Mechanism of the *E. coli* τ Processivity Switch during Lagging-Strand Synthesis

Frank P. Leu,² Roxana Georgescu,¹,² and Mike O'Donnell¹,2,*
¹Howard Hughes Medical Institute
²Rockefeller University
1230 York Avenue
New York, New York 10021

Summary

The *E. coli* replication machinery employs a β clamp that tethers the polymerase to DNA, thus ensuring high processivity. The replicase also contains a processivity switch that dissociates the polymerase from its β clamp. The switch requires the τ subunit of the clamp loader and is regulated by different DNA structures. At a primed site, the switch is “off.” When the replicase reaches the downstream primer to form a nick, the switch is flipped, and τ ejects the polymerase from β. This switch has high fidelity for completed synthesis, remaining “off” until just prior to incorporation of the last nucleotide and turning “on” only after addition of the last dNTP. These actions of τ are confined to its C-terminal region, which is located outside the clamp loading apparatus. Thus, this highly processive replication machine has evolved a mechanism to specifically counteract processivity at a defined time in the lagging-strand cycle.

Introduction

The replicative DNA polymerase of *E. coli*, DNA polymerase III holoenzyme (Pol III holoenzyme), is rapid and highly processive (Kelman and O’Donnell, 1995). Its processivity, in excess of 50 kb, derives from the ring-shaped β sliding clamp that encircles DNA and tethers the polymerase to DNA during synthesis (Kong et al., 1992; Stukenberg et al., 1991). The β clamp is a homodimer of crescent shaped monomer units, and the ring is alternately opened and closed at one interface for assembly onto DNA by the γ complex clamp loader (γτβδ³γ⁴β⁵) within the holoenzyme structure (Turner et al., 1999). Pol III holoenzyme contains two molecules of DNA polymerase III core (α, DNA polymerase; ε, 3’-5’ exonuclease; δ) for simultaneous synthesis of both strands of the DNA helix. These core polymerases are crosslinked to the single clamp loader via the two τ subunits (McHenry, 1982; Studwell-Vaughan and O’Donnell, 1991; Jeruzalmi et al., 2002). The γ and τ subunits are encoded by the same dnaX gene; τ (71 kDa) is the full-length product, and γ (47 kDa) is truncated by a translational frameshift. The 24 kDa C-terminal sequence of τ binds both the core and DnaB helicase (see Figure 1A) (Kim et al., 1996; Studwell-Vaughan and O’Donnell, 1991; Gao and McHenry, 2001a, 2001b).

Continuous synthesis of the leading strand at a replication fork fits nicely with the picture of a highly processive polymerase with a ring riding behind it to hold it down on DNA. However, due to the antiparallel structure of the double helix, the polymerase on the lagging strand must exhibit behavior that contrasts with such high processivity. The lagging strand is synthesized as short 1–3 kb Okazaki fragments. Primase initiates each fragment by synthesis of a short RNA primer. As the polymerase extends the primer, it synthesizes DNA in the direction opposite fork movement even though it travels with the fork due to its connection to both DnaB (through τ) and the leading polymerase (through the shared clamp loader). These opposite motions are thought to be resolved by formation of a DNA loop (Figure 1A) (for overview, see Komberg and Baker, 1992). As the DNA is extended, the loop grows until the polymerase bumps into the RNA primer of the fragment it made previously (Figure 1B). At this point, the polymerase must dissociate from the DNA in order that it may start extension of the next Okazaki fragment. Given the very low copy number of Pol III holoenzyme (10–20 copies/cell) and the need for 2000–4000 Okazaki fragments, this lagging-strand polymerase must be rapidly recycled.

Pol III holoenzyme rapidly cycles to new primed sites despite being held to DNA by a protein ring (O’Donnell, 1987; Stukenberg et al., 1994). Our earlier studies have shown that the polymerase is highly processive during chain extension, but upon completing DNA, it rapidly disengages from its β clamp and the DNA; the orphaned β clamp is left behind on the duplex product (as illustrated in Figure 1B). As synthesis proceeds, the clamp loader can catalytically load another β clamp on a new RNA primed site, leaving it there while the polymerase finishes a fragment. The lagging-strand polymerase, upon completing a fragment, dissociates from β and rapidly hops to the new β clamp (Figure 1B).

This report outlines the mechanism of this “processivity switch.” The results demonstrate that the τ subunit of the clamp loader is required for the processivity switch and that the switch proceeds with extremely high fidelity. The polymerase retains high affinity to its clamp even when only one nucleotide remains to be incorporated. Upon closing the gap to a nick, the switch is turned “on,” and τ separates the polymerase from β with a half-life of about 1 s. The processivity switch requires only the C-terminal domain of τ, which retains DNA binding activity and is positioned outside the clamp loader apparatus.

Results

The Processivity Switch Requires Synthesis to the Very Last Nucleotide

Our previous studies utilized large circular bacteriophage templates to demonstrate that Pol III holoenzyme remains tightly bound to β during synthesis, but upon going full circle and bumping into the 5’ end of the initiating primer, the polymerase pops off the β clamp (O’Donnell, 1987; Stukenberg et al., 1994; Stewart, 2001).
The enzyme synthesizes DNA so fast (~1 kb/s) that it remained possible that the processivity switch triggers at some point prior to finishing DNA, causing Pol III* to lose affinity for β-DNA, but due to its speed, it may simply complete the template before actually falling off.

To determine the point at which the processivity switch is tripped, we designed an experimental system to test the stability of Pol III*. (Pol III holoenzyme lacking only β) with β on a primed site having a defined gap as illustrated in Figure 2. Two DNA oligonucleotides are hybridized to an M13 derivative containing an 18 bp insert to which the latent origin binding protein of Epstein Barr virus, EBNA-1, binds with high affinity. Oligo #1 hybridizes to the ssDNA such that the EBNA-1 site is positioned at the 3' terminus. We have shown previously that EBNA-1 blocks the assembly of β onto this site (Yao et al., 2000). Oligo #2 is positioned 5' to Oligo #1 to produce a ssDNA gap between the two oligos. In the presence of EBNA-1, the only primed site available for assembly of the holoenzyme is at the gap on Oligo #2. The 5' sequence of Oligo #1 can be shortened or lengthened to produce gaps of defined size.

The stability of Pol III holoenzyme on different sized gaps was determined by assembly of 3H-Pol III* and 32P-β on these substrates followed by gel filtration analysis on a large-pore resin that resolves protein bound to the large DNA from proteins not bound to DNA. One potential caveat to this study is the presence of a 3'-5' exonuclease activity (ε subunit) within Pol III*, which could change the gap size by degrading the 3' terminus. To prevent this, we reconstituted Pol III* using an ε mutant that lacks exonuclease activity. This nuclelease-deficient Pol III* behaves with β like wild-type Pol III* (Yao et al., 2000; Giovann and McHenry, 2001).

DNA substrates with gaps that vary from 40 nucleotides to only 1 were used. The results in Figure 2B reveal that 3H-Pol III* remains stably attached to β on all these gapped DNA substrates—even on the substrate with only a single nucleotide gap. A control experiment using the ssDNA template containing only Oligo #2 provides a similar amount of 3H-Pol III* bound to β on DNA (data not shown). In another control, we designed a substrate that closes the gap to produce only a nick. Due to the processivity switch, Pol III* should not remain associated with β on DNA at a nick, and this result is confirmed in this system (Figure 2B). To ensure that β was attached to all these DNAs, including the nicked substrate, we used 32P-β in these experiments in order that its presence on DNA could be examined. The results confirm that similar amounts of β are attached to each of these
DNA substrates, even the one containing only a nick (Figure 2C).

In summary, these results show that the trigger of the processivity switch has very high fidelity for completed DNA and remains switched “off” even when there is only one nucleotide to be incorporated.

Characterization of the Processivity Switch

The next few experiments take advantage of this system to examine the processivity switch and its requirements. First, we performed a simple control in which we placed "H-Pol III"/β on the DNA with a one nucleotide gap and then added either dCTP, the last nucleotide to be incorporated, or dGTP, which should not be incorporated. The result, in Figure 3A, demonstrates that addition of the correct nucleotide to fill the gap elicits Pol III* dissociation, but the incorrect nucleotide does not.

Next, we asked whether the last nucleotide must be incorporated for Pol III* dissociation or if nucleotide binding is sufficient. For this experiment, we synthesized a 3‘ dideoxy terminated Oligo #2 and used it in combination with an Oligo #1 that produces a single nucleotide gap. Upon addition of dCTP, the polymerase should bind and pair it to the template dG, but will be unable to incorporate it due to the 3‘ dideoxy terminated 3‘ terminus at the gap. The result, in Figure 3B, shows that the processivity switch is not flipped upon adding dCTP, and Pol III* remains bound to the β clamp on DNA. Hence, the full catalytic reaction is necessary before the switch is flipped “on.”

The processivity switch may be powered by the energy of ATP. To test whether ATP is needed, Pol III*/β was assembled onto DNA with a one nucleotide gap and then ATP was removed using hexokinase and glucose. If ATP is needed, then Pol III* should remain bound to DNA after addition of dCTP to fill the gap. However, the results show that Pol III* still dissociates from DNA (Figure 3C), and therefore, ATP is not required. Perhaps the energy of incorporating the very last nucleotide is coupled to the switching process. To test this, we placed Pol III*/β onto DNA containing a 15 nucleotide gap and treated it with hexokinase/glucose to remove ATP. Then, instead of filling the gap by adding dNTPs, we added the correct nucleotide to fill the gap elicits Pol III* dissociation, but the incorrect nucleotide does not. DNA 15-mer complementary to the gap sequence. The result, in Figure 3D, shows that Pol III* dissociates upon addition of the 15-mer, indicating that the energy of nucleotide incorporation is not required to trigger the processivity switch. As a control, a noncomplementary DNA 15-mer was added; Pol III* did not dissociate.

Speed of the Processivity Switch

During lagging-strand replication, Okazaki fragments are produced every few seconds. Hence, the polymerase must rapidly disengage from β upon completing
Figure 3. Characterization of the Processivity Switch

(A) Correct incorporation is required. ^3H-Pol III* and β are assembled onto a one-nucleotide gap (top panel), then either the correct nucleotide (dGTP, middle panel) or incorrect nucleotide (dCTP, bottom panel) is added before gel filtration analysis.

(B) dNTP pairing is insufficient. Oligo #2 terminated with a 3’ dideoxynucleotide was used to construct a single-nucleotide gap. Stability of ^3H-Pol III* with β was examined by gel filtration either after mock treatment (top panel) or after treatment with dCTP (bottom panel).

(C) ATP is not required. After assembly of ^3H-Pol III* and β onto a one-nucleotide gap, ATP was removed using hexokinase and glucose, and then the reaction was treated either with dCTP (bottom panel) or without further addition (top panel).

(D) Oligonucleotide gap filling. ^3H-Pol III* and β were assembled onto a 15 nucleotide gap, and ATP was removed as in (C). Then a DNA 15-mer was added that was either complementary (bottom panel) or not complementary (top panel) to the gap before analysis by gel filtration.

Each section of DNA. The gel filtration experiments in Figures 2 and 3 are rather time consuming, requiring several minutes from start to finish. Therefore, the actual speed at which the switch separates polymerase from β cannot be addressed. To examine the processivity switch in real time, we adapted the system to solid phase for observation of polymerase dissociation from β-DNA by surface plasmon resonance. In the experiment of Figure 4, a template with a 10 nucleotide gap was assembled using synthetic oligos, one of which contained biotin at one end for attachment to a strepavidin chip. To prevent the holoenzyme from simply sliding off the other end of the linear DNA, we positioned an EBNA-1 site at the free end and added EBNA-1 (see scheme,
The processivity switch is rapid. The time course for Pol III* dissociation from DNA was determined by surface plasmon resonance. A 5'-biotin-labeled 10-nucleotide gap DNA was anchored to a strepavidin-coated sensor chip. The free DNA end contains an EBNA-1 site, and EBNA-1 was added to block polymerase from sliding off over the end. Pol III* and β were injected over the chip to assemble the holoenzyme on the immobilized DNA (injection 1). The second injection contained either dTTP (dotted line) or all four dNTPs (solid line). (A) Immobilized system. (B) Time course.

Figures 5A and 5B show the individual elution profiles of either core or β in the presence of the primed template. In Figure 5C, core and β were mixed with the DNA, and the result shows the expected core·β·primed DNA complex. Experiments using radiolabeled DNA confirm that the primed template comigrates with core·β (data not shown). Repeating this experiment using duplex DNA containing a single nick, to mimic a completed reaction, shows no difference; core·β remains stuck together (Figure 5D). Thus, either the processivity switch doesn’t function in this experimental design, or another subunit is required.

Next, we added different clamp loader subunits to reactions containing core, β, and nicked DNA to determine whether one of the clamp loader proteins is required to trigger the processivity switch and dissociate core from β. Whereas addition of γ, δ, δ', or χβ complex made no difference (Figures 5E–5H), the τ subunit had the effect of dissociating core from β (Figure 5I). The τ subunit directly interacts with core polymerase and therefore results in core·τ complex, which elutes earlier than core. Next, we repeated the experiment using the primed template. In this instance, the processivity switch should not be triggered, and core·τ should remain intact on the DNA. Indeed, the result demonstrates that the β clamp comigrates with core·τ when the primed template is used (Figure 5J).

To quantify the affinity between core and β, and the effect of τ and different DNA structures on this interaction, we labeled β at Cys333 with the Oregon Green fluorophore (βOG) and used it as a probe of complex formation (Figure 6). The fluorescence intensity of βOG increases upon complex formation with either core or core·τ, and thus provides a signal to measure the Kd.
Figure 5. The τ Subunit Is Required to Separate Core from β

Mixtures of core and β with linear synthetic primed or nicked DNAs were analyzed by gel filtration on a Superose 12 column followed by analysis of the fractions by SDS PAGE. (A) Core + primed DNA; (B) β + primed DNA; (C) core + β + primed DNA; (D) core + β + nicked DNA; (E–I) core + β + nicked DNA + (E) τ, (F) δ, (G) δ', (H) χβ, or (I) τ. (J) Analysis of core + β + τ + primed DNA. Column fractions are indicated on the top gel, and subunits are identified to the right of each gel.

value of β interaction with core-τ complex. In the presence of the synthetic primed template, the K_d value for interaction of core-τ with β was approximately 16 nM (Figure 6A). It is unlikely that the fluorescent moiety alters the affinity of β for core, as it is located on the opposite face of β from the site of interaction with core (Naktinis et al., 1996) and retains full activity with Pol III*.

Next, we constructed a synthetic duplex having a single nucleotide gap. The K_d value for interaction of β with core-τ complex on the single nucleotide gap DNA was 28 nM, only about 2-fold weaker compared to the primed template (Figure 6B). However, in the presence of the nicked DNA, the affinity between β and core-τ decreased dramatically to a K_d value of higher than 600
Figure 6. DNA Structure Modulates \( \tau \) Action in the Processivity Switch

The \( \beta \) subunit was labeled with Oregon Green on Cys333 to follow interaction with core-\( \tau \) at equilibrium using fluorescence. Titration of core-\( \tau \) into solutions of \( \beta \) containing gel-purified synthetic DNA templates produced a fluorescence enhancement. Shown in (A)–(C) are fluorescent emission scans taken during titration of core-\( \tau \) into \( \beta \) with either primed DNA (A), one-nucleotide gap DNA (B), or nicked DNA (C). (D) A titration of core (no \( \tau \)) into \( \beta \) with nicked DNA. Below each fluorescent analysis is a plot of fluorescent intensity at 517 nm versus concentration of core-\( \tau \) or core.

nM (Figure 6C). When \( \tau \) is absent, the \( K_d \) value of the \( \beta \)-core interaction is \( \sim 18 \text{ nM} \) in the presence of nicked DNA (Figure 6D). Thus, the \( \tau \) subunit is required to decrease the affinity between core and \( \beta \) over 20-fold on a nicked DNA substrate.

**The Processivity Switch Requires Only the C-Terminal Section of \( \tau \)**

The \( \tau \) subunit binds core polymerase and DnaB and is a DNA binding protein, yet neither of these properties apply to \( \gamma \), which is essentially the N-terminal 2/3 of \( \tau \). Hence, the DNA and core binding activities of \( \tau \) probably reside in the C-terminal 24 kDa sequence unique to \( \tau \).

A study of the C-terminal sequence of \( \tau \) has confirmed that this is sufficient to bind core and DnaB (Gao and McHenry, 2001a, 2001b). Below, we show that this section of \( \tau \) (referred to herein as \( \tau_c \)) also binds DNA and is sufficient to provide the processivity switch that separates core from \( \tau \) on completed DNA. Further, \( \tau_c \) displays no detectable affinity for \( \gamma \), indicating that it may act as an independent unit from the N-terminal 2/3 of \( \tau \) that participates with \( \gamma \), \( \delta \), and \( \delta' \) in the clamp loading reaction.

In Figure 7A, we used \( \beta^{\omega} \) to measure its affinity to core-\( \tau_c \) in the presence of either the synthetic primed template or the duplex containing a single nick. The \( K_d \)
Figure 7. The C-Terminal Section of τ Activates the Processivity Switch

(A) The Kd of core-τc to βα in the presence of primed DNA (left side) is much lower than in the presence of nicked DNA (right side).

(B) τc retains DNA binding activity. τc was analyzed by gel filtration in the absence or presence of 32P-end-labeled primed DNA. Comparison of the Coomassie-stained SDS-polyacrylamide gels shows that DNA shifts the position of τc, indicating that τc binds the DNA. Autoradiography of a native gel analysis of the column fractions shows the 32P-DNA is gel shifted in fractions containing τc.

(C) τc has no detectable affinity for γ upon analysis by gel filtration (Superose 12) at high concentration (100 μM τc and 30 μM γ, as monomer).

(D) A sequence near the C terminus of γ is disordered in the crystal structure, indicating it may be a flexible linker connecting τc to γ. Flexibility is consistent with the many prolines in this sequence.

The processivity switch requires DNA recognition. To determine whether τc contains DNA binding activity, τc was mixed with 32P-labeled primed DNA, and then the mixture was analyzed by gel filtration. Column fractions were analyzed by denaturing SDS-PAGE to identify τc.
and in a native gel followed by autoradiography to detect the \(^{32}\)P-DNA (Figure 7B). The result shows that \(\tau_c\) with DNA elutes earlier than \(\tau_c\) without DNA, indicating formation of a \(\tau_c\)-DNA complex. The autoradiogram of the native gel confirms that the \(^{32}\)P-DNA comigrates with \(\tau_c\), and that the \(^{32}\)P-DNA-\(\tau_c\) complex is retarded in the gel relative to unbound \(^{32}\)P-DNA (Figure 7B).

Next, we asked whether \(\tau_c\) binds to \(\gamma\) by mixing the two proteins at high concentration, followed by analysis for complex formation between them by gel filtration (Figure 7C). The two proteins do not comigrate, indicating that they probably do not form strong contacts with one another within full-length \(\tau\). This experiment has been repeated in the presence of primed DNA and nicked duplex DNA, but \(\gamma\) and \(\tau_c\) still do not comigrate (data not shown). Shown in Figure 7D is a sequence near the C-terminal region of \(\gamma\) that is disordered in the \(\gamma\) complex crystal structure, implying it may be a disordered peptide that links \(\tau_c\) to the rest of \(\tau\). Consistent with disorder in the \(\gamma\) complex structure, the sequence contains many prolines and relatively few hydrophobic residues. This peptide is located near the C terminus of \(\gamma\) (see scheme in Figure 7D). Taken together with the lack of detectable binding between \(\tau_c\) and \(\gamma\), the structural data provide evidence that this peptide may act as a flexible tether that links \(\tau_c\) to the clamp loader.

**Discussion**

**A Precise Processivity Switch with High Fidelity for Completed DNA**

Pol III holoenzyme binds primed DNA with high affinity as a result of its ring-shaped \(\beta\) subunit (Kuriyan and O’Donnell, 1994). However, a chromosomal replicase must be capable of more than simply remaining on DNA for highly processive synthesis. This is especially true on the lagging strand, where DNA synthesis is discontinuous. Here, the polymerase needs to balance this processive grip with an ability to pop off DNA when it completes an Okazaki fragment so that it can be available for synthesis of the next fragment. The scarcity of Pol III holoenzyme in the cell makes this balancing act all the more important. The speed of the replication fork (\(\sim 1 \text{ kb/s}\)), coupled with the 1–3 kb length of Okazaki fragments, indicates that these fragments are synthesized about every 1–3 s. How can a processive replicase, bound to DNA by a ring, recycle itself from DNA in such rapid fashion?

This report illustrates that polymerase recycling occurs by a processivity switching mechanism that has exquisite fidelity for completion of an Okazaki fragment. The enzyme retains a tight grip to \(\beta\) on a primed template, even when the ssDNA template strand is a single nucleotide in length. Only when the gap has been completely filled to a nick does the enzyme dissociate, leaving the \(\beta\) clamp on the DNA.

**The Mechanics behind the Processivity Switch**

What underlies the intelligence of this smart switching process? We have discovered herein that the \(\tau\) subunit of the clamp loader is responsible for modulating the core-\(\beta\) interaction, and it does so in a fashion that is highly responsive to the DNA structure. As explained above, provided even a single nucleotide remains in the template strand, \(\tau\) does not disengage the \(\beta\) ring from the DNA polymerase. But when no ssDNA remains (i.e., a nick), the \(\tau\) subunit severs the core-\(\beta\) interaction. Exactly how does \(\tau\) sense the DNA structure and transduce this signal that modulates the affinity between core polymerase and \(\beta\)? Although the detailed mechanism behind this switch will require further biochemical studies, and ultimately crystal structures, a few possible scenarios can be proposed from this work.

The \(\tau\) subunit has DNA binding activity, and we demonstrate here that the activity resides in the C-terminal sequences unique to \(\tau\) (i.e., missing in \(\gamma\)) and thus is in the same general area as the core polymerase binding sequences of \(\tau\). It therefore seems quite possible that \(\tau\) contains the DNA recognition activity that distinguishes the complete product DNA from primed template. A possible mechanism by which \(\tau\) functions is illustrated in Figure 1B, in which the DNA binding site of \(\tau\) is in close proximity to the primed template binding site of the \(\gamma\) subunit of core polymerase, perhaps even forming a part of its substrate binding site. In this model, where \(\tau\) is situated near the DNA binding site of \(\alpha\), it may help core interact with the primed template DNA structure and will be ideally situated to recognize whether replication is completed, whereupon it acts to dissociate core from \(\beta\). Alternatively, the DNA binding domain of \(\tau\) may be some distance from the polymerase active site yet still sense the DNA structure of the primer template. In this case, when Pol III holoenzyme finishes a template, it may slide with \(\beta\) on DNA, allowing \(\tau\) to come into position and check whether the template is complete.

Core has considerable affinity for \(\beta\), even at a nick. Thus one may ask whether core functions in the cell with \(\beta\) but without \(\tau\). We have examined this issue and find that core without \(\tau\) is not as stable as core on DNA compared to core in the presence of \(\tau\) (Stukenberg and O’Donnell, 1995). This is indicated by low yields of core on \(\beta\)-DNA complex during gel filtration and the requirement for much lower ionic strength in the column buffer. As gel filtration is a nonequilibrium technique, we presume the decreased stability reflects a more rapid \(K_{\text{off}}\) of core from \(\beta\)-DNA relative to core-\(\tau\) (core could have a more rapid \(K_{\text{on}}\) for \(\beta\) than core-\(\tau\)). The decreased stability between core and \(\beta\) in the absence of \(\tau\) may obviate the need for a processive switch when \(\tau\) is not present. However, the resulting synthesis may be less processive and could leave ssDNA gaps that would need to be filled by subsequent polymerase action by core or other DNA polymerases. Regardless, during replication core probably functions within the context of being bound to \(\tau\), as the C-terminal sequences of \(\tau\) are essential for cell viability (Blinkova et al., 1993). Therefore, a processive switch is needed to facilitate departure of Pol III from its \(\beta\) clamp at the end of each fragment.

Analysis of replication fork operations in vitro indicates that under some conditions the lagging-strand polymerase can dissociate from DNA prior to completion of an Okazaki fragment (Li and Marians, 2000). This premature cycling is observed upon initiating lagging-strand replication after the leading strand polymerase has been allowed a “head start” in synthesis. Premature polymerase recycling may be useful to the cell when the leading strand polymerase gets too far ahead of the lagging-strand polymerase. It is important to note that
since DnaB also binds τ, this proximity to core may affect cycling behavior at a replication fork.

What Aspect of the DNA Structure Triggers the Processivity Switch?

This study demonstrates that attachment of the polymerase to β on DNA decreases dramatically in going from a single nucleotide gap to a nick. Perhaps τ sterically collides with the 5′ terminus as it approaches the duplex DNA junction. This collision may then trigger τ to separate core from β. We have examined whether polymerase dissociation requires recognition of the 5′ terminus in our earlier studies in which the holoenzyme, upon going full circle around a singly primed M13 ssDNA, collides with the 5′ terminus and rapidly cycles to a challenge template. These studies show that rapid polymerase cycling still occurs using a variety of 5′ structures, including 5′ OH, 5′ phosphate, 5′ RNA triphosphate, 5′ biotin, and 5′ psoralen, indicating that recognition of the chemical moieties at the 5′ position does not trigger the processivity switch (Stukenberg et al., 1994; Stewart, 2001). It is possible that τ recognizes the different biophysical properties of a nicked and gapped DNA. Even a single nucleotide gap would introduce a very large degree of freedom between two relatively stiff duplex sections, whereas a nick would allow stacking between the bases at the nick, which may greatly decrease the flexibility of this structure.

Regardless of what may trigger the polymerase to dissociate from its clamp, it may not occur exactly at the nick. Previous experiments have demonstrated that the holoenzyme, upon encountering a short region of duplex DNA (15–400 bp) in its path, slides over the duplex region to locate the next 3′ terminus and proceeds to extend it in a processive fashion (O’Donnell and Komberg, 1985). This type of behavior indicates that polymerase dissociation does not occur immediately upon completing the template. The current study demonstrates that the processivity switch is rapid, but it is quite possible that the polymerase, while it remains attached to β, scans the duplex before it fully disengages from β.

Implications for a Processivity Switch in Other Systems

The use of a clamp and clamp loader generalizes to all cells, prokaryotic, eukaryotic, archaeal, and at least in one case, to a bacteriophage (Davey et al., 2002). Do their clamp loaders contain a subunit that interacts with the polymerase and provides it with a processivity switch for lagging-strand replication? We have examined two other bacterial systems, Streptococcus pyogenes and Aquifex aeolicus, and in both cases the τ subunit interacts with the replicative polymerase (Bruck et al., 2002; Bruck and O’Donnell, 2000). Moreover, there are reports of direct interaction between the T4 clamp loader (gene protein 44/62 complex) and the T4 DNA polymerase (gene protein 43) (Alberts et al., 1983; Kaiboord and Benkovic, 1995). Although the connection between the clamp loader and DNA polymerase is not as tight in solution for these other systems relative to E. coli core and τ, it is thought that the clamp loader is stably attached within the replisome apparatus at a replication fork (Munn and Alberts, 1991; Waga and Stillman, 1994). Whether the clamp loader-polymerase interaction acts as a processivity switch in these other systems has not been addressed. However, in all these diverse systems, the polymerase/clamp complex is processive, yet the lagging strand is replicated discontinuously. Hence, a processivity switch for recycling of polymerase on the lagging strand would appear to be a general requirement.

Studies in the T4 system demonstrate that gene 43 DNA polymerase binds tight to its clamp while stalled on a template due to nucleotide omission, but the polymerase rapidly disengages from its clamp upon finishing a template (Hacker and Alberts, 1994). Although the T4 clamp is not as stable on DNA as β, it is probably left on DNA after polymerase dissociates, as it has been shown to have a residence time on duplex DNA in the absence of polymerase (Fu et al., 1996). Indeed, the T4 clamp activates RNA polymerase for late gene transcription, a process that is tightly coordinated with DNA replication (Herendeen et al., 1992). Taken together, these data suggest that the processivity switch may be central to the process by which transcription and replication are coordinated in DNA viruses. Specifically, after completing a lagging-strand fragment, the DNA polymerase dissociates, leaving the clamp on DNA where it can activate transcription of late genes.

Studies of the eukaryotic replication machinery have not examined polymerase recycling, but the similarity to E. coli Pol III holoenzyme is quite striking. For example, the PCNA chain folding pattern is the same as in E. coli β, the PCNA trimer has high stability on duplex DNA like β, and PCNA is actively unloaded from DNA as in the E. coli system (Gulbis et al., 1996; Krishna et al., 1994; Yao et al., 1996). Further, the RFC clamp loader interacts with DNA polymerase δ, albeit weakly (Yuzhakov et al., 1999), and there is evidence that RFC travels with DNA polymerase δ during chain extension (Tsurimoto and Stillman, 1991). Given the similarity between the E. coli, yeast, and human systems, it seems likely that the eukaryotic lagging-strand polymerase contains a processivity switch (Stillman, 1994). Further study of the processivity switching mechanism in E. coli, eukaryotes, and other systems, including crystal structure analysis of the fine details that are most certainly involved in the process, remains an exciting avenue for the future.

Experimental Procedures

Materials

Bio-Gel A15m agarose was from Bio-Rad; restriction enzymes were from NEB; DNA oligonucleotides were from Gibco BRL. Oligonucleotides were gel purified. Buffer A is 10 mM Tris-HCl, 300 mM NaCl, and 30 mM sodium citrate (final pH 8.0). Buffer B is 20 mM Tris-HCl (pH 7.5), 4% (w/v) glycerol, 0.1 mM EDTA, 40 μg/ml bovine serum albumin (BSA), 5 mM DTT, 1 mM ATP, and 8 mM MgCl₂. Buffer C is 20 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 4% (v/v) glycerol, 100 mM NaCl, 0.5 mM EDTA, 100 μg/ml BSA, and 2 mM DTT. Buffer D is 20 mM Tris HCl (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, and 1 mM DTT.

Proteins

Proteins were purified as described: α, ε, γ, τ, δ, ξ, φ, and ι (Onrust et al, 1995), β (Kong et al., 1992), and SSB and EBNA-1 (Yao et al., 2000). The γ complex (Onrust and O’Donnell, 1993), ²H-Pol III, core, and αα (Stukenberg and O’Donnell, 1999) were reconstituted from pure subunits and purified as described, except the ε subunit was mutated (D12A, E14A) to eliminate the 3′-5′ exonuclease activity.
**DNA**

**Defined Gaps on Phage Templates**

M13mp19EBNA-1 is M13mp19 containing an 18 bp EBNA-1 binding site (Yao et al., 2000). Phage was purified, and ssDNA was extracted as described (Yao et al., 2000). Two DNA oligonucleotides were annealed to M13mp19EBNA-1 ssDNA to produce defined gaps between them. The holoenzyme assembles at the 3' end of Oligo #2 (31-mer, map position 6291–6381). The 3' terminal 18 nucleotides of Oligo #1 contain the EBNA-1 site, and the 5' sequence ranged from 44 to 84 nucleotides to produce ssDNA gaps of 40, 15, 3, 2, 1, or 0 nucleotides, when annealed to M13mp19EBNA-1 ssDNA with Oligo #2. Hybridization reactions contained 15 pmol ssDNA and 375 pmol of each oligo in 20 μl buffer A.

**Oligonucleotide Templates**

The nicked duplexes were assembled upon mixing 8 nmol of DNA 62-mer (5'-ccc ATC gTA TAC gAA gAgT tgA gCT gA gT gT gC gT aCT gAT gCT ATg CTg CT-3') and 40 nmol each of 28-mer (5'-GCC gTc gAc Tcc Tct gCT gAT aCA gT gG-3') and 34-mer (5'-Agc TAg gCT gAT gAgT gAg gCT gAg TAg gCT gA g-3') in 100 μl buffer B. Reactions were shifted to 23°C, and then incubated 3 hr at 23°C. The reaction was adjusted to 60 mM Tris-Cl (pH 7.5), 20 mM MgCl₂. The reaction was warmed to 95°C, slowly cooled to 23°C, and then incubated at 23°C. The resulting substrate contains a 10 nucleotide sequence of the EBNA-1 binding site.

**Stability of Pol III**

Stability of Pol III was measured using the EBNA-1 binding site (Yao et al., 2000). The reaction mixture contained 12 nM Pol III, 96 nM DNA, and 0–120 nM core in 60 μl buffer D on ice. Reactions were shifted to 22°C for 15 min, and then 51 μl was placed in a 3 x 3 mm cuvette. Excitation was at 490 nm, and emission was monitored from 500–600 nm in a PTL Quantumaster spectrofluorimeter. Fluorescence emission at 517 nm was used for analysis. Data points were fit according to the simple model, A → B ↔ AB, using the Origin software (Microcal). Analysis of core interaction with Pol III was performed as described above with the exception that 0.36 nmol core was incubated with 0.72 nmol at 15°C for 15 min before use.

**Surface Plasmon Resonance**

Duplex DNA (5'-GGG TAG CAT ATG TTC CTT CCC GAA TTA ACT GGC CGT CTT TTT ACA ACG TCG TCA TGG AAA CCC TGG CTG TAC CCA ACT T-3') containing a 10 nucleotide gap was constructed upon mixing 0.5 nmol of a gel-purified 8' biotin end-labeled 79-mer, 5 nmol of a 39-mer (5'-AAA ACG ACG GCC AAT GTA TGG AAA GAG ATG TCC CTA GCA TCT G-3') that anneals to the 3' sequence of the 79-mer, and 5 nmol of a 30-mer (5'-AAG TTG GTG AAC AGG CCT GTC TTC CCA GTC-3') that anneals to the 3' sequence of the 79-mer. DNAs were mixed in 250 μl of buffer A, brought to 95°C, and then cooled to 23°C. Recombinant T7 DNA polymerase was then used to extend the 3' end of the ssDNA gap, bracketed by a 5' biotin and a 3' EBNA-1 binding site.

The DNA (250 fmol as 79-mer) was passed over a Streptavidin-coated sensor chip in a 30 μl injection lasting 7 min. This resulted in an increase of 170 response units (RU). EBNA-1 (3 pmol) was then injected over the sensor chip in 30 μl SPR buffer in another 7 min injection, resulting in a further rise of ~180 RU. Next, DNA Pol II (700 fmol), β clamp (1 pmol), 1 mM ATP, and 4.2 pmol of dTTP, in 30 μl SPR buffer, was injected over the chip for 7 min. This resulted in a rise of 220 RU. Immediately after this, 30 μl SPR buffer containing either 4.2 nmol of dTTP or 4.2 nmol of each of the 4 dNTPs was injected. The rate of Pol III dissociation from DNA was determined using the BioEvaluation 2.0 software assuming a simple dissociation reaction. Control reactions included a test for β dependence, a test for ATP dependence, and a test for DNA dependence. In each case, less than 15 RU accumulated upon injection of Pol III**β**.

**Acknowledgments**

This work was supported by the HHMI and a grant from the NIH (GM38839).

Received: October 10, 2002

Revised: November 26, 2002
References

 phage T4 in vitro system. Cold Spring Harb. Symp. Quant. Biol. 47, 
 655–668.

 but only γ is essential. J. Bacteriol. 175, 6018–6027.

Bruck, I., and O’Donnell, M. (2000). Reconstitution of the DNA repli- 
 cation apparatus of Streptococcus pyogenes, a gram positive or- 

Bruck, I., Yuzhakov, A., Yurieva, O., Jeruzalmi, D., Skangalis, M., 
 thermostable DNA polymerase III holoenzyme from an extreme thermo- 

 tors and switches: AAA+ machines within the replisome. Nat. Rev. 

 motif of Escherichia coli dnaQ: defective proofreading and invisibility 

 Dynamics of DNA- tracking by two sliding-clamp proteins. EMBO J. 
 15, 4414–4422.

 coli replication proteins through distinct domains: partial proteolysis 
 of terminally tagged γ to determine candidate domains and to assign 
 domain V as the γ binding domain. J. Biol. Chem. 276, 4433–4440.

 coli replication proteins through distinct domains: domain IV located 
 within the unique C terminus of γ, binds the replication fork helicase, 
 DnaB. J. Biol. Chem. 276, 4441–4446.

 holoenzyme: an asymmetric dimeric replicative complex with lead- 
 ing and lagging strand polymerases. Cell 105, 925–934.

 Escherichia coli DNA polymerase III holoenzyme is dissociated into 
 monomers upon binding magnesium (II)2+. Biochemistry 27, 5210– 
 5215.

 (1996). Structure of the C-terminal region of p21waf1/cip1 com- 
 plexed with human PCNA. Cell 87, 297–306.

Hacker, K.J., and Alberts, B.M. (1994). The rapid dissociation of the 
 T4 DNA polymerase holoenzyme when stopped by a DNA hairpin 
 helix. A model for polymerase release following the termination of 

 and sliding clamps in one holoenzyme particle. V. Four different 
 transcriptional enhancer whose function imposes a requirement that polymerase-clamp complexes on DNA. J. Biol. Chem. 
 269, 29971–29982.


 proteins. II. Characterization of β and γ’. J. Biol. Chem. 268, 11766– 
 11772.


Li, X., and Marians, K.J. (2000). Two distinct triggers for cycling of 
 the lagging strand polymerase at the replication fork. J. Biol. Chem. 
 275, 34757–34765.

McHenry, C.S. (1982). Purification and characterization of DNA poly- 

 accessory proteins form an ATP-dependent complex on a primer- 

 of a chromosomal replication machine: two DNA polymerases, a 
 clamp loader, and sliding clamps in one holoenzyme particle. II. 
 Intermediate complex between the clamp loader and its clamp. J. 

 in a replication machine defined by an internal competition for pro- 
 tein rings. Cell 84, 137–145.

 and mediate rapid cycling of DNA polymerase III holoenzyme from 

 asse III holoenzyme of Escherichia coli in replication of a multiprimed 

 proteins. II. Characterization of δ and δ’. J. Biol. Chem. 268, 11766– 
 11772.

Onrust, R., Finkelstein, J., Turner, J., Naktinis, V., and O’Donnell, M. 
 polymerases, a clamp loader, and sliding clamps in one holoenzyme 
 particle. III. Interface between two polymerases and the clamp 

Stewart, J. (2001). Dynamics of the DNA pol III holoenzyme of E. 
 coli: β clamp opening by the β subunit and dissociation of the pol 

 78, 725–728.

 the twin polymerase of DNA polymerase III holoenzyme. J. Biol. 
 Chem. 266, 19833–19841.

 mosomal replication machine: two DNA polymerases, a clamp loader, 
 and sliding clamps in one holoenzyme particle. V. Four different 
 polymerase-clamp complexes on DNA. J. Biol. Chem. 270, 13384– 
 13391.

 Mechanism of the sliding β-clamp of DNA polymerase III holo- 
 enzyme. J. Biol. Chem. 266, 11328–11334.

 tion for lagging strand replication: polymerase hopping among DNA 

 for SV40 DNA replication in vitro. I. DNA structure-specific recogni- 
 tion of a primer-template junction by eukaryotic DNA polymerases 

 The internal workings of a DNA polymerase clamp-loading machine. 
 EMBO J. 18, 771–783.

 revealed by reconstitution of SV40 DNA replication in vitro. Nature 
 369, 207–212.
