Supporting Information

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SI Text

Sensitivity of Helicase Sliding to KCl Concentration. We tested the effect of KCl on helicase sliding/ unwinding *in vitro* by carrying out the reaction with the triplex 99B-54B-45R* (the asterisk indicates the location of 5′ P32 label) in the standard helicase buffer [20 mM Tris·HCl (pH 7.6), 10 mM Mg acetate, 5 mM ATP, 5 mM DTT, 8% glycerol, 50 mg/ml BSA] and the same buffer supplemented with 50, 100, or 200 nM KCl. The data showed (Fig. S1) that 150 mM KCl reduced the activity to 15% of the control, whereas at 200 mM KCl, the activity was essentially eliminated. Is this salt inhibition caused by the presence of the helicase minus other proteins of the replisome? The answer is in the negative because we and others have carried out reconstitution of replication initiation, elongation and termination *in vitro* at precisely this concentrations and observed robust arrest of the replication fork (1). Furthermore, addition of 0.15M KCl to the reaction mixture causes significant inhibition of replication.

It should be noted that the base flipping and enhancement of Tus–*Ter* stability was shown to occur at higher salt concentration and that with <40 mM salt used in the reaction buffer the difference between DNA substrate that did not have an unpaired CG6 at *Ter* and the one that had the unpaired C6 and another four to five residues in the single-stranded state did not show any appreciable difference in the off-rate of Tus from *Ter* (2). Despite this lack of presumptive base flipping, we observed robust replication fork arrest at the Tus–*Ter* complex *in vitro* (1). This observation provides supporting evidence that base flipping is unlikely to be the primary cause of polar replication termination.

DnaB Sliding Did Not Induce a Transient Denaturation Bubble Between the Arrested DnaB and the Tus–*Ter* Complex. Although the experiments described above showed no net melting of the dsDNA on which helicase sliding had occurred, we wanted to determine whether a transient denaturation bubble (or a persistent bubble according to model II) might have been generated and trapped between the sliding helicase and the Tus–*Ter* complex. Such a bubble, if present, could potentially cause base flipping, thereby forming a structure required by model II. Although *a priori*, the relative free rotation of the DNA duplex (without a constrained end) with respect to the sliding helicase should have dissipated any helical stress that might have promoted duplex melting, we still wanted to ascertain whether such a denaturation bubble could have formed at a Tus–*Ter* complex on the 99B-54B*-45R triplex, after incubation with Tus followed by DnaB and ATP. For a positive control, we constructed the triplex 111B-66B*-45R with a preformed bubble of five unpaired bases by using the oligos 111B, 66B and 45R (Table S1; see Fig. S2 *A* and *B*). We wanted to detect a transient bubble, if any, on the 99B-54B*-45R-Tus-DnaB complex by reacting the melted base pairs, with either 1%, 5% or 10% HCHO or 2 mM $KMnO_4$, to prevent snapping back of the unpaired bases upon removal of the bound proteins. We used sensitivity to digestion by the single-strand-specific S1 nuclease to monitor any "fixed" melted DNA in the triplexes (3).

The S1 sensitivity of the 5-nt preformed bubble on the triplex 111B-66B*-45R reassured us that the single-stranded region was accessible to S1 nuclease after HCHO (or $KMnO_4$) treatment of the protein–DNA complexes (Fig. S2). Therefore, we reasoned that under similar experimental conditions, any base-pair melting of an equivalent extent induced by helicase sliding on the nonbubble triplex would be similarly detected. Autoradiograms of the products after S1 digestion showed that, although the triplex with the preformed bubble was sensitive to S1 nuclease digestion, there was no detectable digestion of the 99–54*-45 triplex after it was allowed to form a complex with Tus and DnaB (Fig. S2) and fixed with 10% HCHO. Even after prolonged exposures, and under experimental conditions in which the substrate with the preformed bubble was almost completely digested with S1, the autoradiograms showed no evidence of a denaturation bubble on the 99B-54B*-45R triplex.

That HCHO was reactive under the experimental conditions was indicated by the formation of significant levels of protein–DNA cross-linked products, especially after treatment with 10% HCHO. We incubated the reaction products at neutral pH at 65°C for 12 h to reverse the cross-links. This was followed by reannealing by slow cooling, deproteinization with proteinase K, removal of the enzyme by self-digestion, and phenol extraction followed by S1 digestion. However, S1-sensitive sites were still not detected in the triplex without a preformed bubble. In summary, the results from these experiments showed that Tus-mediated arrest of helicase sliding under our experimental conditions did not generate a detectable denaturation bubble on the double-stranded substrate DNA at or near *Ter* (Fig. S2).

Presence of a Seven-Base Denaturation Bubble Containing the Unpaired C6 Does Not Elicit Stronger Polar Arrest of DnaB in Comparison with the Triplex Without Such a Bubble. Although the present work shows that the base-flipping DNA fork does not play a significant role in the determination of the effectiveness and polarity of replication fork arrest at *Ter*, we wondered whether these steps might occur *in vivo* and play an incremental role in the termination of replication. To investigate this possibility we reasoned that if a 7-bp bubble (mismatched bases) structure containing that included the GC pair at position 6 and the next residue in *Ter* (Fig. S3 *A* and *B*) were to be provided at the *Ter*–Tus complex, if base flipping occurred and caused enhanced stability of the complex, it should lead to significantly more robust helicase assay in comparison with the 99B-54B-45R triplex that has no such bubble.

We constructed the 7-bp bubble-containing triplex by annealing the 111A, 66, 45* (Table S1) oligos and performed helicase sliding/ unwinding under the same experimental conditions. Six independent sets of experiments were performed by two of the authors (D.B. and M.S.). The data (Fig. S3*C*) shows no significant enhancement of helicase block in the bubbled substrate in comparison with the one without the bubble. From these results we conclude that base flipping and fork formation did not play a significant role in helicase arrest *in vitro*.

Preparation of Cross-Linked Triplexes and Helicase Sliding/Melting Assays. We wanted to investigate whether DNA strands that were held together by interstrand cross-links immediately preceding C6 would still promote helicase sliding and its arrest by the Tus–*Ter* complex. To do this experiment, it was necessary to use site-directed interstrand cross-linking chemistry that was least likely to cause significant distortions of the DNA duplex geometry that might sterically block DnaB sliding. For this purpose, we synthesized a 24-mer containing **1** (24ϕ-Se^3-dTTP) at the positions corresponding to the T nucleotides at positions 4 and 5 of the bottom strand of *Ter* (4). This oligonucleotides was used to construct the triplex 99B-24ϕ-Se^3-30XL-45R* (Fig. 5*A*) and induced cross-linking by oxidation with 10 mM Na-periodate for

4 h at room temperature (≈22°C). The chemical reaction pathway is shown in Fig. 5*B*. The yield of the cross-linked product (which migrated in the gel just above the 99B* marker) varied from ≈40% to >95% (Fig. 4*C*).

We confirmed that product extracted from the gel was cross-linked by boiling and quenching in ice followed by nondenaturing analytical 12% PAGE. The boiling-quenching cycle did not cause any separation of the covalently cross-linked oligos but rather caused them to snap back as revealed by their characteristic lower mobility in comparison with the labeled 99-mer. The gel pattern also showed no detectable contamination of the noncross-linked 99-mer by free 99B* (data not shown).

Subsequently, a triplex substrate was constructed by annealing the purified 99B*-24-ϕ-Se^3 duplex with molar excess of unlabeled 30XL and either labeled or unlabeled 45R (Fig. 4*A Top* and *Middle*). We also constructed the control substrate with *Ter* in the nonblocking orientation by annealing and cross-linking the 26-mer ϕ-Se^R with the labeled 99NB* upper strand followed by hybridization of the partial duplex with a molar excess of the 17-mer and 45R (see Fig. 5*A Bottom*). Although, oxidation with Na-periodate resulted in excellent yields of site-directed interstrand cross-links, exposure of bases, especially in the single-stranded regions, to the oxidizing agent caused some technical problems by interfering with the proper annealing of the 30XL and 45R to the cross-linked duplex. This resulted in a substantial number of substrate molecules in which helicase progression was not responsive to Tus because *Ter* in these molecules apparently remained in the single-stranded state and did not bind to Tus. Because it was difficult to monitor helicase arrest by measuring the release of 45R* reporter against such a background, we decided instead to measure the percentages of input triplex that was protected from helicase action as a function of increasing ratios of Tus/*Ter*.

The measurements of the percentage of the input triplex protected from helicase activity in both orientations of the *Ter* as a function of increasing molar ratio of Tus/*Ter* are shown with the standard error bars in Fig. 4*D*. The data were collected from four separate experiments for each orientation of *Ter*. The results show that interaction of Tus with *Ter* in the blocking orientation impeded helicase progression despite the presence of two interstrand cross-links immediately preceding the C6. The helicase progression in the control substrate with *Ter* in the reverse orientation was much less responsive to increasing Tus concentration. The locations of the cross- link in the blocking orientation (Fig. 4*A Top* and *Middle*) were such that a denaturation bubble such as that required by model II could not have formed on such a triplex.

Oligonucleotides and Triplex Construction. The nonderivatized oligonucleotides were obtained commercially. Some were repurified by denaturing PAGE. The noncross-linked standard triplexes were constructed by annealing ≈400 fmol of a labeled oligo with 200 pmol of the complementary unlabeled oligos in 10 mM Tris, 1 mM EDTA, pH 8.0 first by raising the temperature to 95°C for 3–4 min followed by slow cooling to room temperature over 4–5 h. The material was passed through a Sephadex G25 spin column prepared by packing prewashed G25 over a layer of fine glass beads poured into a 1.5-ml microcentrifuge tube with a needle hole at the bottom.

Oligonucleotides containing phenyl selenide were prepared as described (4), annealed in 0.1 M NaCl in 10 mM Na phosphate buffer, pH 7.6 to other oligos as follows. One hundred picomoles of labeled 99-mer was mixed with 200 pmol of the 24-mer F-Se oligo and incubated at 55°C for 30 min in a microcentrifuge tube that was then inserted into a float and kept in a glass beaker with 500 ml of water at 55°C and allowed to cool slowly for 2 h to room temperature in an insulated Styrofoam box. The mixture was then kept at 4°C for 30 min, freshly prepared Na-periodate (500 mM) was added to a final concentration of 10 mM, and the incubation continued for 4 h at room temperature. The material was desalted through a G25 spin column and then purified by 7 M urea–15% PAGE. The cross-linked product was excised, eluted, and precipitated with ethanol with carrier tRNA. The product was then annealed to other oligos in 10 mM Tris, 1 mM EDTA (pH 8) and 100 mM NaCl by a cycle of brief heating to 90°C followed by slow cooling.

Enzymes. DnaB and Tus were purified as described (5).

S1 Nuclease Analysis. The labeled 55B* and 66B* were repurified by a preparative 7 M urea–15% PAGE, eluted from the gel, and annealed to form the triplexes. The triplexes were incubated with Tus at a molar ratio of 1:40 that was determined to be sufficient to protect ≈60% of the triplex from melting by DnaB in a polar mode.

DnaB at a concentration that melted at least 70% of the input DNA in 10 mM Hepes (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 8% glycerol, 50 mg/ml BSA, and 5 mM ATP was added to the substrate–Tus–*Ter* complex and incubated at 37°C for 15 min. The reaction mixture was treated with HCHO to 1%, 5%, or 10% or $KMnO_4$ to 2 mM for 15 min at 37°C, deproteinized with proteinase K and phenol and chloroform (standard procedure), precipitated with 50 mg/ml carrier tRNA, and then digested with S1 nuclease (at various concentrations of the enzyme in 50 mM Na acetate (pH 4.6), 1 mM Zn acetate, 200 mM NaCl, 50 mg/ml BSA, recovered by alcohol precipitation, and analyzed by 7 M urea–15% PAGE.

1. Abhyankar MM, Zzaman S, Bastia D (2003) Reconstitution of R6K DNA replication *in vitro* using 22 purified proteins. *J Biol Chem* 278:45476–45484.
2. Mulcair MD, *et al.* (2006) A molecular mousetrap determines polarity of termination of DNA replication in *E. coli*. *Cell* 125:1309–1319.
3. Zzaman S, Bastia D (2005) Oligomeric initiator protein-mediated DNA looping negatively regulates plasmid replication *in vitro* by preventing origin melting. *Mol Cell* 20:833–843.
4. Hong IS, Ding H, Greenberg MM (2006) Radiosensitization by a modified nucleotide that produces DNA interstrand cross-links under hypoxic conditions. *J Am Chem Soc* 128:2230–2231.
5. Khatri GS, MacAllister T, Sista PR, Bastia D (1989) The replication terminator protein of *E. coli* is a DNA sequence-specific contra-helicase. *Cell* 59:667–674.

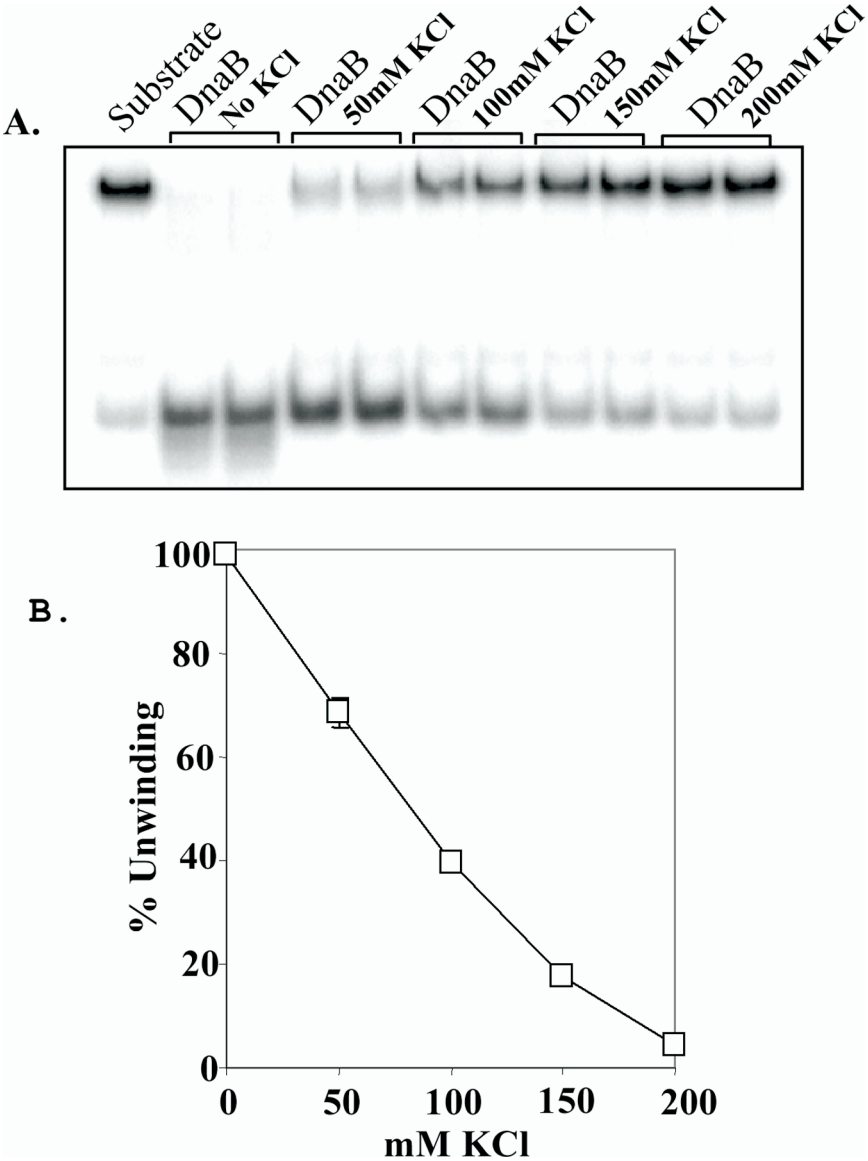

Fig. S1. Effect of KCl concentrations on DnaB activity. (*A*) Autoradiogram showing in duplicate the effect of 50, 100, 150, and 200 mM KCl on sliding of DnaB on the 99B-54B-45R* triplex. (*B*) Quantification of the data shown in *A* with a phosphorimager.

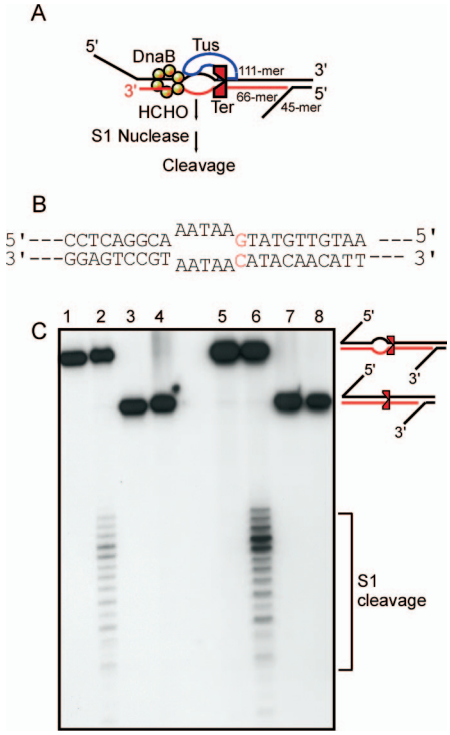

Fig. S2. Arrest of DnaB sliding by the Tus–*Ter* complex did not result in the formation of a denaturation bubble at *Ter*. (*A*) Diagram showing the structure of the triplex with five mismatched base pairs that was used as a control. (*B*) Nucleotide sequence of the bubble that did not include the GC6 pair (shown in red). (*C*) Representative autoradiogram of S1 digestion of the 99B-54B*-45R and 111B-66B*-45R triplexes that were incubated with or without Tus (molar ratio of 40:1) followed by DnaB and ATP. Lane 1, 111B-66B8–45R triplex without S1; lane 2, the same as in lane 1 but with S1; lane 3, 99B-54B*-45R without S1, lane 4, the same as in lane 3 but with nuclease; lane 5, 111B-66*-45R -Tus-DnaB complex without S1; lane 6, the same as in lane 5 but with S1. Lanes 7 and 8 are equivalent to lanes 2 and 3, respectively, but with Tus and DnaB. Note that no S1-sensitive site was observed after incubating 99B-54B*-45R with Tus and DnaB after fixation with 10% HCHO and reversal of cross-links.

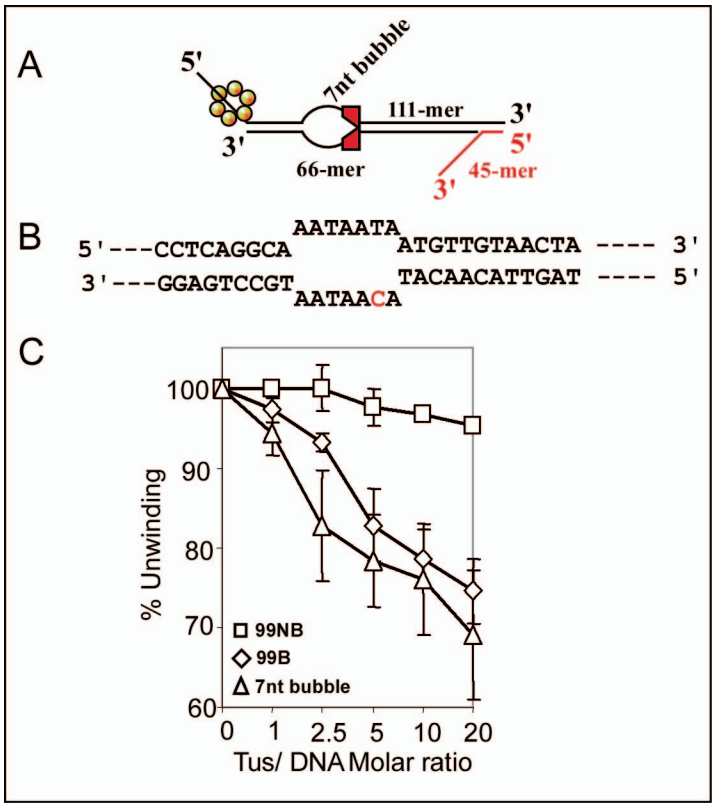

Fig. S3. Effects of a 7-bp denaturation bubble that included GC6 on polar helicase arrest. (*A*) Triplex was formed by annealing the oligos 111B′- 66B-45R* (see Table S1). (*B*) The detailed sequence about the 7-nt-long denaturation bubble in the triplex with the C6 residue (shown in red). (*C*) Arrest of helicase translocation caused by WT Tus on the triplexes 99B-54B-45R*; 99NB-54NB-45R* that had no unpaired bases and on 111B′-66B-45R* that had the 7-nt unpaired bubble at *Ter*. The data represent the average of six independent experiments, and the data points are shown with the standard errors of the measurements.

Table S1. Oligo sequences

99B	5′GGCATCTGCAGGCAGGAAGAGCGAAAACCTCGTCGGCCTCAGGCAA*AATAAGTATGTTGTAACTAAAGT*/AGGCAGCCTCGACGGGGA CGCGCTC CTCGG 3′
99NB	5′GGCATCTGCAGGCAGGAAGAGCGAAAACCTCGTCGGCCTCAGGC*ACTTTAGTTACAACATACTTATT*/TTAGGCAGCCTCGACGGGGACGCGCTCCT CGG 3′
54B	5′CCGTCGAGGCTGCCT*ACTTTAGTTACAACATACTTATT*/TTGCCTGAGGCCGACG 3′
54NB	5′CCGTCGAGGCTGCCTAA*AATAAGTATGTTGTAACTAAAGT*/GCCTGAGGCCGACG 3′
45R	5′CCGAGGAGCGCGTCCTCCAAAAGCGAGAAGGACGGACGTCTACGG 3′
111B	5′GGCATCTGCAGGCAGGAAGAGCGAAAACCTCAGGAGGGACAGCGTCGGCCTCAGGCAAAATAA*GTATGTTGTAACTAAAGT*/AGGCAGCCTCGAC GGGGACGCGCTCCTCGG 3′
66B	5′CCGTCGAGGCTGCCT*ACTTTAGTTACAACATA*/CAATAATTGCCTGAGGCCGACGCTGTCCCTCCTG 3′
115NB	5′GGCATCTGCAGGCAGGAAGAGCGAAAACCTCAGGAGGGACAGCGTCGGCCTCAGGCA*CTGCCTACTTTAGTTACAACATA*/CAATAAAGCGCCCTC GACGGGGACGCGCTCCTCGG 3′
70NB	5′CCGTCGAGGGCGCTAATAA*GTATGTTGTAACTAAAGTAGGCAG*/TGCCTGAGGCCG`ACGCTGTCCCTCCTG 3′
Mflp1	5′GGCATCTGCAGGCAGGAAGAGCGAAAACCTCGTCGGCCTCAGGCAAAATAA*TTATGTTGTAACTAAAGT*AGGCAGCCTCGACGGGGACGCGCTCC TCGG 3′
Mflp2	5′CCGTCGAGG*CTGCCTACTTTAGTTACAACATAA*/TTATTTTGCCTGAGGCCGACG 3′
111′B	5′GGCATCTGCAGGCAGGAAGAGCGAAAACCTCAGGAGGGACAGCGTCGGCCTCAGGCAAAATAATA*ATGTTGTAACTAAAGTAGGCAG*/CCTCGAC GGGGACGCGCTCCTCGG 3′
30-XL	5′ CCGTCGAGGCTGCCTACTTTAGTTACAACA 3′
24-mer Φ-Se^{B}	5′TACTΦTΦATTTTGCCTGAGGCCGACG 3′
26-mer Φ-Se^{NB}	5′AAGTΦATΦGTTGTAACTAAAGTGCCTGA
20-XL	5′ CCGTCGAGGCTGCCTAAAAT 3′

The Ter sequence in each oligo is italicized and underlined. TΦ, phenyl-Selenide T.