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Dynamic DNA Helicase-DNA Polymerase Interactions Assure Processive Replication Fork Movement

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SUMMARY

A single copy of bacteriophage T7 DNA polymerase and DNA helicase advance the replication fork with a processivity greater than 17,000 nucleotides. Nonetheless, the polymerase transiently dissociates from the DNA without leaving the replisome. Ensemble and single-molecule techniques demonstrate that this dynamic processivity is made possible by two modes of DNA polymerase-helicase interaction. During DNA synthesis the polymerase and the helicase interact at a high-affinity site. In this polymerizing mode, the polymerase dissociates from the DNA approximately every 5000 bases. The polymerase, however, remains bound to the helicase via an electrostatic binding mode that involves the acidic C-terminal tail of the helicase and a basic region in the polymerase to which the processivity factor also binds. The polymerase transfers via the electrostatic interaction around the hexameric helicase in search of the primer-template.

INTRODUCTION

The replisomes of *Escherichia coli* and its bacteriophages T4 and T7 mediate leading- and lagging-strand synthesis in a processive manner; the replisomes resist dissociation from the fork when challenged by dilution (Debyser et al., 1994; Kadyrov and Drake, 2001; Kim et al., 1996). However, for both T4 and T7, the replisome-associated DNA polymerases can exchange with free DNA polymerase without affecting processivity (Johnson et al., 2007; Yang et al., 2004). A similar exchange has been observed in *E. coli* where an error-prone DNA polymerase could exchange with DNA polymerase III that had encountered a lesion in the DNA (Indiani et al., 2005). This dynamic nature of protein-protein interactions allowing for polymerase exchange challenges the traditional definition of processivity (Joyce, 2004). Apparently, a processive replisome as defined by dilution experiments can contain DNA polymerases that undergo multiple binding events.

The bacteriophage T7 replisome provides a simple system to dissect the reactions that contribute to the processivity of the replisome. With only four proteins, it can advance the replication fork in a mechanism that mimics more complex systems (Benkovic et al., 2001; Johnson and O'Donnell, 2005; Lee et al., 1998). Gene 5 DNA polymerase (gp5), encoded by the phage, in complex with its processivity factor, *E. coli* thioredoxin (trx), is responsible for nucleotide polymerization. The hexameric gene 4 protein (gp4) has a helicase domain responsible for DNA unwinding and a primase domain that catalyzes the synthesis of RNA primers for the initiation of Okazaki fragments. Gene 2.5 single-stranded DNA (ssDNA) binding protein (gp2.5) removes secondary structure in ssDNA and plays a role in the coordination of the other replication proteins (Lee et al., 1998; Marintcheva et al., 2006).

T7 gp5 displays low processivity, dissociating from the primer after the polymerization of less than 20 nucleotides (Tabor et al., 1987). The processivity factor, trx, binds to a unique 76 residue segment at the tip of the thumb region of gp5 (Figure 1) (Bedford et al., 1997; Doublie et al., 1998), hence termed the thioredoxin-binding domain (TBD), and increases the processivity to hundreds of nucleotides per gp5/trx binding event (Tabor et al., 1987). Single-molecule experiments have shown that the average processivity of gp5/trx is 700 nucleotides per cycle (Wuite et al., 2000). Strand-displacement synthesis on double-stranded DNA (dsDNA) carried out in conjunction with the gp4 helicase is even more processive with greater than 17 kilobases (kb) polymerized per binding event (Lee et al., 2006a). Obviously the interaction of the DNA polymerase with the helicase translocating on the lagging strand has a dramatic effect on the stability of the proteins and their processivity (Lee et al., 2006a; Stano et al., 2005). The two proteins are known to interact physically (K_D = 90 nM) through an electrostatic interaction mode via the acidic C-terminal tail of gp4 and the basic TBD of gp5 (Hamdan et al., 2005; Notarnicola et al., 1997). We have recently shown that when the polymerase is bound to a primer-template in a polymerizing mode, gp4-gp5/trx interaction is extremely stable with

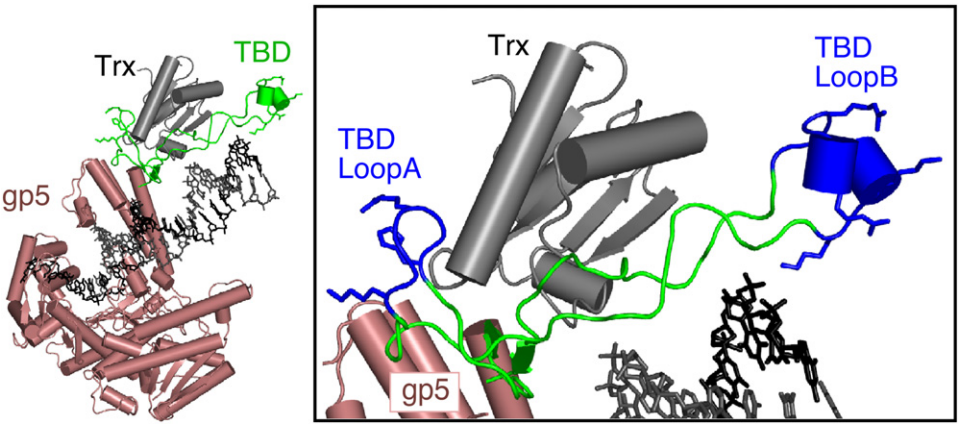

Figure 1. Crystal Structure of T7 gp5/trx Bound to Primer-Template and an Incoming Nucleotide

Gp5 is shown in pink and trx in gray. The DNA is depicted as sticks with the template in black and the primer in gray. Trx binds to the unique 76 residue segment at the tip of the thumb (TBD) (green). The inset shows an enlargement of the TBD and indicates the position of the two basic loops within TBD. Loop A is formed by residues 275–285. Loop B is formed by residues 299–314. In the current study we have substituted alanine for residues H276, K278, and R281 in loop A to generate gp5-loopA/trx. In loop B, we have replaced residues K302, K304, R307, and R310 with alanine to generate gp5-loopB/trx. Seven basic residues in loops A and B (H276, K278, R281, K302, K304, R307, and R310) are changed to alanine to generate gp5-loopAB/trx.

a half-life of more than 10 min (Hamdan et al., 2005; Lee et al., 2006a); this stable interaction does not involve the C-terminal tail of gp4.

How then can such a high processivity be maintained when DNA polymerases in solution exchange with the actively synthesizing polymerase? We speculate that the high processivity occurs even as gp5/trx undergoes multiple transient dissociations from the primer-template. The dissociation would be transient in the sense that, in the absence of free DNA polymerase, the same gp5/trx reinitiates synthesis at the primer-template from which it dissociated. In this model the polymerase, upon dissociation from the primer, undergoes a conformational change that switches its binding mode to gp4 from that of the tight binding polymerization mode to the weaker electrostatic binding mode. Nonetheless, gp5/trx remains bound to gp4 albeit perhaps to other subunits of the hexameric helicase. If free DNA polymerase is present in solution, it can also establish itself on one or more of the subunits of gp4 and thus bind to the primer-template during one of the transient dissociations of the synthesizing polymerase.

We have used a combination of ensemble and single-molecule techniques to obtain definitive evidence for the model described above. We show that two basic loops in the TBD are critical for the electrostatic interaction of gp5/trx with gp4, but not for the tight binding observed when gp5/trx is in a polymerization mode. Both modes of interaction are essential for the observed processivity of the replisome during strand-displacement synthesis. The results support a dynamic interaction of the polymerase with the helicase and provide a mechanism for assuring processivity of synthesis of both the leading and lagging strands even when there is dissociation of the replicating polymerase. The two modes of binding could provide for the retention of the lagging-strand polymerase within the replisome during its recycling from a completed Okazaki fragment to a new primer.

RESULTS

Two Solvent-Exposed Loops in the TBD of Gp5 Bind Gp4 When Gp5/trx Is Not Bound to a Primer-Template

The TBD of gp5 binds trx, the processivity factor, with high affinity (K_D = 5 nM) (Bedford et al., 1997; Tabor et al., 1987). The binding of trx structures the TBD such that basic residues face the DNA binding cleft through which the duplex portion of the primer-template passes (Doublie et al., 1998) (Figure 1). However, the TBD is also the site of interaction of gp4; deletion of TBD (gp5ΔTBD) reduces the binding affinity of gp5/trx to gp4 by 90-fold (Hamdan et al., 2005). The question therefore arises as to what effect the binding of gp5/trx to a primer-template has on the binding of gp4. In order to address this problem, it is important to have gp5 proteins containing altered TBDs that are defective in binding gp4, but not in binding trx. Our earlier studies relied upon using gp5ΔTBD that cannot bind trx (Hamdan et al., 2005).

Because the interaction of gp4 with gp5/trx requires the acidic C-terminal tail of gp4 (Notarnicola et al., 1997), it seemed likely that the interaction was at least partially electrostatic. Inspection of the crystal structure of gp5/trx (Doublie et al., 1998) revealed a number of basic residues located in the TBD that neither interact with trx nor DNA. As shown in Figure 1, these residues are located in two solvent-exposed loops composed of portions of the TBD. Loop A, residues 275–285, contains four basic residues, and loop B, residues 299–314, contains six basic residues. Using in vitro mutagenesis, we replaced the basic residues that are not critical for binding trx and DNA

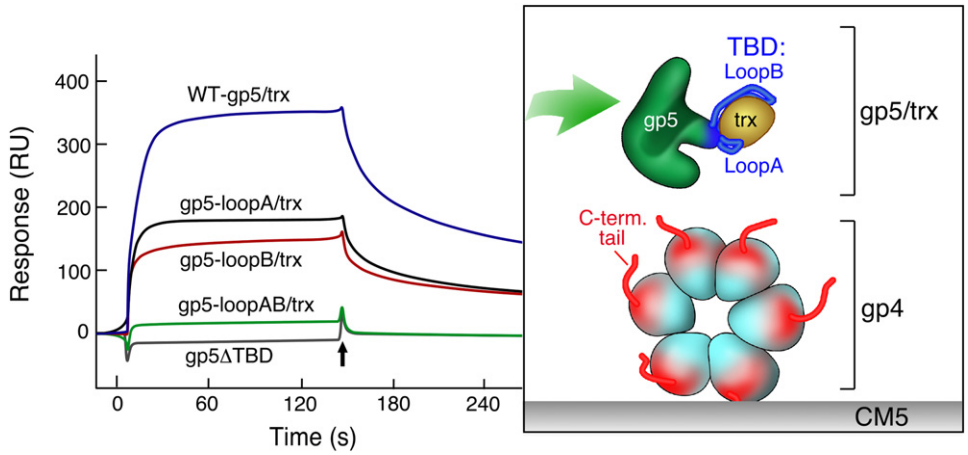

Figure 2. Binding of gp4 to gp5/trx Variants in Absence of Primer-Template

Gp4 is immobilized via its amine groups to the sensor chip CM-5, and gp5/trx variants are flowed over as shown in the inset. Binding studies were carried out as described in Experimental Procedures. Three thousand response units of gp4 are coupled to the chip, and the concentration of the gp5/trx variants in the flow buffer was 0.2 μM. A control flow cell lacking gp4 is used to subtract the RU resulting from nonspecific interaction and bulk refractive index; the coupling step is omitted, and the baseline is adjusted to zero. The end of injection of gp5/trx variants is indicated by an arrow. Gp5/trx variants used in this study are wild-type gp5/trx, gp5-loopA/trx, gp5-loopB/trx, gp5-loopAB/trx, and gp5ΔTBD. These proteins are depicted in Figure 1 and in the text.

with alanine. Three altered gp5 proteins emerged from this screen. In one construct, three of the residues (H276, K278, and R281) in loop A were simultaneously changed to alanine (gp5-loopA/trx). A second construct was made in which four of the basic residues in loop B (K302, K304, R305, and R310) were simultaneously replaced with alanine (gp5-loopB/trx). In addition, a gp5 was constructed in which alanine replaced the seven basic residues in loops A and B (gp5-loopAB/trx). These substitutions had minimal effect on the binding of gp5 to trx and DNA as measured by polymerase activity on primed M13 ssDNA. Gp5-loopA/trx, gp5-loopB/trx, and gp5-loopAB/trx had 125%, 35%, and 50% activity relative to wild-type gp5/trx. When secondary structure in the M13 ssDNA was removed with *E. coli* SSB protein, the altered proteins were indistinguishable from wild-type protein in both rate of nucleotide polymerization and processivity (see Figures S1A and S1B in the Supplemental Data available with this article online). Attempts to eliminate the additional three basic residues in loops A and B led to a significant decrease in rates and processivity (data not shown).

We have used surface plasmon resonance (SPR) to quantitatively measure the interaction of the wild-type and genetically altered gp5 proteins bound to trx with gp4 (Figure 2) in the absence of DNA. In these experiments gp4 was immobilized to a carboxymethyl-5 (CM-5) sensor chip and then gp5/trx variants were flowed over (Figure 2). We have recently used this procedure to calculate a K_D of 90 nM for the binding of gp4 to wild-type gp5/trx (Hamdan et al., 2005). Gp5-loopA/trx and gp5-loopB/trx both show decreased amounts of protein bound relative to wild-type gp5/trx. Nonetheless, at the end of the injection of protein and after washing with buffer, some gp5-loopA/trx and gp5-loopB/trx remain bound to the immobilized gp4. When the basic residues in both loops are eliminated, there is little binding of gp5-loopAB/trx to gp4. However, as seen in Figure 2, a small amount of binding above that observed with gp5 lacking the TBD is seen. We have shown that trx itself can bind weakly to gp4 (K_D = 130 μM) and this residual binding could result from such an interaction because gp5ΔTBD cannot bind trx (Hamdan et al., 2005). In a control experiment, gp5/trx was immobilized on the surface via a thioredoxin-monoclonal antibody and gp4 was injected over it: a drastic reduction in the binding of gp4 to gp5-loopAB/trx relative to wild-type gp5/trx was observed (data not shown).

TBD of Gp5 Is Not Critical for Binding Gp4 When Gp5/trx Engages a Primer-Template

As shown above, basic residues located in two loops of the TBD bind gp4 via its acidic C-terminal tail. Because the TBD interacts with DNA during DNA synthesis, it is likely that the interaction of gp5/trx with DNA would influence the ability of gp5/trx to interact with gp4. We were surprised to find that gp5/trx that was bound to a primer-template in a polymerizing mode bound gp4 with an extremely high affinity, an interaction that did not involve the C-terminal tail of gp4 (Hamdan et al., 2005). The two binding modes were also demonstrated using single-molecule technique where a single copy of gp5/trx and gp4 could be preassembled only when the two proteins are bound in a polymerizing mode (Lee et al., 2006a).

In order to determine whether the TBD is involved in the binding of gp4 to gp5/trx bound to DNA, we have examined, using SPR, the binding of gp5-loopAB/trx to gp4 when the former is in complex with a primer-template.

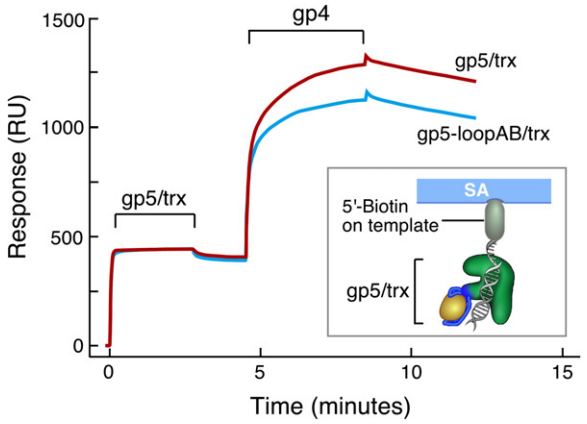

Figure 3. Binding of gp4 to gp5/trx and gp5-LoopAB/trx in Presence of Primer-Template

The primer-template strand, which contains a biotin at the 5′ end of the template strand, is immobilized on SA-sensor chip. Gp5/trx or gp5-loopAB/trx is injected, followed by gp4. Binding studies were carried out as described in Experimental Procedures. One hundred response units of the biotinylated primer-template is coupled to the surface. Gp5/trx or gp5-loopAB/trx is injected at a concentration of 0.2 μM in a flow buffer containing 1 mM dGTP and 10 μM ddATP: a saturating 1:1 binding condition between gp5/trx and primer-template. The 100 response units resulting from the coupling of the primer-template was subtracted from the baseline. Gp4 is injected at a concentration of 0.7 μM (monomer) in flow buffer containing 0.1 mM ATP and 2 mM dGTP. The start and end of injections of gp5/trx and gp4 are indicated.

We formed a stable complex of either wild-type gp5/trx or gp5-loopAB/trx and a primer-template as described previously (Hamdan et al., 2005), by terminating the primer with a 2′,3′-dideoxynucleotide (ddAMP in this experiment) and providing the next dNTP specified by the template (dGTP in this experiment). The 5′ end of the template is biotinylated to enable the immobilization of the primer-template to a streptavidin (SA) sensor chip (Figure 3). Both gp5/trx and gp5-loopAB/trx bind equally well to the immobilized primer-template (Figure 3). The high affinity of both polymerases to the primer-template is apparent from the lack of dissociation during the extensive washing of the complex.

Upon flowing gp4 over the immobilized complex, both wild-type gp5/trx and gp5-loopAB/trx form equally stable complexes with gp4 as shown by the slow dissociation after the injection of gp4 is completed (Figure 3). In control experiments we found no detectable binding of gp4 to the immobilized DNA alone (Hamdan et al., 2005), and gp4 alone and gp4 bound to ssDNA both bind similarly to gp5/trx-DNA (Figure S2). These results complement those obtained in a similar experiment in which we found normal binding of gp4-CΔ17 lacking its C-terminal tail to an immobilized gp5/trx-DNA complex (Hamdan et al., 2005). These studies together demonstrate that gp5/trx bound to a primer-template interacts with gp4 in a manner distinct from that observed when gp5/trx is free in solution. The sites of this unique interaction have not yet been identified. The binding of gp4 to DNA, on the other hand, does not modulate its binding to gp5/trx; gp4 bound to ssDNA utilizes its C-terminal tail to bind gp5/trx (Notarnicola et al., 1997); the TBD of gp5 is required for this interaction (data not shown).

Both TBD of Gp5 and C-Terminal Tail of Gp4 Are Required for Leading-Strand Synthesis

The tight binding mode of gp5/trx and gp4 observed when gp5/trx is bound to DNA is likely to be critical during leading-strand synthesis. Yet we had previously found that the C-terminal tail of gp4, a motif required for the weaker mode of binding in the absence of DNA, is also required for efficient strand-displacement synthesis (Lee et al., 2006b; Notarnicola et al., 1997). Why would this electrostatic interaction be important for leading-strand DNA synthesis? As shown in Figure 4A, we have examined strand-displacement synthesis catalyzed by gp5/trx and gp4 via a rolling circle mechanism on M13 circular template. The dependence of leading-strand DNA synthesis by wild-type gp5/trx on gp4 helicase is apparent; no DNA synthesis is observed in the absence of gp4 (Figure 4A). In the presence of gp4, the length of the product is greater than 30 kb as revealed by alkaline agarose gel electrophoresis (Figure 4B).

The C-terminal tail of gp4 is required for optimal strand-displacement synthesis (Lee et al., 2006b; Notarnicola et al., 1997). The rate of DNA synthesis with gp4-CΔ17 is one-third that observed with the wild-type protein (Figure 4A). More informative is the distribution of products separated by alkaline agarose gel electrophoresis (Figure 4B, lanes 22–26). Not only is the amount of product less but there is, in contrast to the reactions with wild-type gp4, a wide distribution of product size in the lower molecular weight range, a distribution best seen at the lower concentrations of protein. Gp5-loopA/trx supports leading-strand synthesis equally as well as the wild-type gp5/trx. The rate of synthesis and the product distributions are similar. Gp5-loopB shows a 1.5-fold decrease in rate of synthesis, but the product distribution is similar to that observed with wild-type enzyme (Figure 4B, lanes 12–16). Gp5-loopAB/trx, on the other hand, catalyzes strand-displacement synthesis 2-fold less than wild-type gp5/trx (Figure 4A), and the product size is more heterogeneous (Figure 4C, lanes 17–21), similar to that observed in reactions carried out with gp4-CΔ17 and wild-type gp5/trx. In the previous section we showed that gp5-loopA/trx, gp5-loopB/trx, and gp5-loopAB/trx showed decreased binding to gp4 but only gp5-loopAB/trx could not form a stable complex. It appears that the interaction of gp4 with either loops A or B is sufficient to support strand-displacement synthesis.

The results obtained with gp5-loopB/trx are particularly informative. Its polymerase activity on primed M13 ssDNA is 3-fold less than the wild-type gp5/trx, a deficiency that could be overcome by the addition of *E. coli* SSB protein to remove secondary structure (Figure S1). However,

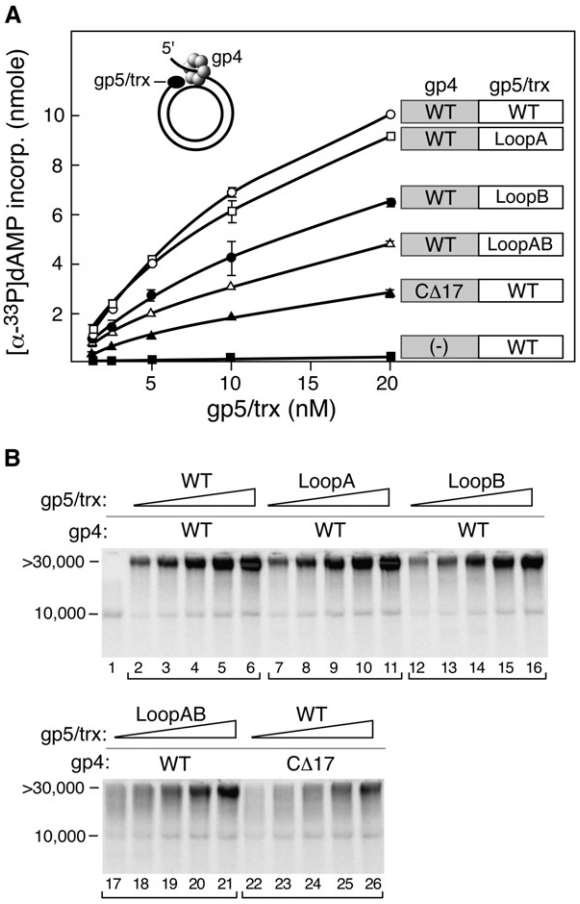

Figure 4. Leading-Strand DNA Synthesis Catalyzed by gp4 and gp5/trx Variants

Strand-displacement synthesis was measured using M13 circular dsDNA with a preformed replication fork as depicted at the top of the figure.

(A) Leading-strand DNA synthesis by gp5/trx variants. The incorporation of [α-^{33}P]dAMP is measured at 37°C for 15 min, as described in Experimental Procedures. Reaction mixtures contain 20 nM DNA, 20 nM gp4 (hexamer), 4 μM trx, and various concentrations of gp5 variants (1.3, 2.5, 5.10, and 20 nM). The gp5 variants used in this study are described in Figure 1. Gp4-CΔ17 is a truncated mutant of gp4 that lacks the 17 C-terminal residues. Error bars correspond to the standard deviation of two independent experiments. The lines connecting the points are for visual purposes.

(B) Radioactive production of leading-strand DNA synthesis. The DNA reaction products from the points shown in (A) were denatured and analyzed by electrophoresis in 0.6% alkaline agarose gel. Synthesis in the absence of gp4 (lane 1) is shown using a gp5/trx concentration of 20 nM.

gp5-loopB/trx and gp4 mediate strand-displacement synthesis, in which case the secondary structure is removed by the helicase. Therefore we believe that the decreased activity seen in the absence of SSB protein does not preclude the use of gp5-loopB/trx in these studies with gp4. Further evidence to support this conclusion is presented below.

Single-Molecule Analysis of Leading-Strand Synthesis Mediated by Gp5-LoopAB/trx and Gp4-CΔ17

Several roles for the electrostatic interaction during leading-strand synthesis can be hypothesized. One possibility is that the assembly of the more stable complex is prevented due to a requirement of the two proteins to associate prior to the binding of the DNA polymerase to the primer-template. On the other hand, the defect may arise during leading-strand synthesis. For example, the rate of DNA synthesis could be decreased or the proteins could dissociate more frequently, resulting in decreased processivity. These questions are difficult to address with ensemble-averaging experiments. Therefore, we have examined leading-strand DNA synthesis mediated by either gp5-loopAB/trx or gp4-CΔ17 using single-molecule technique.

The strand-displacement assay using single-molecule technique has been described previously (Lee et al., 2006a) and is depicted schematically in Figure 5A. Bacteriophage λ duplex DNA (48.5 kb) containing a replication fork is attached to a glass flow cell via the 5′ end of one strand whose 3′ end is linked to a 2.8 μm-sized bead. A constant laminar flow is applied such that the resultant drag on the bead stretches the DNA molecule with a force of 3 pN. At this force the elasticity of the DNA is determined by entropic contributions and thus does not influence protein interactions with DNA (Lee et al., 2006a). The ssDNA, due to coiling, is shorter than dsDNA at low stretching forces (<6 pN). Consequently, the conversion of dsDNA to ssDNA as a result of leading-strand synthesis can be monitored through a decrease in length of DNA (Figure 5A). The change in lengths of individual DNA molecules is measured by imaging the beads and tracking their positions.

To assure the assembly of gp4 and gp5/trx at the replication fork, both of these proteins were flowed over the immobilized DNA for 15 min at high concentrations. The presence of dNTPs allowed assembly of the proteins at the fork, but the absence of Mg^{2+} prevented any unwinding or DNA synthesis. After removal of free protein by washing the flow cell with buffer, the reaction is initiated by flowing buffer containing Mg^{2+} and the four dNTPs. With wild-type gp5/trx and gp4, leading-strand synthesis proceeded at a rate of 164 ± 8 nucleotides per s with a processivity of 17 ± 2 kb (Figure 5B). This processivity is less than that reported from the bulk assay (Figure 4), because no reinitiation of leading-strand synthesis is possible if the polymerase dissociates. Substitution of wild-type gp5/trx with gp5-loopAB/trx had no effect on the rate of nucleotide polymerization but did result in a 3-fold decrease in processivity to 6 ± 3 kb per binding event. Substitution of gp4-CΔ17 for wild-type helicase resulted in a similar decrease in processivity (5 ± 1 kb) and a 40% reduction in rate to 96 ± 10 nucleotides per s (Figure 5B). The decrease in rate observed with gp4-CΔ17 is not surprising because independent measurements of its rate of unwinding in the absence of DNA polymerase

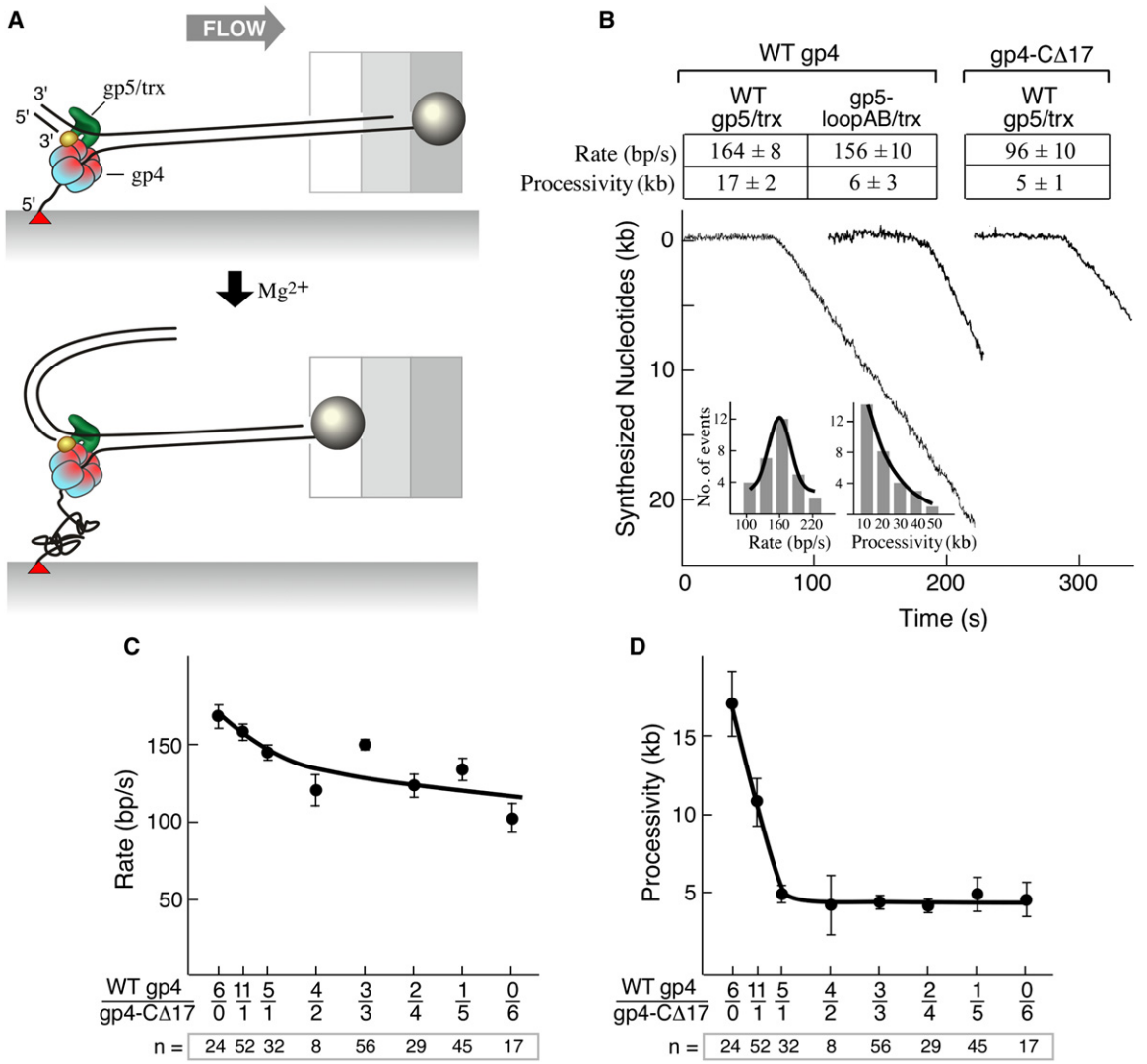

Figure 5. Single-Molecule Measurement of Leading-Strand DNA Synthesis

(A) Experimental design. Duplex λ DNA (48.5 kb) is attached to the surface of the flow cell via the 5′ end of the fork using biotin-SA interaction, and the 3′ end is attached to a paramagnetic bead using digoxigenin-anti-digoxigenin interaction. Gp5/trx and gp4 are preassembled at the replication fork in the presence of dNTP but in the absence of Mg^{2+}. Free proteins are removed by washing the flow cell with buffer that does not contain proteins. The reaction is started by the addition of Mg^{2+} and dNTP. The positions of the beads are recorded and analyzed as described in Experimental Procedures. The conversion of the dsDNA to ssDNA results in shortening the DNA and the movement of the bead against the flow direction.

(B) Rate and processivity of leading-strand synthesis of gp5/trx and gp4, gp5-loopAB/trx and gp4, and gp5/trx and gp4-CΔ17. Examples of single-molecule trajectories for leading-strand synthesis are shown. Rate and processivity were calculated by fitting the distributions of individual single-molecule trajectories using Gaussian and exponential decay distributions, respectively (fitted histograms shown are for gp5/trx and gp4). Thirty single events are used to calculate rate and processivity for gp5/trx and gp4, 12 events for gp5/trx-loopAB and gp4, and 14 events for gp5/trx and gp4-CΔ17.

(C) Rate of leading-strand synthesis at different stoichiometries of gp4 and gp4C-Δ17.

(D) Processivity of leading-strand synthesis at different stoichiometries of gp4 and gp4C-Δ17. Number of events used to calculate rate and processivity are indicated. The lines connecting the points are for visual purposes. Error bars correspond to errors in fitting the rate and processivity distributions with Gaussian and exponential decay functions, respectively.

also reveal a reduction of ~50%; the processivity of unwinding, however, is unaffected (J.-B.L., C.C.R., and A.M.v.O., Harvard Medical School, unpublished data). Results similar to that observed with gp5/trx and gp4-CΔ17 were obtained with gp5-loopAB/trx and gp4-CΔ17 (data not shown). This observation is particularly interesting because synthesis by gp5-loopAB/trx and gp4-CΔ17 is drastically reduced relative to wild-type gp5/trx and gp4-CΔ17 using the bulk assay. The single-molecule measurements demonstrate that the source of the deficiency in the bulk assay arises from only an assembly problem.

The single-molecule experiments revealed that the lower-molecular-weight DNA products of leading-strand synthesis seen by gel analysis (Figure 4B) arose from a decrease in processivity. These experiments demonstrate that an elimination of the electrostatic interaction involving the TBD of gp5/trx or the C-terminal tail of gp4 does not interfere with the more stable interaction that occurs when gp5/trx is bound to a primer-template; processivity is still in the 5 kb range. However, due to the random nature of the distribution of events in an activity such as processivity (Figure 5B), discrete populations of events at 5 kb increments in the wild-type gp5/trx and gp4 cannot be observed. The ability of gp5-loopAB/trx to catalyze extensive strand-displacement synthesis with gp4 under condition where there is no free trx or polymerase supports our previous conclusion that this alteration per se does not affect the ability of this enzyme to catalyze strand-displacement synthesis.

Requirement of C-Terminal Tails on All Subunits of Gp4 Hexamer for Extensive Processivity of Leading-Strand Synthesis

Hexamers of gp4 in which all six subunits lack a C-terminal tail display a 3-fold reduction in the processivity of leading-strand synthesis (Figure 5). We were curious as to the effect on processivity of having hexamers in which only one or a few subunits lack a C-terminal tail. In the experiment summarized in Figures 5C and 5D, gp4-CΔ17 and wild-type gp4 were mixed at various concentrations to yield oligomers containing different ratios of the two proteins. Previous studies have shown random mixing of subunits (Lee and Richardson, 2002), and therefore a Poisson distribution of subunits in the mixed oligomers is expected. The same single-molecule analysis presented in Figures 5A and 5B was then used to measure the rates and processivity of strand-displacement synthesis in conjunction with gp5/trx. As the ratio of wild-type gp4 to gp4-CΔ17 increases, there is a small increase in the rate of synthesis indicative of the reduced rate of unwinding by gp4-CΔ17 (Figure 5C). However, no change in processivity was observed until all six subunits were wild-type gp4 (Figure 5D). Any hexamer containing one or more subunits of gp4-CΔ17 had a processivity of ~5 kb whereas wild-type gp4 had the expected processivity of 17 kb. An alternative explanation is that gp4-CΔ17 binds more strongly to DNA than does wild-type gp4, thus giving rise to the assembly of hexamers only containing gp4-CΔ17. In this case, only the experiment with wild-type gp4 present exclusively would give rise to the presence of wild-type proteins in the hexamer. To rule out this possibility, we performed this experiment with a mixture of wild-type gp4 and gp4-CΔ17 at a ratio of 11:1. The appearance of a processivity that is between the values observed in the presence and absence of gp4-CΔ17 (Figure 5D) is consistent with half the hexamers displaying a high processivity, and the other half a low processivity. This observation confirms the notion of a purely random composition of the hexamers. We conclude that all six subunits of gp4 must have a C-terminal tail to obtain high processivity. Severity of mixing gp4-CΔ17 with wild-type gp4 on leading-strand synthesis is shown in the bulk (Lee et al., 2006b). However, because both rate and processivity change simultaneously upon mixing gp4-CΔ17 with wild-type gp4, the bulk assay would merely report a gradual decrease in synthesis upon mixing. Consequently, the stoichiometric requirement of the C-terminal tail of gp4 can only be obtained by single-molecule technique.

Exchange of DNA Polymerases during Leading-Strand Synthesis Depends on the Interaction between the C-Terminal Tail of Gp4 and the TBD of Gp5

We recently reported that gp5/trx could exchange with gp5/trx that was catalyzing strand-displacement synthesis (Johnson et al., 2007). Remarkably, the exchange occurs without affecting the rate of DNA synthesis or its processivity as measured by challenge with excess primer-template. How then does one reconcile the high processivity of gp5/trx and gp4 helicase observed during strand-displacement synthesis using ensemble (Johnson et al., 2007) and single-molecule techniques (Lee et al., 2006a)? By necessity it would appear that the replicating gp5/trx must on occasion transiently dissociate from the primer without entering into solution. Otherwise, in dilution and single-molecule experiments, any gp5/trx entering into solution would be well below the concentration required for rebinding. If the polymerase remains associated with the replisome, then gp4 provides an attractive anchor for the transiently associated protein. Gp4 is firmly bound to the lagging strand and contains six subunits, any one of which could interact with gp5/trx. The interaction we postulate would be the electrostatic interaction involving the C-terminal tail of gp4 and loops A and B of the TBD of gp5.

In order to see whether this electrostatic interaction between gp5/trx and gp4 is necessary for the exchange of DNA polymerase during leading-strand synthesis, we have examined the ability of gp5-loopAB/trx to mediate this reaction. In the experiment shown in Figure 6, leading-strand synthesis is initiated using gp5/trx and gp4 on M13 dsDNA containing a replication fork as shown in the inset to Figure 4. The DNA polymerase used to initiate DNA synthesis is gp5-Y526F/trx in which tyrosine 526 is replaced with phenylalanine. Gp5-Y526F/trx catalyzes processive DNA synthesis (Johnson et al., 2007) but differs from wild-type gp5/trx in that it incorporates ddNTP inefficiently; wild-type gp5/trx incorporates ddNTP almost as well as it does dNTPs (Tabor and Richardson, 1995). Once synthesis has commenced, a 10-fold excess of wild-type gp5/trx is added to the reaction followed 1 min later by sufficient ddGTP to inhibit wild-type gp5/trx, but not gp5-Y526F/trx (Figure 6A). As shown previously (Johnson et al., 2007), all DNA synthesis ceases upon addition of the ddGMP. In a control experiment the addition of the wild-type gp5/trx without the addition of ddGMP had no effect (Figure 6A). We conclude that the

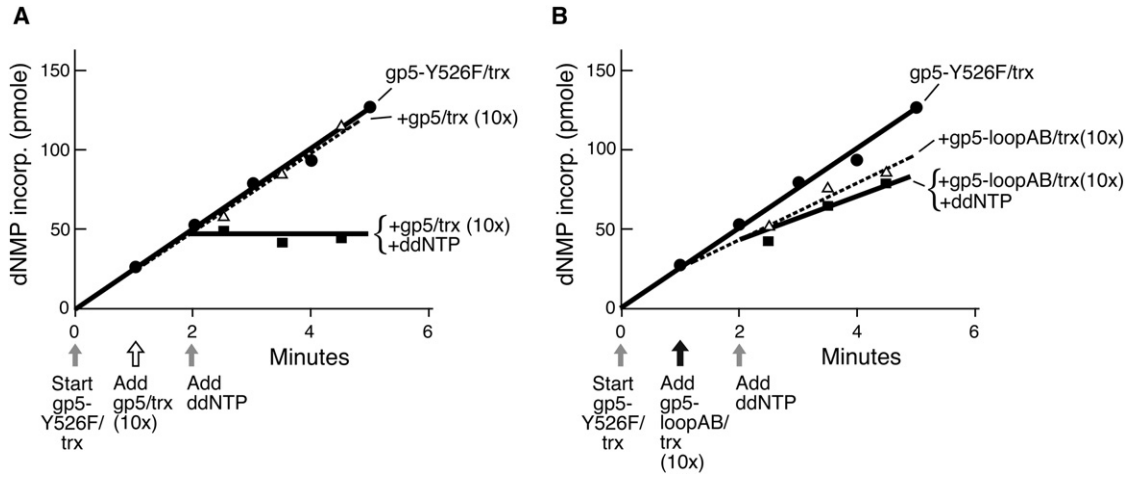

Figure 6. Exchange of gp5/trx during Leading-Strand DNA Synthesis

The ability of gp5-loopAB/trx to exchange with gp5-Y526F/trx at the replication fork is monitored using the M13 dsDNA having a replication fork described in Figure 4. The reaction mix contains 2 nM M13 dsDNA, 16 nM gp4 (hexamer), 16 nM gp5-Y526F, and 5 μM trx.

(A) Ability of gp5-Y526F/trx to exchange with wild-type gp5/trx from solution. Leading-strand DNA synthesis is initiated by gp5/trx-Y526F, an active-site mutant of gp5/trx that incorporates ddNTP poorly. After 1 min, 10-fold excess of wild-type gp5/trx is added. After another minute, the reaction is challenged with ddGTP. Synthesis in the presence of 10-fold wild-type gp5/trx but without the addition of ddGTP is shown by the dotted line. In the absence of ddGTP, the addition of 10-fold wild-type gp5/trx does not interfere with DNA synthesis by the highly processive gp5/trx-Y526Y. When ddGTP is added after 2 min DNA synthesis stops.

(B) Ability of gp5-Y526F/trx to exchange with gp5-loopAB/trx from solution. The experimental design is similar to that described above except that 10-fold of gp5-loopAB/trx instead of wild-type gp5/trx was added after 1 min.

polymerase in solution can exchange with that at the replication fork.

In the same experiment, if the reaction is challenged with a 10-fold excess of gp5-loopAB/trx, there is significantly less of a decrease in DNA synthesis upon addition of ddGTP. A slight decrease is also observed in the absence of ddGTP, indicative of some of the nonprocessive gp5-loopAB/trx exchanging with the replicating enzyme. Nonetheless, most of the replicating gp5-Y526F/trx failed to exchange with gp5-loopAB/trx. Gp5-loopAB/trx is inhibited to similar levels by ddNTP as wild-type gp5/trx (data not shown). Because gp4-CΔ17 is not processive in supporting leading-strand synthesis, we could not address the exchange by starting synthesis with gp4-CΔ17 and gp5/trx and then compete with gp5-Y526F/trx.

In order to obtain direct evidence that the exchange of DNA polymerases is mediated by the electrostatic interaction between gp4 helicase and gp5/trx from solution, we preassembled a complex of gp4 and gp5/trx in a polymerizing mode (Figure 3) and measured the ability of gp4 to bind gp5/trx. Wild-type gp5/trx, but not gp5-loopAB/trx, binds to a gp4-gp5/trx-DNA complex (Figure S3).

DISCUSSION

In the current study we demonstrate that gp5/trx binds to gp4 via two modes. When gp5/trx and gp4 are catalyzing leading-strand synthesis, the two proteins are tightly bound at sites we have not yet identified. When gp5/trx is not bound to a primer-template the mode of binding switches to one of lower affinity. This second mode involves an interaction of the acidic C-terminal tail of the helicase with two basic loops in the TBD of gp5. This interaction is enhanced by the binding of trx to TBD, probably resulting from a conformational change that exposes loops A and B. The two modes together provide for a processivity greater than 17 kb per binding event on duplex DNA (Lee et al., 2006a). If the second, electrostatic mode is lost, as observed with gp4-CΔ17 or gp5-loopAB/trx, processivity decreases to ~5 kb. Thus it appears that the tight mode of binding is sufficient for 5 kb of synthesis but that gp5/trx does on occasion disengage from the primer.

How does the electrostatic interaction increase processivity? In one scenario the two modes simply function together and increase the overall affinity. The binding of gp4 to gp5/trx observed when gp5/trx is bound to DNA is so tight that it would mask the weaker electrostatic interaction. Without a structure of a gp4-gp5/trx complex we cannot rule out the possibility that the electrostatic interaction gives rise to conformational changes that then allow for the tighter binding mode when gp5/trx engages DNA. Inasmuch as the high processivity observed when both binding modes are operable occurs in a solution without free additional proteins, it seems unlikely that the electrostatic mode increases the apparent processivity by facilitating just the assembly of the gp4-gp5/trx complex.

We favor a model in which gp5/trx switches to the electrostatic mode whenever it disengages the primer

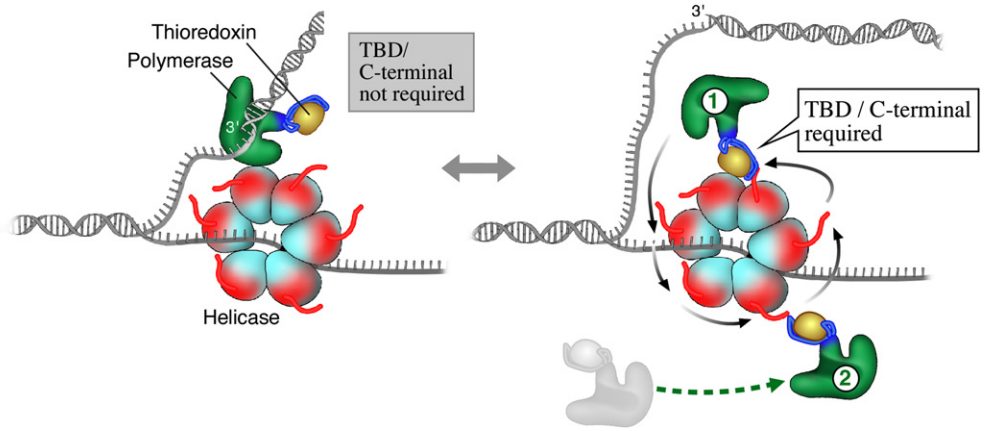

Figure 7. Two Binding Modes Mediate gp5/trx and gp4 Interaction at the Replication Fork

While synthesizing DNA, gp5/trx and gp4 interact with a tight polymerizing mode that is independent of the C-terminal tail of gp4 or the TBD of gp5/trx. This tight binding mode leads to the synthesis of 5 kb of DNA on average prior to the dissociation of gp5/trx from DNA. The electrostatic binding mode can retain the dissociated gp5/trx in close proximity to DNA (1) and/or recruit gp5/trx from solution. The latter provides an explanation for the ability of DNA polymerases to exchange at the replication fork without affecting processivity (2). The polymerase or polymerases bound in the electrostatic mode move sequentially from one C-terminal tail of the hexameric gp4 to another in search of the primer-template.

(Figure 7). In this model gp5/trx could then bind to any of the six subunits of the hexamer, remain at the replisome, and then rebind to the same primer from which it had recently dissociated. If the time between disengagement and reattachment to the primer is sufficiently rapid, it would not be detected as a separate event in the single-molecule experiments.

The latter model also provides an explanation for the ability of gp5/trx molecules in solution to exchange with the synthesizing gp5/trx without affecting processivity (Johnson et al., 2007). A similar exchange of DNA polymerases at the bacteriophage T4 replication fork has also been observed (Yang et al., 2004). Based on the model of the helicase serving as intermediary in the delivery of gp5/trx to an adjacent primer, gp5/trx in solution should be able to bind to one of the subunits of the translocating helicase via the electrostatic mode of binding (Figure S3). If at any time the synthesizing polymerase transiently dissociates from the primer, then it would essentially be in equilibrium with the other polymerase bound to the helicase. Thus, upon rebinding there would be an equal opportunity for either the original polymerase or the newly recruited polymerase to rejoin the primer-template (Figure 7).

An interesting observation is that the C-terminal tail of all six subunits of gp4 hexamer must be able to interact with gp5/trx in order to retain the dissociating replicative polymerase within the replisome. We suspect that this requirement arises from a necessity for the bound gp5/trx to move sequentially from one subunit to another in search of the primer to which it eventually binds. Whether this is related to a rotating helicase itself is not known.

Why does gp5/trx disengage from the primer every 5 kb? It is possible that its association with gp4 via the tight mode of interaction is subjected to torsional strain of the primer-template resulting from rotation of the helicase. Transient dissociation of the polymerase from the primer would allow for the relaxation of the tension and realignment of the polymerase and helicase at the replication fork. It is also possible that lesions of unknown identity and origin occur infrequently in duplex DNA and could induce the dissociation. Such an event could actually be beneficial in the replisome bypassing the lesion with only transient dissociation of the polymerase.

We have not yet examined the ability of the lagging-strand DNA polymerase to exchange with free DNA polymerase or identified the binding modes of the lagging-strand DNA polymerase with gp4. It seems likely that the lagging-strand polymerase, bound to and extending a primer, would bind to gp4 via the same tight binding that occurs with the leading-strand DNA polymerase. Likewise, the potential for the electrostatic mode of binding should also exist for the lagging-strand polymerase. The two modes of binding of gp5/trx to gp4 may, in fact, be more important for the replication of the lagging strand. Inasmuch as the lagging-strand polymerase must dissociate from the primer every few thousand nucleotides or whenever it completes an Okazaki fragment, the ability to transiently remain bound to the replisome would be essential for processivity. Alternatively, if the signal for the replication loop containing the nascent Okazaki fragment to resolve arises from the synthesis of a new primer then the synthesizing polymerase could accompany the Okazaki fragment and complete its synthesis. In this scenario a new gp5/trx already assembled via the electrostatic mode on the helicase would initiate synthesis of the new Okazaki fragment. Again, apparent processivity would be maintained.

Most replicative DNA polymerases function within a multiprotein complex with accessory subunits maintaining

several interactions between the holoenzyme and other replication proteins (Johnson and O'Donnell, 2005). In *E. coli*, the polymerase subunit, α, interacts with the helicase, DnaB, indirectly via the C-terminal region of its accessory protein τ; domain V of τ interacts with α and domain IV of τ interacts with DnaB (Gao and McHenry, 2001a, 2001b). Interestingly, as is the case for the gp4-gp5/trx interaction, α appears to interact with τ in two different binding modes depending on whether α is bound to a primer-template or not (De Saro et al., 2003; Leu et al., 2003). Although two regions in α were identified to interact with τ, it is unclear whether they act independently (Gao and McHenry, 2001b). Nonetheless, one can envision that they can function in a similar way to the two binding modes of gp4-gp5/trx to assure processive DNA synthesis. In the T7 replisome, the two binding modes are also utilized to recruit polymerases from solution into the replisome. In *E. coli* and T4 replisomes, however, the exchange of DNA polymerases appears to be mediated by the polymerase sliding clamp: a protein known to act as a platform for the delivery of proteins that act on DNA (de Saro et al., 2004).

EXPERIMENTAL PROCEDURES

Mutagenesis of T7 Gene 5, Protein Expression, and Purification

Three site-directed point mutants of gene 5 were constructed using polymerase chain reaction with plasmid pGP5-3 having gene 5 (Tabor et al., 1987). The first has H276, K278, and R281 replaced with alanines (gp5-loopA/trx). The second has K302, K304, R307, and R310 replaced with alanines (gp5-loopB/trx). The third has H276, K278, R281, K302, K304A, R307, and R310 replaced with alanines (gp5-loopAB/trx). The identity of all clones was confirmed using DNA sequencing. Gp5 variants were overproduced in *E. coli* strain A307(DE3) that does not express trx and then purified as described (Tabor et al., 1987). Gp4, gp4-CΔ17 (Notarnicola et al., 1997), and trx (Tabor et al., 1987) were purified as described.

Surface Plasmon Resonance

SPR analysis was performed using a Biacore-3000 instrument (Uppsala, Sweden). Three thousand response units (RU) of gp4 were coupled to CM-5 chip at concentration of 100 μg/ml in 10 mM Na-acetate (pH 4.5) as described previously (Hamdan et al., 2005). A control flow cell was activated and blocked in the absence of protein to subtract nonspecific interactions and bulk refractive index. Binding studies were performed at 20°C by injecting gp5/trx variants at concentration of 0.2 μM at a flow rate of 40 μl/min in buffer A (20 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 5 mM DTT, 250 mM K-glutamate, and 0.005% [v/v] Tween-20). Complexes of gp5 variants with trx are formed by mixing gp5 variants with trx at a 1:40 molar ratio.

The binding of gp4 to gp5/trx in the presence of primer-template was investigated as previously described (Hamdan et al., 2005). The template (5′-biotin-TTCCCCCTTGGCACTGGCCGTCGTTTTCACG-3′) and the primer (5′-CGTGAAAACGACGGCCAGTGCCA-3′) strands were annealed and 100 RU coupled to SA chip. Free SA was then blocked with free biotin. Binding studies were carried out in buffer B (20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 2.5 mM DTT, 200 mM K-glutamate, 1% [w/v] glycerol, and 0.5 mM dGTP) at a flow rate of 10 μl/min. Gp5/trx or gp5-loopAB/trx was injected at 0.2 μM in buffer B containing 10 μM ddATP and 1 mM dGTP, to incorporate the dideoxynucleotide at the 3′ end of the primer strand. Gp4 at 0.7 μM (monomeric) was injected in buffer B containing 0.1 mM ATP and 2 mM dGTP. As a control, a flow cell blocked with biotin was used to measure the nonspecific interaction and bulk refractive index.

Strand-Displacement DNA Synthesis

M13 circular dsDNA having a 5′ tail was used to monitor strand-displacement DNA synthesis. A replication fork was constructed by annealing M13 mGP1-2 ssDNA to an oligonucleotide (5′-T_{36}AATTCG TAATCATCATGGTCATAGCTGTTTCCT-3′) having 30 bases complementary to the M13 ssDNA and 36 bases forming a 5′ tail. Then gp5/trx was used to convert the ssDNA circle to dsDNA circle. Phenol/chloroform was used to remove gp5/trx. Strand-displacement synthesis was performed at 37°C in a reaction (16.5 μl) containing buffer C (40 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10 mM DTT, 50 mM potassium glutamate [pH 7.5], and 0.1 mg/ml BSA); 500 μM each of dATP, dCTP, dGTP, and dTTP; 0.05 μCi [α-^{33}P]dATP; 20 nM dsM13; 20 nM gp4 (hexamer); 4 μM trx; and 1.2–20 nM gp5. Gp5, trx, and gp4 were incubated in ice for 30 min, the rest of the components were then added, and the reaction was started by transfer to 37°C. After 15 min, the reaction was stopped with EDTA (150 mM). DNA synthesis was monitored by the amount of [α-^{33}P]dAMP incorporated into the DNA (Tabor et al., 1987). To visualize the product of DNA synthesis, the DNA products that contain [α-^{33}P]dAMP were denatured and analyzed by electrophoresis in alkaline 0.6% agarose gel.

Single-Molecule Measurements

Phage λ DNA molecules containing a replication fork are attached with the end of one strand to the glass surface of a flow cell via biotin-SA link and with the other end to a 2.8 μm paramagnetic bead (Dynal) via digoxigenin-anti-digoxigenin as described previously (Lee et al., 2006a). To prevent nonspecific interactions between the beads and the surface, a 3 pN magnetic force was applied upward by positioning a permanent magnet above the flow cell. Beads were imaged with a CCD camera with a time resolution of 250 ms, and the centers of their positions for every acquisition time point were determined by particle-tracking software (Semasopht). The replication reaction in the flow cell is as follows: tethered DNA molecules are incubated for 15 min with 18 nM gp4 (hexameric) and 18 nM gp5/trx in buffer C containing 700 μM each of dATP, dTTP, dCTP, and dGTP. A 20 flow-cell volume of a protein-free replication buffer is used to wash the free proteins. DNA synthesis is started by introducing buffer C containing 10 mM $MgCl_2$ and 700 μM dNTPs. For data analysis, after particle tracking, the traces were corrected for residual instabilities in the flow by subtracting traces corresponding to tethers that were not enzymatically altered. Bead displacements were converted into numbers of nucleotides synthesized using the known length difference between ssDNA and dsDNA at our experimental conditions (Lee et al., 2006a). Leading-strand synthesis by different ratios of wild-type gp4 and gp4-CΔ17 was carried out as described above. Gp5/trx was used at 20 nM, and wild-type gp4 and gp4-CΔ17 were added to a final hexameric concentration of 20 nM.

Exchange of Gp5/trx during Leading-Strand Synthesis

The exchange reactions were performed on M13 circular dsDNA described above at 37°C in a reaction (40 μl) containing buffer C; 500 μM each of dATP, dCTP, dGTP, and dTTP; [^{3}H]dTTP (10 Ci/mmol); and 2 nM M13 dsDNA. Reactions were initiated with addition of 16 nM gp4 (hexamer), 5 μM trx, and 16 nM gp5-Y526F/trx. After 1 min, a 10-fold excess of either wild-type gp5/trx or gp5-loopAB/trx was added to the reaction, followed by an addition of 50 μM ddGTP at 2 min. Aliquots (6 μl) were removed every minute, and the reaction was stopped with EDTA (25 mM final). DNA synthesis was monitored by [^{3}H]dTMP incorporation into the DNA as described above.

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.molecule.org/cgi/content/full/27/4/539/DC1/.

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