A solution to release twisted DNA during chromosome replication by coupled DNA polymerases

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Chromosomal replication machines contain coupled DNA polymerases that simultaneously replicate the leading and lagging strands. However, coupled replication presents a largely unrecognized topological problem. Because DNA polymerase must travel a helical path during synthesis, the physical connection between leading- and lagging-strand polymerases causes the daughter strands to entwine, or produces extensive build-up of negative supercoils in the newly synthesized DNA. How DNA polymerases maintain their connection during coupled replication despite these topological challenges is unknown. Here we examine the dynamics of the Escherichia coli replisome, using ensemble and single-molecule methods, and show that the replisome may solve the topological problem independent of topoisomerases. We find that the lagging-strand polymerase frequently releases from an Okazaki fragment before completion, leaving single-strand gaps behind. Dissociation of the polymerase does not result in loss from the replisome because of its contact with the leading-strand polymerase. This behaviour, referred to as ‘signal release’, had been thought to require a protein, possibly primase, to pry polymerase from incompletely extended DNA fragments. However, we observe that signal release is independent of primase and does not seem to require a protein trigger at all. Instead, the lagging-strand polymerase is simply less processive in the context of a replisome. Interestingly, when the lagging-strand polymerase is supplied with primed DNA in trans, unpicking it from the fork, high processivity is restored. Hence, we propose that coupled polymerases introduce topological changes, possibly by accumulation of superhelical tension in the newly synthesized DNA, that cause lower processivity and transient lagging-strand polymerase dissociation from DNA.

Coupled leading- and lagging-strand polymerases, as observed in phage and bacterial replisomes, have marked implications for the topology of the daughter DNA products. The topological problem is based on the a priori fact that polymerases generate a helical product, and thus they must travel a helical path or the DNA product must turn behind them. For example, a rotating leading-strand polymerase will take the attached lagging-strand polymerase and wind the DNA duplex 360° around the axis of the leading strand, forming precatenanes in the daughter helices (Fig. 1a, left). At the rate of E. coli replication (650 base pairs per second) rotating polymerases quickly result in an impossible tangle. Alternatively, if the DNA products rotate instead of the polymerases, negative supercoils accumulate (Fig. 1a, right). Superhelical tension on the lagging strand may be relieved by rotation of single-stranded DNA (ssDNA), but ssDNA-binding (SSB) proteins form large superstructures, which probably constrain swivel motion. The energy generated by only three or four supercoils is sufficient to disrupt protein–protein and protein–DNA interactions. Thus topological tension could disrupt the replisome, unless the tension is periodically released. The supercoils produced by coupled polymerases behind the fork are negative supercoils, which can be resolved by Topo I and Topo III. However, Topo III is non-essential, and the viability of Topo I mutants has been controversial. Hence, topoisomerases may participate, but are insufficient to remove negative supercoils produced by coupled replisomes.

Interestingly, single-molecule studies demonstrate highly processive DNA synthesis (>100 kilobases (kb)) in the absence of topoisomerases, implying that replisomes possess an intrinsic solution to the problem of coupled replication. Indeed, it was originally proposed that the topological problem could be solved by transient release of one polymerase of a coupled replisome from DNA (Fig. 1b), enabling the negative supercoils on both strands to relax. If the leading polymerase detaches from DNA, it will rebind the same primer terminus for continued extension. If the lagging polymerase dissociates, it will reattach to the same Okazaki fragment and complete it provided a new primed site is not yet formed. During extension of longer Okazaki fragments a new priming event is more likely to occur; thus a dissociated lagging polymerase may rebind the new RNA primer, leaving the original Okazaki fragment incomplete (Fig. 1b). In fact incomplete Okazaki fragments have been observed in bacterial and phage systems.

Premature polymerase dissociation is referred to as ‘signal release’ because it has been presumed that the lagging-strand polymerase is ‘signalled’ by a replisome component to dissociate and alleviate supercoil tension.

To gain insight into the topological challenge of coupled DNA replication, we developed assays to study signal release. The E. coli replisome is assembled on a 5’ biotinylated rolling circle substrate and then attached to streptavidin beads (Fig. 2a). The substrate has
Figure 2 | Signal release does not require primase and correlates with increasing Okazaki fragment length. a. The replisome, consisting of DNA helicase (blue), Pol III* (one clamp loader (green) that binds three Pols (yellow); only two Pols are shown for clarity) and β-clamps (red) is assembled on a 5′-biotinylated rolling circle DNA, then attached to beads. After unbound proteins are removed, replication is initiated by adding primase, on a 5′-32P-labelled deoxyribonucleotide triphosphates. Reactions are quenched and treated with S1 to cleave gaps left by signal release, before analysis on alkaline gels. The newly synthesized leading strand (purple) is the template for lagging-strand synthesis (blue), initiated by RNA primers (red). b. Replication reactions using [α-32P]dATP either before (lane 1) or after (lane 2) S1 analysis, or using [γ-32P]dATP (lane 3). c. Reactions in the absence of primase, using 800 nM DNA 20-base oligonucleotides to prime the lagging strand. d. Titration of DNA 20-base oligonucleotides into reactions. e. Plot of Okazaki fragment length versus percentage signal release.

only three nucleotides on either strand, allowing either leading or lagging strand to be labelled depending on which radioactive nucleotide is used. Replication is initiated after wash steps to remove unbound proteins, and ssDNA gaps left by signal release are detected by treating the α-32P-labelled deoxyxyribonucleotide triphosphate ([α-32P]dATP)-labelled lagging-strand template with S1 endonuclease to digest the gaps (Supplementary Fig. 1). If all Okazaki fragments are completely extended, the product will be S1-resistant. Conversely, 100% signal release will leave gaps between every Okazaki fragment, and S1 digestion will yield products of similar size to Okazaki fragments (Supplementary Fig. 2). An intermediate length of DNA will result if signal release is less than 100%.

The S1 analysis shows an intermediate-sized [α-32P]dATP-labelled DNA (Fig. 2b, lane 2) that migrates between the length of undigested DNA (lane 1) and the size of Okazaki fragments (lane 3). Size analysis of S1-treated products shows that about one-third of all Okazaki fragments are incomplete (Supplementary Fig. 3 and Methods). To validate the assay, several controls were performed (Supplementary Fig. 4): (1) S1 digestion eliminates ssDNA produced in a reaction without primase (that is, no lagging-strand synthesis); (2) S1 does not cleave double-strand Okazaki fragments; (3) ssDNA gaps produced by signal release can be filled in by addition of DNA polymerase II (Pol II); (4) the presence of complete Okazaki fragments is confirmed using ligase.

Earlier studies implied that primase triggers polymerase signal release5,6. To test this hypothesis, we performed the reaction in the complete absence of primase, using exogenously added DNA primers to initiate Okazaki fragments. However, the result was unchanged (Fig. 2c). Studies in the T4 system suggest that a loaded clamp triggers polymerase release7. Although clamps cannot be omitted without negating lagging-strand synthesis altogether, titrations of the clamp (with or without clamp loader) do not influence signal release (Supplementary Fig. 5). Notably, the assays of Fig. 2 remove excess (unbound) polymerase-clamp loader (that is, Pol III*) and DnaB helicase, and show that primase, soluble polymerase, clamp loader and helicase are not required to trigger polymerase dissociation.

We find that increasing Okazaki fragment size by lowering DNA primer concentration results in more frequent signal release (Fig. 2d, e and Supplementary Fig. 6). Okazaki fragments of 1 kb give only 20% signal release whereas 5 kb Okazaki fragments give 80% signal release. Hence, small Okazaki fragments are more likely to be completed than long Okazaki fragments, implying that the lagging-strand polymerase is not very processive. In contrast, studies on model lagging-strand templates show the polymerase has high intrinsic processivity and extends a primed 5.4 kb φX174 ssDNA without dissociating8,9. To exclude the possibility that this result was an artefact of using DNA primers, we altered Okazaki fragment size by changing the concentration of primase8, but the results were upheld (Supplementary Fig. 7).

That signal release correlates with long Okazaki fragments was confirmed using Pol II to fill in ssDNA gaps, demonstrating that ssDNA gaps are associated with long Okazaki fragments and yield an average gap size of 800 nucleotides (Supplementary Fig. 8).

To test further for a protein circuit that lowers processivity by triggering polymerase release, we designed an assay to measure lagging-strand polymerase processivity directly. Because priming at a replication fork occurs randomly, producing heterogeneous-sized products, we attached a 5.4 kb primed φX174 circular ssDNA to the lagging-strand polymerase in trans before initiating replication (Fig. 3a). Considering that all replisomal proteins were present, protein-induced signal release should still have occurred, and the length of

Figure 3 | Lagging-strand polymerase processivity is restored using a separate DNA molecule. a. The replisome is assembled on DNA as in the legend to Fig. 2a. Unbound proteins are washed away before addition of primed φX174 5.4 kb ssDNA. B. Reactions are initiated in the presence (lanes 1–12 and 19–24) or absence (lanes 1–6 and 13–18) of primase. Supernatants containing φX174 replication products (lanes 1–12) are separated from bead-bound rolling circle products (lanes 13–24).
A time-course of replication showed a most surprising result (Fig. 3b). The 5.4 kb ϕX174 DNA was fully replicated by the lagging polymerase (Fig. 3b, lanes 1–6 and 13–18), in striking contrast to the low processivity during Okazaki fragment synthesis (that is, 80% dissociation by 5 kb; Fig. 2d, e). We obtained similar results in the presence or absence of primase, confirming that neither primase nor primed sites trigger signal release (Fig. 3b, lanes 7–12 and 19–24).

The main difference in reactions with or without the primed ϕX174 DNA in trans is the connection of the two polymerases through the DNA, indicating that the connection of two polymerases to the same DNA molecule causes a decrease in lagging-strand polymerase processivity. One possible non-protein source is the topological strain DNA molecule causes a decrease in lagging-strand polymerase processivity. One possible non-protein source is the topological strain of DNA in turn 

Notably, previous studies of signal release that suggested a protein trigger are also consistent with polymerase release triggered by torsional constraints owing to coupled replication1–2. For example, in T4, 4 APRIL 2013 | VOL 496 | NATURE | 121

Figure 4 | Single-molecule total internal reflection fluorescence microscopy. a, The replisome is assembled on the rolling circle DNA and attached to a lipid bilayer. Replication is initiated upon flowing replication buffer at 10 μl min⁻¹. b, DNA curtain produced at 10 μl min⁻¹. c, Normalized line plot intensity of a representative DNA strand. Diagrams at the top illustrate dsDNA and ssDNA regions. d, Analysis as in c, but with Pol III⁺ in the buffer flow. e, Quantification of line plot analysis at indicated flow rates (n = 9 ± s.d. for 10 μl min⁻¹ or n = 13 ± s.d. for 100 μl min⁻¹). f, DNA curtain at 100 μl min⁻¹. g, Line plot analysis as in c, at 100 μl min⁻¹. h, Histogram relating pixel intensity to dsDNA at 10 μl min⁻¹ (light blue) and 100 μl min⁻¹ (dark blue). i, Scheme at 100 μl min⁻¹.
increasing the concentration of clamps may accelerate clamp assembly on new RNA primers and increase the probability that a released polymerase binds the new RNA primed site. In the T7 system, additional primase may enhance priming frequency providing more opportunity for a dissociated polymerase to bind a new site. Negative supercoiling is required for a variety of DNA metabolic actions, including replication, recombination and transcription. Negative superhelicity is a property of genomic DNA in all organisms and seems constant over the entire genome. Hence it is conceivable that coupled leading-/lagging-strand replication provides a method by which negative supercoils can be placed into DNA across the entire genome without topoisomerase action.

Our data suggest that signal release is caused by DNA topology and thus may provide a topoisomerase-independent solution to the topological problem incurred by coupled DNA polymerases during replication. The results demonstrate that (1) signal release does not require primase, (2) the release process is stochastic implying an inherent low processivity of the lagging polymerase, (3) signal release requires connection of both polymerases to the same DNA and (4) addition of DNA in trans eliminates signal release in the presence of all replisomal proteins. Despite the correlation of premature signal release to the topological problem inherent in coupled polymerases, the problem of coupled polymerase action is not definitively solved in this paper and will require further studies.

METHODS SUMMARY

Reactions contained 100 fmol 5'-biotinylated 100-base oligonucleotide rolling circle DNA 18, 60 μM deoxyadenosine triphosphate (dATP) and deoxyguanosine triphosphate (dGTP), 50 μM ATP-γ-S, 4 pmol DnaB, 2.5 pmol β2, and 0.5 pmol Pol III* in 20 μl reaction buffer. Reactions were incubated 5 min at 37 °C before immobilization on streptavidin-magnetic beads. Beads were washed three times in 500 μl replication buffer containing 30 μM dATP, dGTP, 12 pmol β2 and 50 μM ATP-γ-S before re-suspending in replication buffer containing 60 μM dCTP, 60 μM dGTP and 2.5 pmol β2. Replication was initiated by adding 0.5 mM ATP, 480 mM SSB, 50 μM NTPs and either 60 μM dATP, 10 μM [α-32P]dTTP, or 60 μM dTTP, 10 μM [α-32P]dATP, and 200 nM primase or DNA 20mer (unless indicated otherwise) for 35 s. For S1 analysis, reactions were quenched, and beads washed twice in Buffer E and once in S1 buffer. Beads were re-suspended in 20 μl S1 buffer. Five-microtitre beads were incubated with 1 U S1 for 7.5 min in 15 μl. Single-molecule total internal reflection fluorescence microscopy was performed as described 19, and is further elaborated in Methods.

Full Methods and any associated references are available in the online version of the paper.

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1. Kornberg, A. & Baker, T. A. DNA Replication 2nd edn (W. H. Freeman, 1992).
2. Ullsperger, C. J., Volgodskii, A. V. & Cozzarelli, N. R. Unlinking of DNA by Topoisomerases during DNA Replication Vol. 9 (Springer, 1999).
3. Hingorani, M. M. & O'Donnell, M. Sliding clamps: a (tailored fit. Curr. Biol. 10, R25–R29 (2000).
4. Wyman, C. & Botchan, M. DNA replication. A familiar ring to DNA polymerase processivity. Curr. Biol. 5, 334–337 (1995).
5. Hamdan, S. M., Loparo, J. J., Takahashi, M., Richardson, C. C. & van Oijen, A. M. Dynamics of DNA replication loops reveal temporal control of lagging-strand synthesis. Nature 457, 336–339 (2009).
6. Li, X. & Marians, K. J. Two distinct triggers for cycling of the lagging strand polymerase at the replication fork. J. Biol. Chem. 275, 34757–34765 (2000).
7. Yang, J., Nelson, S. W. & Benkovic, S. J. The control mechanism for lagging strand polymerase recycling during bacteriophage T4 DNA replication. Mol. Cell 21, 153–164 (2006).
8. Johnson, A. & O'Donnell, M. Cellular DNA replicases: components and dynamics at the replication fork. Annu. Rev. Biochem. 74, 283–315 (2005).
9. Postov, I., Piter, B. J. & Cozzarelli, N. R. Is this the best thought we have thought: the twisted story of replication. Bioessays 21, 805–808 (1999).
10. Chastain, P. D. I., Makarov, A. M., Nossal, N. G. & Griffith, J. Architecture of the replication complex and DNA loops at the fork generated by the bacteriophage T4 DNA polymerase III holoenzyme. J. Biol. Chem. 267, 21276–21285 (2002).
11. Vologodskii, A. V., Lukashin, A. V., Anshelevich, V. V. & Frank-Kamenetskii, M. D. Fluctuations in superhelical DNA. Nucl. Acids Res. 6, 967–982 (1979).
12. Lee, F. P., Georgescu, R. & O'Donnell, M. Mechanism of the E. coli tau processivity switch during lagging-strand synthesis. Mol. Cell. 11, 315–327 (2003).
13. Espeli, O. & Marians, K. J. Untangling intracellular DNA topology. Mol. Microbiol. 52, 925–931 (2004).
14. Stockum, A., Lloyd, R. G. & Rudolph, C. J. On the viability of Escherichia coli cells lacking DNA topoisomerase I. BMC Microbiol. 12, 26 (2012).
15. Stupina, V. A. & Wang, J. C. Viability of Escherichia coli topA mutants lacking DNA topoisomerase I. J. Biol. Chem. 280, 355–360 (2005).
16. Yao, N. Y., Georgescu, R. E., Finkelstein, J. & O'Donnell, M. E. Single-molecule analysis reveals that the lagging strand increases replisome processivity but slows replication fork progression. Proc. Natl Acad. Sci. USA 106, 13236–13241 (2009).
17. Tanner, N. A. et al. Real-time single-molecule observation of rolling-circle DNA replication. Nucleic Acids Res. 37, e27 (2009).
18. O'Donnell, M. E. & Kornberg, A. Complete replication of templates by Escherichia coli DNA polymerase III holoenzyme. J. Biol. Chem. 260, 12884–12889 (1985).
19. McHenry, C. S. DNA replicas from a bacterial perspective. Annu. Rev. Biochem. 80, 403–436 (2011).
20. Wu, C. A., Zechner, E. L., Reems, J. A., McHenry, C. S. & Marians, K. J. Coordinated leading- and lagging-strand synthesis at the Escherichia coli DNA replication fork. J. Biol. Chem. 287, 14047–14052 (2012).
21. Marko, J. F. & Siggia, E. D. Stretching DNA. Macromolecules 28, 8759–8770 (1995).
22. Dorman, J. C. DNA supercoiling and bacterial gene expression. Sci. Prog. 89, 151–166 (2006).
23. Travers, A. & Muskhelishvili, G. A common topology for bacterial and eukaryotic transcription initiation? EMBO Rep. 8, 147–151 (2007).
24. Benyajati, C. & Worcel, A. Isolation, characterization, and structure of the folded interphase genome of Drosophila melanogaster. Cell 9, 393–407 (1976).
25. McHenry, C. S. DNA replicas from a bacterial perspective. Annu. Rev. Biochem. 80, 403–436 (2011).

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.O.D. (odonnel@rockefeller.edu).
Analysis of lagging-strand polymerase processivity on primed phiX174 ssDNA in trans. Replisomes were assembled on the biotinylated rolling circle substrate as described above. After the wash steps, beads were re-suspended in 20 μl replication buffer containing 8 nM phiX174 ssDNA (5.4 kb) primed with a DNA oligonucleotide (5’-CAAGCAGTAACTCGTCTTATCAAG-3’). 2.4 pmol SSB1, 1 pmol β2, 0.08 pmol γ-complex, 60 μM dCTP and 60 μM dGTP for 2 min at 37 °C. Beads were immobilized, washed and replication was initiated as described above.

Data analysis. Lanes in alkaline gels were scanned using a phosphorimager with ImageQuant software. The intensity of radioactive signal at each pixel was normalized to the corresponding molecular mass to correct for the fact that larger products incorporate more radiolabel. Size markers were used as a reference in the gel. Percentage signal release was calculated from the size difference of S1-treated leading- and lagging-strand products (fit with a single Gaussian distribution as described in the legend to Supplementary Fig. 3) using the following calculation:

\[ \text{SR} \% = \frac{\text{Size (1g)}}{\text{Size (1d+S1)}} \times 100 \]

where SR = signal release; size (1g) = size of lagging-strand product; size (1d + S1) = size of S1 digested leading-strand product (that is, lagging-strand template).

Single-molecule replication assays. Replisomes were assembled onto 530 fmol (10.5 nM) 3' biotinylated 100-base oligonucleotide rolling circle DNA by first dilution 18 pmol DNAhe helicase (365 nM) in 50 μl Buffer A (20 mM Tris-HCl, pH 7.5, 5 mM DTT, 40 μg ml−1 BSA, 4% glycerol) containing 8 mM MgOAc2 and 0.25 mM ATP, followed by incubation for 30 s at 37 °C. Then a 25 μl solution of Buffer B containing 60 μM of each dATP, dGTP and dTTP and 8 mM MgOAc2, was added followed by a further 1 min at 37 °C. To immobilize the replisome–DNA complex in the flow cell, 1 μl of the above solution was diluted 1000-fold into 1 ml of Buffer B (8 mM MgOAc2, 60 μM each of dCTP and dGTP, and 50 nM Yo-Pro1 in Buffer A), then the diluted reaction was passed through the flow cell at 500 μl min−1 for 30 s, and a further 2 min at 10 μl min−1. DNA replication was initiated upon flowing Buffer A containing 60 μM of each deoxyribonucleotide triphosphate, 250 μM each of CTP, TTP and UTP, 1 mM ATP, 462 mM SSB, 100 mM primase, 50 mM β2, 50 mM potassium glutamate, 50 mM Yo-Pro1, 0.8% glucose, 0.01% β-mercaptoethanol, 0.57 mM glucose oxidase and 2.1 U catalase. The flow rate was either 10 μl min−1 or 100 μl min−1, as indicated. After 10 min of replication, the flow rate was adjusted to 100 μl min−1 to analyze replication products.

Intensity analysis of the DNA products. To correct for flow disturbances in our analysis, we averaged between 30 and 40 frames for each DNA strand (over two adjacent pixels); the same operation was performed for an adjacent empty region to serve as background correction that took into consideration the signal variation of non-uniform intensities shown by the bilayer. In addition to this background subtraction, we performed a signal normalization. As the DNA strand grows longer and extends further away from the biotin–streptavidin anchoring point in the lipid bilayer, there is an additional ‘slow-drift’ in intensity that is due to higher strand mobility into the flow. Therefore we normalized the slow-drift intensity signal to 100% dsDNA by applying a rank-order filter, a method best suited for removing shot noise. This method finds the specified percentile of data in the data window around each point in the data set (here the corrected line plot intensity) and replaces that point with the percentile. We analysed the data using Matlab software.

26. Yao, N., Hurwitz, J. & O’Donnell, M. Dynamics of beta and proliferating cell nuclear antigen sliding clamps in traversing DNA secondary structure. J. Biol. Chem. 275, 1421–1432 (2000).

27. Ohrrut, R., Finkielstein, J., Turner, J., Naikinis, V. & O’Donnell, M. Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. III. Interface between two polymerases and the clamp loader. J. Biol. Chem. 270, 13366–13377 (1995).

28. Bailey, S., Wing, R. A. & Steitz, T. A. The structure of T. aquaticus DNA polymerase III is distinct from eukaryotic replicative DNA polymerases. Cell 126, 893–904 (2006).

4. Georgescu, R. E. et al. Mechanism of polymerase collision release from sliding clamps on the lagging strand. EMBO J. 28, 2981–2991 (2009).