T7 priming-sequence (3'-CTGGG, Supplementary Table 1). Efficient synthesis of RNA primers from 2mer to 5mer by T7 replisome (T7 gp4 and T7 DNA polymerase) was observed on the priming fork (Fig. 1a and Supplementary Fig. 1) with half-life of ~0.5 s and yield >60% (Fig. 1a, right). T7 gp4 alone also makes RNA primers on this priming fork, but at ~10-fold

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Author Contributions M.P. purified T7 gp5, T7 gp4 and constructed DNA substrates for the priming loop studies, and obtained and analyzed all the ensemble DNA synthesis and primer synthesis experiments; S.S. developed robust single molecule assays for observing DNA unwinding and priming loop formation, and obtained and analyzed all single molecule data; I.D. and G.P. performed the ensemble unwinding experiments; M.P., S.S., S.S.P., and T.H. designed the experiments, analyzed the data, and wrote the manuscript.

slower rate (Supplementary Fig. 1), consistent with polymerase assistance of the helicase rate¹⁴. An average 46% yield of primer synthesis with forks of different lengths and sequence (Supplementary Table 2) indicates that T7 replisome lays down primers on newly unwound lagging-strand template with a high efficiency. In addition, the newly made primers are elongated through lagging-strand DNA synthesis (Supplementary Fig. 1).

All-or-none DNA strand separation assays^{15, 16} under primer synthesis conditions show that T7 replisome unwinds the priming fork and the control fork (without the priming-sequence) with similar rate constants at all [dTTP] (Supplementary Fig. 2). Single-molecule FRET (Fluorescence resonance energy transfer) unwinding assays¹⁷ show an increase in fluorescence intensity of Cy3 (donor, green) and decrease in Cy5 (acceptor, red) due to DNA unwinding/synthesis (Fig. 1b), and the priming and control forks show similar FRET decrease time Δt of 0.4 ± 0.027 and 0.37 ± 0.022 s, respectively. By comparing the histograms of FRET values visited during the unwinding reactions between the T7 replisome reaction and the ~ 3 fold slower reaction by T7 gp4 alone (Supplementary Fig. 3), we further confirmed that the T7 replisome does not pause during primer synthesis (Supplementary Fig. 6). If the T7 replisome paused for several seconds every time a primer is made¹², our single molecule analysis would have detected the pausing events.

To investigate if DNA synthesis was occurring concomitant with primer synthesis, the kinetics of strand displacement DNA synthesis was measured on the priming and control forks under the same reaction conditions as in Fig. 1a. In the high resolution sequencing gels used to analyze the DNA synthesis kinetics, any pausing of the T7 replisome activity due to primer synthesis would be detected as an accumulation of specific DNA products in the priming fork reactions, but not in the control. However, no unusual accumulation of intermediate DNA products due to replisome pausing was observed with the priming fork template compared to the control (Fig. 1c). Consistent with this result, the average DNA elongation rate on priming (126 ± 9 nt/s) and control (113 ± 8 nt/s) forks are similar (Fig. 1d). No pausing was detected on longer forks (Supplementary Figs 1 and 8), or forks of different GC content or at different [dNTPs] (Supplementary Table 3). Finally, coupled leading-strand and lagging-strand DNA synthesis measured by the rolling-circle assay with T7 gp2.5 showed no effect of the lagging-strand synthesis on the leading-strand synthesis rate (Supplementary Fig. 9). Similar observations have been made with the T4 replication proteins¹⁸, although a recent study of *E. coli* replication reports a different result¹⁹. Overall, our results indicate that DNA synthesis continues uninterrupted while RNA primers are laid down, and the leading-strand polymerase does not slow as a function of primase activity or due to any of the steps during lagging-strand polymerase recycling.

Since DNA synthesis continues uninterrupted while primers are being synthesized, our results predict that the nascent lagging-strand template should loop out between the covalently linked helicase and primase domains of T7 gp4 (Fig. 2a)²⁰. The formation of such a priming loop during DNA synthesis was probed using single molecule FRET experiments: Cy3 and Cy5 fluorophores were introduced 40 bp apart on the lagging-strand template of the surface-attached DNA fork (Fig. 2b). Before DNA is unwound, no FRET is observed due to the long 40-bp distance between the fluorophores (Fig. 2b, 2c, (i)). As T7 replisome unwinds the dsDNA, the donor Cy3 shows an increase in intensity (green trace in Fig. 2c, top panel) due to change in environment from protein-induced fluorescence enhancement²¹ and DNA strand separation²² (Fig. 2b, (ii)). Continued DNA unwinding brings the priming-sequence and the donor nearby close to the primase domain, where they are held in place. The replisome continues unwinding the DNA while the primase domain is engaged with the priming-sequence; therefore, at some point in time, the acceptor comes close to the donor (Fig. 2b, (iii)) and detected as an increase in FRET (Fig. 2c, (iii) top and middle panels), providing evidence for the formation of a priming-loop. 40 molecules out of

~ 75 with fluorescently active donor and acceptor showed priming loop formation. With continued unwinding, the priming-loop grows in size and the donor and acceptor move apart (Fig. 2b, (iv)), which is detected as a decrease in FRET (Fig. 2c, (iv) top and middle panels). Finally, the total fluorescence signal disappears due to reaction completion and release of the fluorescently labelled DNA strand from the surface.

The control fork shows an increase in donor intensity (green) (Fig. 2c, bottom panel), but no increase in acceptor intensity or FRET (more than 200 molecules analyzed). The donor intensity time (Fig. 2d, bottom panel), the time between the jump in donor intensity and the total signal disappearance, is the same for priming and control DNAs, indicating that both DNAs are unwound with the same rate. The average time for FRET increase (Fig. 2d, top panel) with the priming fork is 3 times longer for the experiments with T7 gp4 only (Supplementary Fig. 11) compared to T7 replisome indicating the assistance of the polymerase in the reactions¹⁴. To test if compaction of unwound ssDNA may give rise to high FRET values²³, we measured FRET between Cy3 and Cy5 separated by 40 nt of ssDNA and found the average FRET value to be 0.2, much lower than those obtained during priming loop formation (Supplementary Fig 12). Overall, these results show that: 1) The primase remains engaged with the priming-sequence while DNA continues to be synthesized, and 2) the nascent lagging-strand forms a priming-loop.

Making primers ahead of time during ongoing DNA synthesis minimizes the delay due to primer synthesis, and keeping the RNA primers in physical proximity to the replicating complex provides a mechanism for efficient priming sequence utilization and hand-off to the polymerase. Nevertheless, lagging-strand polymerase dissociation, primer hand-off, and initiation of a new Okazaki fragment synthesis events take time that can potentially delay synthesis of the lagging-strand. Since the leading-strand replisome does not slow or pause during primer synthesis, the question remains as to how the lagging-strand polymerase overall keeps up with the leading-strand polymerase. We therefore tested an alternative model, proposed by Alberts²⁴ some 20 years ago, that the leading-strand polymerase simply moves with an overall slower rate than the lagging-strand polymerase.

The transient state kinetic assays allow us to measure the DNA synthesis rates very precisely. To measure the rate of DNA synthesis as catalyzed by the lagging-strand polymerase, we used a primer/template DNA substrate coated with T7 gp2.5 that mimics the already unwound lagging-strand template. To measure the rate of leading-strand synthesis by the T7 replisome, we used a fork substrate that contained the same template sequence in the dsDNA. T7 DNA polymerase copies the gp2.5 coated ssDNA template at a 30% faster (188 ± 10 nt/s) rate than the T7 replisome (132 ± 10 nt/s) (Fig. 3a, b). Faster rate of DNA synthesis by T7 DNA polymerase as compared to the replisome rate was observed with *E. coli* SSB coated ssDNA template (158 ± 10 nt/s) and without any single-strand binding protein (200 ± 8 nt/s) (Supplementary Fig. 13 and Supplementary Table 3). That the T7 replisome moves more slowly than the DNA polymerase alone is consistent with the DNA synthesis rate being limited by the helicase's speed¹⁴. From multiple experiments we estimate that T7 DNA polymerase alone copies the ssDNA template on an average 38% faster than the T7 replisome (Supplementary Table 3). Thus, the leading-polymerase will take on an average 6–7s longer than the lagging-strand polymerase to copy 3000 bp of DNA, the average length of an Okazaki fragment. Unless physical coupling slows its rate, the lagging polymerase will reach the end of the previously made Okazaki fragment with 6–7s to spare to pick up a new primer and initiate another round of Okazaki fragment synthesis.

Not observing replisome pausing in our studies contrasts previous reports^{12, 13} that suggested that T7 replisome pauses during primer synthesis. DNA synthesis in these

previous studies was measured indirectly by following the shortening of the overall length of DNA as dsDNA was converted to coiled ssDNA. In the presence of ATP+CTP, 5–6 s intervals were observed with no change in DNA length, which was attributed to replisome stopping. We propose that these pauses are not caused by replisome stopping, but by the conversion of ssDNA back to dsDNA due to uncoupled lagging-strand synthesis. Although reactions were washed, contaminating polymerase catalyzing uncoupled DNA synthesis including those tethered via T7 gp4²⁵ could not be ruled out. Under conditions where excess polymerase was present, Lee et al¹² observed transient loops in addition to pauses. It could not be ruled out that the pausing and looping pattern was caused by separate Okazaki fragment synthesis events. Those that were coupled showed loop release and those that were uncoupled showed the pausing behaviour.

Based on our studies, we propose that T7 replisome does not pause during primer synthesis or any of the steps of lagging-strand synthesis. Instead, the following synergistic mechanisms exist to coordinate strand synthesis: a) Primers are made ahead of time during ongoing DNA synthesis; hence, primer synthesis itself does not delay lagging-strand synthesis; b) the primer is kept in physical proximity to the replication complex via a priming loop that assures efficient primer utilization and hand-off to the lagging-strand polymerase (Fig. 4); and c) the lagging-strand polymerase copies the ssDNA template at a faster rate²⁴ providing extra time for the recycling steps. In addition to moving at a faster rate multiple lagging-strand polymerases could work at the same time to complete lagging-strand synthesis in a shorter time²⁶. Under certain conditions, the lagging-strand polymerase may jump to a new primer before the completion of the Okazaki fragment, thereby leaving gaps that can be filled in later²⁷. Since the basic mechanism of dsDNA replication is conserved from phage to human^{1, 2}, the mechanisms revealed from studies of the T7 replication proteins are broadly applicable to the more complex replication complexes of bacteria and eukaryotes.

METHOD SUMMARY

Ensemble kinetic assays

T7 gp4²⁸ and T7 DNA polymerase²⁹ (T7 gp5/*E. coli* thioredoxin) were preassembled on the DNA with dTTP and EDTA in replication buffer (50 mM Tris Cl pH 7.6, 40 mM NaCl, 10% glycerol) and the reactions were initiated with the addition of MgCl₂, rest of the dNTPs, with or without ATP, CTP, or dT₉₀ trap. Primer synthesis and DNA synthesis kinetics was measured using the rapid quenched-flow instrument (KinTek Corp) and products were resolved on 24 or 25% acrylamide/urea sequencing gel. DNA synthesis kinetics were fit to the polymerization model (Supplementary Information).

Single molecule FRET assays

Single molecule FRET experiments to measure unwinding and priming loop formation were performed on a wide-field total-internal-reflection fluorescence microscope with 30-ms time resolution and imaged via a CCD camera (iXon DV 887-BI, Andor Technology)³⁰. The Cy3 and Cy5 fluorophores were internally labeled on the dT via a C6 amino linker. Gel-based DNA synthesis reactions were performed to confirm that the fluorophores on the DNA did not affect DNA synthesis (Supplementary Fig. 7). The priming loop substrates were prepared by ligating donor and acceptor labelled DNAs (Supplementary Information). FRET was calculated as the ratio of the acceptor intensity and the total (acceptor + donor) intensity after correcting for cross-talk between the donor and acceptor channels and subtracting the background. For Fig. 1c and Fig. 2, the initiation of FRET change and its saturation were scored by visual inspection of the donor and acceptor intensities demonstrated as a robust method (Supplementary Fig. 5), and the calculated FRET efficiency and the time difference

between the two points was assigned as Δt . Photobleaching of the fluorophores was at least 10 times slower than the unwinding time, and no unwinding-like signal was observed without Mg^{2+} addition.

METHODS

Proteins and DNA

T7 gp4A' and gp5 (exo-) proteins were purified as described previously^{28, 29}. Thioredoxin of *E. coli* was purchased from Sigma-Aldrich. Protein concentration was calculated by UV absorption (in 8M guanidine hydrochloride) using the extinction coefficients at 280 nm $0.0836 \mu M^{-1}cm^{-1}$ for T7 gp4A' and $0.13442 \mu M^{-1}cm^{-1}$ for T7 gp5 (exo-). Oligodeoxynucleotides (Supplementary Table 1) were purchased from Integrated DNA Technology (Coralville, IA) or Sigma-Aldrich and PAGE purified before use. Substrates for the stopped-flow and the gel unwinding assays had an amino linker at the 5'-end of their bottom strands which was labeled with 5-(and-6)- Carboxy fluorescein succinimidyl ester [(5/6)-FAM, SE] using the procedure from Molecular Probes with carbonate buffer. Proteins were preassembled on the DNA before reaction start: T7 gp4 was added to the fork substrate with dTTP and EDTA in the replication buffer and incubated on ice for 30 min. For assembling T7 replisome, T7 DNA polymerase (T7 gp5 and *E. coli* thioredoxin in 1:5 proportion mixed for 5 min 22°C in replication buffer containing freshly made 5 mM DTT²⁹) was added to T7 gp4 and DNA mix and further incubated at room temperature for 30 min.

RNA primer synthesis assay

The protein-DNA complex was loaded in one syringe of the rapid quenched flow instrument. The second syringe contained $MgCl_2$, ATP, CTP mixed with a trace amount of $\alpha^{32}P$ -CTP, dATP, dCTP, dGTP, and dT₉₀ trap in the replication buffer (50 mM Tris Cl pH 7.6, 40 mM NaCl, 10% glycerol). Reaction was initiated by mixing equal volumes of the two solutions at 18 °C and quenched after various time intervals with 300 mM EDTA. Primers generated in reaction were resolved on 25% acrylamide/3M urea sequencing gel with 1.5×TBE buffer running the gel only 3/4th of the sequencing gel length. The gel was imaged on a Typhoon Phosphorimager and the products were analyzed using the ImageQuant 5.0 software. The yield of RNA primer synthesis was determined from the radiolabelled CTP incorporation in the 2- to 5-mer RNA taking into account the number of C's in the primers.

DNA synthesis kinetics

T7 replisome-fork DNA or T7 DNA polymerase-primer/template DNA complex was loaded in one syringe of the quenched-flow instrument. The second syringe contained dATP, dCTP and dGTP, with or without NTPs (ATP and CTP), $MgCl_2$ and trap (where applicable, see Supplementary Table 3) in replication buffer. Reactions were initiated by rapidly mixing equal volumes of the two solutions, and quenched after various intervals with 300 mM EDTA. The quenched solution was loaded on 22 or 24% acrylamide/7M urea sequencing gel with 1.5×TBE buffer. The gel was imaged by Phosphorimager and each of the DNA bands were quantified using the ImageQuant software. The time courses of the individual DNA product formation and decay were fit to the polymerization model using *mgfit* (<http://gfit.sourceforge.net>) to obtain the individual nucleotide addition rate constants from which the average DNA primer elongation rate was calculated.

Single molecule FRET experiments and data analysis

Biotin was attached at 5' end of the DNA strand during DNA synthesis. Cy3 NHS-ester and Cy5 NHS-ester (GE Healthcare) were internally labeled to the dT of single-stranded DNA strands via a C6 amino linker (modified by IDT). A quartz microscope slide (Finkenbeiner) and coverslip were coated with polyethyleneglycol (m-PEG-5000, Laysan Bio Inc.)¹⁷ 30 and biotinylated-PEG (biotin-PEG-5000, Laysan Bio Inc.). Measurements were performed by using a flow chamber that was assembled as follows. After the assembly of coverslip and quartz slide³⁰, a syringe was attached to an outlet hole on a quartz slide through a tubing. All the solution exchanges were done by putting the solutions (0.1 ml) in a pipette tip and affixing it in the inlet hole followed by pulling the syringe. The solutions were added in the following order. Neutravidin (0.2 mg/ml, Pierce) was applied to the surface and washed away with T50 buffer (10 mM Tris, 50 mM NaCl, pH 8). Biotinylated DNA (~50 to 100 pM) in T50 buffer was added and washed away with imaging buffer (10 mM Tris, 50 mM NaCl, 0.1 mg/ml Glucose Oxidase, 0.02 mg/ml Catalase, 0.8% dextrose, and Trolox, pH 8)³¹. For replisome measurements, T7 gp4 (50 nM hexamer) and T7 DNA polymerase (gp5/thioredoxin) (50 nM) were loaded on the DNA with 2 mM dTTP, 5 mM DTT, 5 mM EDTA in imaging buffer, and incubated for 10 min. After few seconds of imaging, the unwinding and polymerase synthesis was initiated by adding 1 mM each of other dNTPs, 1 mM ATP, 1 mM CTP, 5 mM DTT and 4 mM free Mg(II) in imaging buffer. All measurements were done at room temperature (23 ± 1 °C).

FRET values were calculated as the ratio between the acceptor intensity and the total (accepter + donor) intensity after correcting for cross-talk between the donor and acceptor channels and subtracting the background. For the unwinding experiment shown in Fig 1c, the initiation of FRET decrease and its saturation were scored via visual inspection of the donor and acceptor intensities and the calculated FRET efficiency, and the time difference between the two points was assigned as the Δt value of each reaction. Once we identify a sustained FRET decrease below 0.5, the first time point at which FRET value drops below the average FRET value before unwinding begins, typically ~0.8, was assigned as the initiation point. Likewise, once we identify a saturation in FRET decrease, the first time point at which FRET reaches the average FRET value in the saturation plateau is assigned as the saturation point. We demonstrate that the error in determining the initial time point of FRET decrease by this method is negligible (Supplementary Fig. 5). The FRET increase time in the priming loop experiment was plotted in the cumulative fraction vs time to indicate the fraction of molecules that have completed the FRET increase up to a given time point. The donor intensity time was the time between the initial donor signal increase and the final disappearance of fluorescence signal, also determined by visual inspection. The donor intensity time was also plotted in the cumulative fraction vs time format. All data were analyzed using scripts written in MATLAB and plotted in Origin.

Supplementary Material

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Acknowledgments

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Figure 1. Primer synthesis occurs concomitant with DNA unwinding and synthesis

a, Sequencing gel shows RNA primers (left) made by T7 replisome (600 nM) on ts40P-50% (300 nM) with dNTPs (1 mM each), CTP (0.5 mM), $\alpha^{32}\text{P}$ -CTP, ATP (1 mM), MgCl_2 (4 mM free), and dT₉₀ trap (3 μM) at 18 °C in replication buffer. Total yield of RNA primers is shown on the right panel. **b**, Representative Cy3 and Cy5 intensity traces showing DNA unwinding by single-molecule FRET with T7 replisome (50 nM), 1 mM each dNTPs, ATP, and CTP, and 4 mM free Mg^{2+} (23±1 °C). Additional traces are shown in Supplementary Fig. 4. The FRET time course and dwell time histograms (right) determinations are described in the Methods. Priming and control substrates show high FRET before unwinding and similar FRET decrease time Δt due to unwinding/synthesis. **c**, Polyacrylamide

sequencing gels show progressive elongation of fluorescein labelled DNA primer by T7 replisome on ts40–50% (left) and ts40P-50% (right) under the same conditions as in (a). The nucleotide incorporation rates and error bars (right panels) were calculated from the global fit to the polymerization model (Supplementary Information), and the average DNA primer elongation rates are shown with the standard error of the mean.

Figure 2. Priming loop: Primase domain maintains contact with the priming-sequence during replication

a, The cartoon shows that while the primase domain remains bound to the priming sequence to make RNA (red), nascent lagging-strand template forms a priming loop. **b**, Fluorescently labeled DNA fork to investigate priming loop. **c**, Top: Cy3 (donor, green) and Cy5 (acceptor, red) intensity time traces during DNA synthesis by T7 replisome. (i) - (iv) labels correspond to the various DNA states in the cartoons on the left. Middle: FRET efficiency vs. time. Bottom: Representative Cy3 and Cy5 intensity traces on the control fork without the priming sequence. **d**, Cumulative fraction vs. the indicated time intervals determined from single molecule time traces. The time intervals measured are marked by the arrows in **c**. 40 molecules from one experiment were used to build the curves (see Methods). Supplementary Fig 10 shows additional representative time traces.

Figure 3. Lagging-strand synthesis is faster than leading-strand synthesis

a, Sequencing gels showing the progressive elongation of fluorescein labeled DNA primer by T7 replisome (200 nM) on 100 nM of ts40 (left) and p/t40 (right) with T7 gp2.5 (5 μM) under the same conditions as in Fig. 1a, without dT₉₀ trap. **b**, The rate constants of individual nucleotide addition steps. Errors were calculated from the global fits to the polymerization model. Standard error of mean are reported for the average DNA primer elongation rate constant.

Figure 4. Model of T7 DNA replication

The leading-strand template (yellow) is copied continuously by the cooperative action of T7 DNA polymerase and T7 gp4 while the lagging-strand template (in blue coated with gp2.5) is synthesized by T7 DNA polymerase through Okazaki fragment synthesis. The physical coupling of the leading and lagging-strand polymerases via T7 gp4 and gp2.5 interactions creates a Trombone loop. The priming loop (coated with gp2.5) is created between the physically linked primase and helicase domains of T7 gp4 due to ongoing DNA synthesis during primer synthesis. The priming loop keeps the nascent primer within physical reach of the lagging-strand polymerase. Upon primer hand-off to the lagging-strand polymerase, the priming loop becomes part of the Trombone loop.