SUPPLEMENTARY INFORMATION

Coupling DNA unwinding activity with primer synthesis in the bacteriophage T4 primosome

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SUPPLEMENTARY RESULTS

a S1: GTT sites during unwinding

b

| streptavidin | biotin | digoxigenin | antidigoxigenin |
|-------------|--------|-------------|----------------|

~1200bp hairpin
sequence C

~600bp hairpin
sequence C

76nt tail
146bp tail

Glass surface

S2: GTT sites during rezipping

Magnetic bead

~600nt tails

Magnetic bead

polymericde with sequence C

Glass surface

c

[dig] = 40nM
[ATP] = 5mM

Loading Time [s]

2,000 3,000 4,000 5,000 6,000

5’ tail size [nt]

30 40 50 60 70 80 90 100

d

whole hairpin (1200bp)

half-hairpin (600bp)

Force [pN]

0 0.25 0.5 0.75 1

0 5 10 12.5 15 17.5 20

Extension [μm]
**Supplementary Figure 1** DNA substrate characterization. (a) Schematic representation of the two DNA hairpins, S1 and S2, used in this study. 5’-GTT priming sites are present only along the 5’ strand accessible during unwinding in the S1 hairpin, and along the 3’ strand accessible during ssDNA translocation in the S2 hairpin. (b) Schematic representation of the DNA hairpin substrate consisting of a 1239 bp hairpin with a 4 nt loop, a 76 nt 5’-biotinylated ssDNA tail, and a 146 bp 3’-digoxigenin labeled dsDNA tail, and the half-hairpin substrate created with a complementary 50-mer oligonucleotide (green) used to reduce the length of the hairpin and increase the length of the 5’ ssDNA tail. (c) The mean helicase loading time as a function of the length of the 5’ ssDNA tail. Assays measured the loading of 40 nM gp41 at saturating ATP concentration on a 6.7 kbp hairpin substrate. Four oligonucleotides complementary to different sequences along the hairpin were used to obtain 5’ ssDNA tails of approximately 2000, 3000, 4500, and 6000 nt. (d) The force-extension curves for the entire S1 hairpin (red) and the S1 half-hairpin (blue) demonstrate that the hairpins remain annealed at a constant extension until the force reaches ~16 pN when the extensions abruptly increase due to the mechanical unzipping of the hairpins and reanneal returning to their initial extension as the force is decreased below 15 pN. The larger initial extension for the S1 half-hairpin is the result of the elastic ssDNA tails of the substrate.
Supplementary Figure 2 Unwinding and translocation velocities depend on particular NTP. (a)
The unwinding and translocation rates, $v_u$ and $v_t$, are computed in segments (30 points long) from
the slope of the unwinding and rezipping phases, respectively. Under priming conditions
(helicase, primase, and rNTPs) the velocity analysis was performed using parts of the trace that
do not display priming signatures (jumps or blocks). (b) Distribution of unwinding rates
measured for the helicase alone in the presence of 5 mM ATP (light red), 1 mM rNTP (light
blue), and 5 mM GTP (light green) or complexed with primase in the presence of 5 mM ATP (dark red) and 1 mM rNTP (dark blue). The mean unwinding rate for each condition is labeled on the graph. The unwinding rate of helicase was not affected by the presence of primase, but surprisingly varied with the particular NTP present; the mean unwinding rate of helicase alone or in complex with primase was 1.5 times greater in the presence of all NTPs or 2.2 times greater in the presence of GTP than in the presence of ATP alone. Similar results were also true for the translocation rate of helicase in the presence of various NTPs (data not shown). The early literature reported that gp41 helicase had both ATPase and GTPase activity. Interestingly, it seems that gp41 helicase is able to unwind DNA faster in the presence of GTP rather than ATP.

(c) The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and total priming events (lower panel) measured in experiments with the wt primosome and the indicated rNTP. The number of molecules ($N_{mol}$) analyzed for each condition is 29, 12 and 9 in order, resulting in 447, 226 and 67 number of enzymatic traces ($N$), respectively. Note that very few blocks in hairpin reannealing and no jumps in DNA extension by the primosome were observed in the presence of only ATP or GTP.
**Supplementary Figure 3** Ensemble primer synthesis and priming only during unwinding. (a) Ensemble assays measuring the incorporation of radiolabeled CTP into pentaribonucleotide primers with the S1 (circles) and S2 (squares) hairpin substrates; the incorporation rates correspond to the synthesis of 0.15 and 0.01 primers per min per primosome, respectively. Two possible explanations for these low priming rates including inefficient primosome loading due to the short 5′ ssDNA tail of the substrate in the absence of the helicase accessory protein gp59; and reannealing of the DNA hairpin behind the primase. (b) The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and total priming events (lower panel) measured in experiments with the wt primosome at the indicated rNTP concentration on either the S1 (blue) or S2 (magenta) hairpin. The number of molecules (N_{mol}) analyzed for each condition is 29, 24, 21, 15, 12 and 10 in order, resulting in 447, 483, 612, 354, 226 and 161 number of enzymatic traces (N), respectively. Note that in the presence of 1 mM CTP priming events were observed only on the S1 hairpin where 5′-GTT priming sites are located during the unwinding phase and not on the S2 hairpin where 5′-GTT priming sites are located during the rezipping phase.
Supplementary Figure 4 Frequency of pauses during unwinding depends on substrate sequence. 

(a) The frequency of pauses during unwinding observed with the wt primosome at the indicated rNTP concentration on the S1 (blue) and S2 (magenta) hairpin substrate. The number of molecules ($N_{mol}$) analyzed for each condition is 29, 24, 21, 15, 12 and 10 in order, resulting in 447, 483, 612, 354, 226 and 161 number of enzymatic traces ($N$), respectively. Note that in the presence of 1 mM CTP the frequency of primosome pausing was independent of the DNA hairpin despite the fact that substrate S2 does not contain functional priming sites in the unwinding phase. (b) Number of pauses during unwinding (red) and the percent GC content of the substrate (blue) are plotted against the corresponding position of the S1 hairpin demonstrating that the primosome occasionally stops unwinding the DNA when it encounters stable GC rich regions of the DNA hairpin substrate. The normalized cross-correlation coefficient for the number of pauses and the percent GC content is 0.705. The priming recognition sequences occur more or less evenly spaced throughout the S1 hairpin (shown in Supplementary Figure 5), whereas the GC rich regions are concentrated at positions 250 to 280 and 560 to 600 on the S1 hairpin.
Supplementary Figure 5 Position of blocks is localized to priming sites on 5′ strand of substrate. Normalized distribution of blocks plotted against the corresponding position along the hairpin substrate measured in wt primosome experiments on the S1 hairpin with ATP and CTP (blue; $N_{mol} = 21, N = 345$) and on the S2 hairpin with all rNTPs (magenta; $N_{mol} = 24, N = 312$). The location of the priming sites available on the 5′ strand for the given rNTP and substrate are indicated by dashed gray lines. The normalized cross-correlation coefficient for the frequency of blocks and the priming site position is 0.59 and 0.62 for the S1 and S2 hairpins, respectively. The uniform yellow distribution represents primase binding randomly along the DNA hairpin. The maximum primer length that may be synthesized at each priming site for the given rNTP condition is shown in the upper panel. Under limited priming conditions with CTP and ATP present, only two 5′-GTT priming sites of the eleven available 5′-GTT priming sites on the S1 hairpin will result in a complete pentaribonucleotide primer being synthesized; the other nine sites will result in aborted primers due to the lack of GTP and UTP. The majority (~70%) of the observed blocks in hairpin reannealing are localized close to the two priming sites corresponding to the synthesis of a pentaribonucleotide primer (left panel). In contrast, a more homogenous distribution of blocks in DNA reannealing at all of the priming sites is observed when all rNTPs
are present, consistent with the fact that all the priming sites can support the synthesis of a complete pentaribonucleotide primer under these conditions (right panel).
Supplementary Figure 6 Pentaribonucleotide primers alone are not responsible for blocks in DNA rehybridization. (a) Example of a DNA extension trace (blue) obtained by repeatedly cycling between high and low applied force (green) in the presence of 1 µM 10-mer RNA primer complementary to a region of the DNA hairpin substrate. Annealing of the RNA oligonucleotide generates blocks in the DNA rehybridization of lifetime $\tau$. RNA oligonucleotides shorter than eight nucleotides did not generate blocks that could be detected within the time resolution of our instrument. (b) Example of the distribution of blocking lifetimes for a 10-mer RNA primer obtained at a reannealing force of ~9 pN fit to an exponential function with a mean blocking time $<\tau>$ = 1.9 ± 0.4 s.
of $1.9 \pm 0.4$ s. (c) The mean blocking lifetime at a reannealing force of ~9 pN varied exponentially with the length of the exogenous RNA primer. (d) The mean blocking lifetime of a 9-mer RNA oligonucleotide varied exponentially with the reannealing force; however, the mean lifetime of blocks in hairpin reannealing generated by the wt primosome was independent of the force (Table 2 in the main text).
Supplementary Figure 7 Mutant gp61(E234Q) primosome recognizes priming sites. The frequency of blocks in hairpin reannealing (upper panel), jumps in extension during unwinding (center panel), and total priming events (lower panel) measured in experiments with the wt (blue) and gp61(E234Q) (green) primosome at the indicated rNTP concentration on the S1 hairpin. The number of molecules ($N_{mol}$) analyzed for each condition is 29, 16, 21, 13, 12 and 14 in order, resulting in 447, 375, 612, 327, 226 and 189 number of enzymatic traces ($N$), respectively. The increased frequency of blocks and jumps observed with the wt primosome compared to the mutant primosome is attributed to the added stability of primase subunit(s) with a synthesized primer making the detection of blocks and jumps easier. Due to our stringent selection criteria for identifying blocks and jumps over the experimental noise, very short or unstable blocks and jumps are underestimated.
a) Time course of primosome association with DNA oligomers.

b) Effect of pentamer addition on primosome association.

c) Leading strand analysis of gp44/62, gp45 primosome association.

-7.25 kb

-d) Effect of rNTPs on primosome extension.

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Supplementary Figure 8 Characterization of the fused primosome. (a) Native polyacrylamide gel analysis of the unwinding activity of wt and fused primosomes at time 30, 60, 90, and 120 s. The fused primosome displayed ~64% of the wt primosome unwinding activity. Previous mutagenesis attempts on gp41 helicase have revealed that the unwinding activity of the protein is highly sensitive to modifications in the protein. (b) Denaturing polyacrylamide gel analysis of the priming activity of 125, 250, 375, and 500 nM wt or fused primosome. The fused primosome displayed ~130% of the wt primosome priming activity. This apparent hyperactivity most likely results from a higher concentration of primosome complex by nature of the fusion over separate primase and helicase proteins with a finite association constant. (c) Denaturing alkaline agarose gel analysis of leading and lagging DNA strand synthesis of 200 nM wt and fused primosome in the presence of 100, 200, 500, and 1000 nM clamp and clamp loader proteins. The ratio of leading to lagging DNA strand synthesis was approximately 50% demonstrating that the two polymerases within the replisome were highly coordinated. However, replisomes containing a fused primosome synthesized longer Okazaki fragments and only ~47% of the total DNA synthesized by wt replisomes. (d) Experimental DNA extension traces corresponding to the activity of the fused primosome in the absence of rNTPs (red) and presence of 1 mM CTP (blue).
Supplementary Figure 9 Translocation velocity during loop formation. (a) The DNA hairpin substrates have long (~600 nt) 5′ and 3′ ssDNA tails containing several 5′-GTT and 5′-GCT priming recognition sites that may be used for priming by the primosome which initially binds randomly at any position along the ssDNA tail. An experimental trace displays DNA loop formation and release during wt primosome translocation along the 5′ ssDNA tail. (b) Several traces displaying loop formation during primosome translocation at 9 pN (left panel) and 5 pN
(right panel) applied force. Traces have been shifted in time and extension for clarity. The translocation velocity measured during loop formation is 0.8 and 0.9 times the average translocation velocity measured during DNA hairpin rezipping at an applied force of 9 pN ($N_{mol} = 9, N = 23$) and 5 pN ($N_{mol} = 10, N = 17$), respectively.
SUPPLEMENTARY METHODS

Construction of gp61-gp41 fusion protein

A fusion protein of the separate T4 gp61 primase and gp41 helicase was constructed. An overlap extension PCR protocol was used to connect the C-terminus of gp61 to the N-terminus of gp41 with a 24 amino acid linker (GGGSEGGSSEGGSSEGGSSEGGS) based on the flexible glycine-rich linker found in the phage minor coat protein g3p. First, the gp61 gene was amplified from the pET-IMPACT(gp61I342G) plasmid\textsuperscript{4} using primers T7 Universal (5′-TAATACGACTCACTATAGGG-3′) and PHL2(-) (5′-CCGGTACCACCATACCTTCACCGCTACCACCACCTTCGCTACCACCCACCCTTAGCATATTTGA-3′) and the gp41 gene was amplified from the pET-IMPACT(gp41) plasmid\textsuperscript{5} using primers PHL2(+) (5′-GCGGTGAAGGTGGTGGTACCGGTGGTGGTAGCGAAGGTGGTGGTAGCATGGTAGAAATTATTCTT-3′) and gp41 Intein Reverse (5′-AACATTGGTACCCGCTTCATCCAGGAATTATTTATTCTT-3′). Second, the entire gp61-gp41 fusion gene was amplified from the previous two PCR products using primers T7 Universal and gp41 Intein Reverse and inserted into the pTYB1 plasmid using the NdeI and SapI restriction sites. The sequence integrity was confirmed by DNA sequencing the entire insert at the Penn State University Nucleic Acid Facility. Additional fusion proteins were constructed with linker sizes of 0, 13, and 18 amino acids; however, upon characterization the fusion protein with the 24 amino acid linker was found to have unwinding, priming, and coordinated leading and lagging strand synthesis activities most similar to the wt T4 primosome.

Characterization of gp61-gp41 fusion protein

*Helicase Unwinding Assay.* The helicase unwinding assays were performed in the standard replication buffer (25 mM Tris-Ac (pH 7.8), 150 mM KOAc, and 10 mM Mg(OAc)\textsubscript{2}) with 50 nM unwinding fork DNA, 500 nM trap ssDNA, 5 mM ATP, 375 nM gp32, 375 nM gp59, and 150 nM each gp41 and gp61 (monomeric concentrations) or 150 nM gp61-gp41 fusion in a typical reaction volume of 60 µL. The reaction was carried out at 37 °C, and aliquots were
removed at the time points indicated and quenched with an equal volume of 250 mM EDTA, 0.2% SDS, and loading buffer (50% glycerol, 1 µg/mL bromophenol blue, 1 µg/mL xylene cyanol FF). Reaction products were separated by 10% PAGE in TBE buffer and analyzed using a PhosphorImager. The sequences of the oligonucleotide substrates were as follows: fork lead (5'-CATCATGCGGACAGTCGACTTCAGATTTACTGTGTCATATAGTACGTATTC AG-3'); fork lag (5'-TAACGTATTCAAGATACCTCGTACTCTGTACTGACTGCGATCCGA CTGTCCCTGCATGATG-3'); and trap (5'-CTGACTGCGATCCGACTGTCCTGCATGATG-3').

The unwinding fork was made by mixing fork lead and fork lag DNA in equal molar amounts and radiolabeling the 5'-ends with T4 Polynucleotide Kinase and [γ-32P]ATP.

**Primase Priming Assay.** Priming reactions were carried out in the standard replication buffer containing 50 nM ssDNA oligo (71-mer), 4 mM ATP, 100 µM each CTP, GTP, and UTP, 20 µCi of [α-32P]CTP, and 125 – 500 nM each gp41 and gp61 (monomeric concentrations) or 125 – 500 nM gp61-gp41 fusion in a typical reaction volume of 20 µL. The reactions were carried out at 37 °C and quenched with an equal volume of 250 mM EDTA and loading buffer (formamide, 1 µg/mL bromophenol blue, 1 µg/mL xylene cyanol FF) after 5 min. Priming products were separated by 22% denaturing PAGE and analyzed using a PhosphorImager. The sequence of the ssDNA oligonucleotide substrate was 5'-AGAGGGAGATTTAGATGAGATGATTGAGGATGGAGATGTTGATGGAGAGATGATGAGATGAGGG-3'.

**Rolling Circle Replication.** The rolling circle replication reactions were performed in the standard replication buffer containing 1.8 nM nicked dsM13 DNA, 50 µM each CTP, GTP, and UTP, 1.5 mM ATP, 50 µM dNTPs, 10 µCi of [α-32P]dGTP, 4 µM gp32, 200 nM gp43, 100 – 1000 nM gp44/62, 100 – 1000 nM gp45, 200 nM gp59, and 200 nM each gp41 and gp61 (monomeric concentrations) or 200 nM gp61-gp41 fusion in a reaction volume of 25 µL. The reactions were quenched after 5 min with an equal volume of 250 mM EDTA and loading buffer (50% glycerol, 1 µg/mL bromophenol blue, 1 µg/mL xylene cyanol FF). The DNA products were separated through 0.8% alkaline-agarose gel electrophoresis (30 mM NaOH and 5 mM EDTA) for 48 h. After running, the gel was neutralized by soaking in TBE buffer and dried onto
a sheet of DE81 paper using first a stack of paper towels and then a vacuum gel-dryer. The dried gel and DE81 filter paper were exposed overnight to a PhosphorImager plate and analyzed using a PhosphorImager.

**DNA Hairpin Substrate Preparation**

The construction of pONE_nick, a pGEM vector containing a 1.1 kbp insert with only two 5′-GTT priming sites on the plus strand is described elsewhere. To make pNo_GTT, the final two 5′-GTT sites in pONE_nick were mutated to 5′-GAA by the standard Quickchange (Stratagene) mutagenesis procedure using the primer pairs: GTT1 forward (5′-GACAAAAACTTTTGAAGCATTTATCAGC-3′) and GTT1 reverse (5′-GCTGATAATGCTTCCAAAGTTTTGTC-3′); GTT2 forward (5′-GGCGAACTCCATAGAATTATGTGTGATCGC-3′) and GTT2 reverse (5′-GCGACTCACATAATTCTATGGAGTTCCGCC-3′). The initial 1.1 kbp insert was constructed from two DNA fragments selected for the limited number of 5′-GTT priming sites on the plus strand; there were no selection criteria for the minus strand or 5′-GCT priming sites. Therefore, the 1.1 kbp insert of pNo_GTT contains no 5′-GTT priming sites and nineteen 5′-GCT priming sites on the plus strand; and twenty 5′-GTT priming sites and nineteen 5′-GCT priming sites on the minus strand.

All oligos required to make both hairpin substrates were ordered from Integrated DNA Technologies (www.idtdna.com). The non-labeled oligos were purified using either 8% or 12% denaturing polyacrylamide gel electrophoresis depending on the length of the oligo followed by desalting. The 5′-biotinylated oligos were purified through binding to Immobilized Monomeric Avidin agarose resin (Pierce) and elution with 2 mM biotin. Before ligation, the hairpin and template oligos of both substrates were phosphorylated using T4 Polynucleotide Kinase. The oligos were annealed at a ten-fold excess over 1.1 kbp insert and ligated using T4 ligase at 4 °C for a minimum of 24 h. The oligos required for hairpin S1 included: hairpin1 (5′-GTCAGATGCTTTTGACGCTC-3′), flap1 (5′-Biotin-AATTGCATGTATTACTTGGTAGGATCGTCATAGCTTTAGCGATTTGGGACACTTCATCAAGACTCCTCCAGAGCGCCGGAGAC.
ATATAGCTACAGGGGCC-3′), and template1 (5′-GGCCCCCTGTAGCTATATGTCTCCGCCCCCCC
CCCTGTGTGTGTGTGTGTTGTGTGTGTGTGTGTTGTGTGTGTGTGTGTGTTGTGTTGCATACTTC
CGGGAACGCAG-3′). The oligos required for hairpin S2 included: hairpin2 (5′-GGCCCGTCA
GATGCCTTTTGGCATCTGAC-3′), flap2 (5′-Biotin-AATTGCATGTATTACTTGGTAGGATC
CGTCATAGCTTTAGCGATTTGGGACACTTCATCAAGACTTCCAGAGCAGCCGGAGAC
ATATAGCTACAGGGGCC-3′), and template2 (5′-CCTGTAGCTATATGTCTCCGCCCCCCC
CCCTGTGTGTGTGTGTGTTGTGTGTGTGTGTGTTGTGTGTGTGTGTGTGTTGTGTTTGGTGCATACTTC
CGGGAACGCAG-3′). Ligated products were purified and extracted from 1% agarose gels using
gel extraction kits (QIAgen). A common primer of sequence 5′-AAAAAAGTGTTGTGTGTG
TTGTTTGGGTTGTTGTTTGGTTGTTGTTGTTTGGTTGTTTGGGTGTTTGTGTGTTGTT
TGCTGCGTTCCCGGAAGTATGC-3′ was used for both substrates. A two-fold excess of
gp43(exo⁻) polymerase was used to fill in the overhang and incorporate multiple digoxigenin-
labeled dUTP nucleotides.

**Optimizing helicase loading conditions**

The time required for the helicase to load and start unwinding the DNA hairpin decreases as the
length of the 5′ ssDNA tail increases (Supplementary Fig. 1c). As the S1 and S2 hairpins are
unwound by helicase, the length of the 5′ ssDNA tail increases from 76 nt initially to a maximum
of 1300 nt. Therefore, helicase binds more rapidly as the substrate is unwound leading to
multiple enzymes being bound on the substrate. To ensure single-enzyme conditions, the helicase
concentration must be well below 100 nM; however, this results in slow initial helicase loading
times. In order to optimize the conditions for helicase loading we have used a complementary
50-mer oligonucleotide that binds near the middle of the hairpin to increase the length of the 5′
ssDNA tail (Supplementary Fig. 1b). Before starting an experiment, the oligonucleotide is
introduced into the chamber at a high concentration, 1 µM. Then, enough force to open the
hairpin, 16 pN, is applied for several seconds allowing the oligonucleotide to hybridize to the
complementary sequence in the hairpin. When the force is relaxed, the hairpin reanneals up to
the position of the oligonucleotide resulting in a substrate with ~600 bp hairpin and long 5’ and 3’ ssDNA tails of ~600 nt. Using this technique, the S1 hairpin is 604 bp long with eleven 5’-GTT and ten 5’-GCT priming sites on the 5’ strand accessible during DNA unwinding, and nine 5’-GCT priming sites on the 3’ strand accessible during rezipping. The S2 hairpin is 605 bp long with nine 5’-GCT priming sites on the 5’ strand accessible during DNA unwinding, and nine 5’-GTT and eight 5’-GCT priming sites on the 3’ strand accessible during rezipping.

**Testing ability of primers to generate blocks in rehybridization of DNA hairpin**

In order to investigate the nature of the DNA rehybridization blocks, we have tested the ability of ribonucleotide primers of various lengths to generate similar blocks in DNA hairpin reannealing. The experimental protocol consisted of introducing 1 µM complementary RNA oligonucleotide of N-length into the sample chamber, applying a force of ~16 pN for several seconds to mechanically unzip the hairpin, and then relaxing the force to a given value, hereafter referred to as the reannealing force, to allow the hairpin to rehybridize checking for the formation of blocks by primer annealing and measuring their lifetime.

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