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

Frank P. Leu,[2] Roxana Georgescu,[1,2]
and Mike O'Donnell[1,2,*]
[1]Howard Hughes Medical Institute
[2]Rockefeller 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 ($\gamma_2\delta\delta'\chi\psi$) 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; θ) 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 Kornberg 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).

*Correspondence: odonnel@mail.rockefeller.edu

A

Replisome Architecture

B

Lagging Strand Processivity Switch
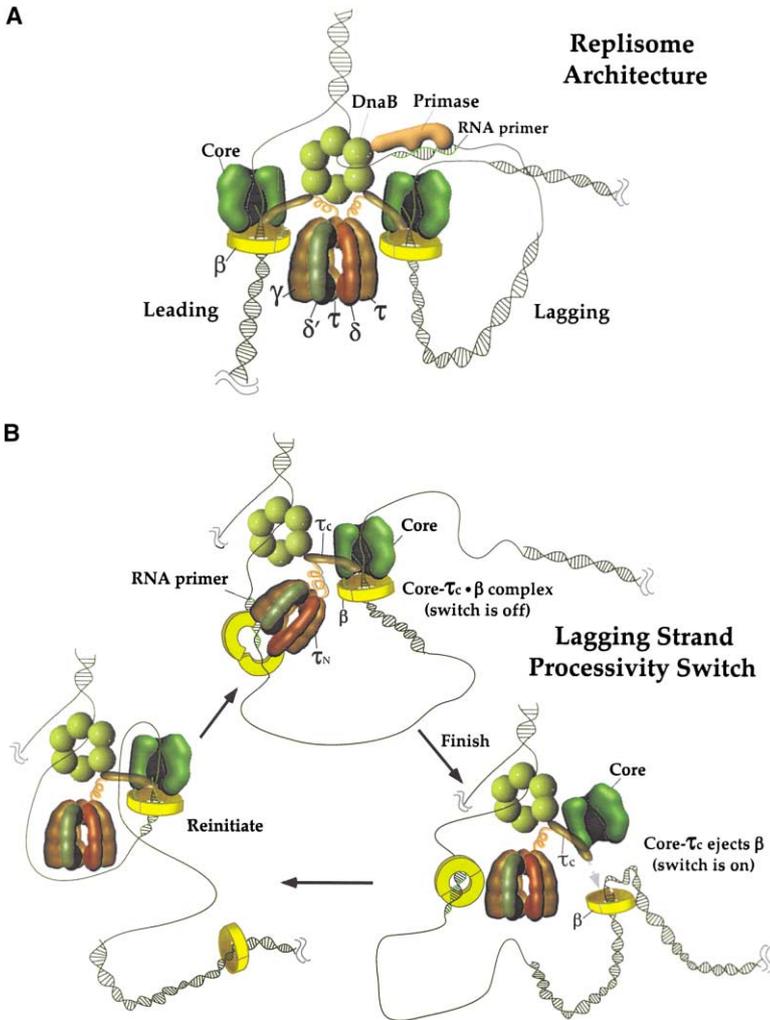
Figure 1. Operation of Pol III Holoenzyme on the Lagging Strand

(A) The architecture of Pol III holoenzyme at a replication fork is illustrated. The single γ complex clamp loader is a pentamer of subunits $\gamma\tau_2\delta\delta'$ and also includes χ and ψ (not shown). Two core polymerases ($\alpha\epsilon\theta$) bind the C-terminal sequences of the τ subunits that protrude from the clamp loader. The core polymerases are held to DNA by β clamps. The DnaB helicase encircles the lagging strand and binds τ. Primase contacts DnaB to initiate RNA primer synthesis.

(B) This panel focuses on the lagging strand and illustrates the findings of this report (see Discussion for details). The top diagram shows the clamp loader placing a β clamp onto a primed site while the lagging-strand polymerase is extending an Okazaki fragment. The C-terminal sequences of τ (τ_c) protrude outside the clamp loading machinery and bind core polymerase, DnaB, and DNA. These τ_c sequences are needed for the processivity switch that controls the affinity of core for β in response to DNA structure. The diagram depicts τ_c as binding the primed template in conjunction with core, although other arrangements are possible (see Discussion). The switch is off during elongation, allowing core to remain associated with β on DNA. In the lower right diagram, the lagging-strand polymerase finishes an Okazaki fragment, thereby flipping the processivity switch and turning τ_c "on" for disengaging core from β. The polymerase then dissociates from β, leaving it behind on the completed fragment. In the bottom left diagram, the lagging-strand polymerase reattaches to β at the new primed site for extension of another Okazaki fragment.

The enzyme synthesizes DNA so fast ($\sim$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 ^{3}H-Pol III* and ^{32}P-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 nuclease-deficient Pol III* behaves with β like wild-type Pol III* (Yao et al., 2000; Glover and McHenry, 2001).

DNA substrates with gaps that vary from 40 nucleotides to only 1 were used. The results in Figure 2B reveal that ^{3}H-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 ^{3}H-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 ^{32}P-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
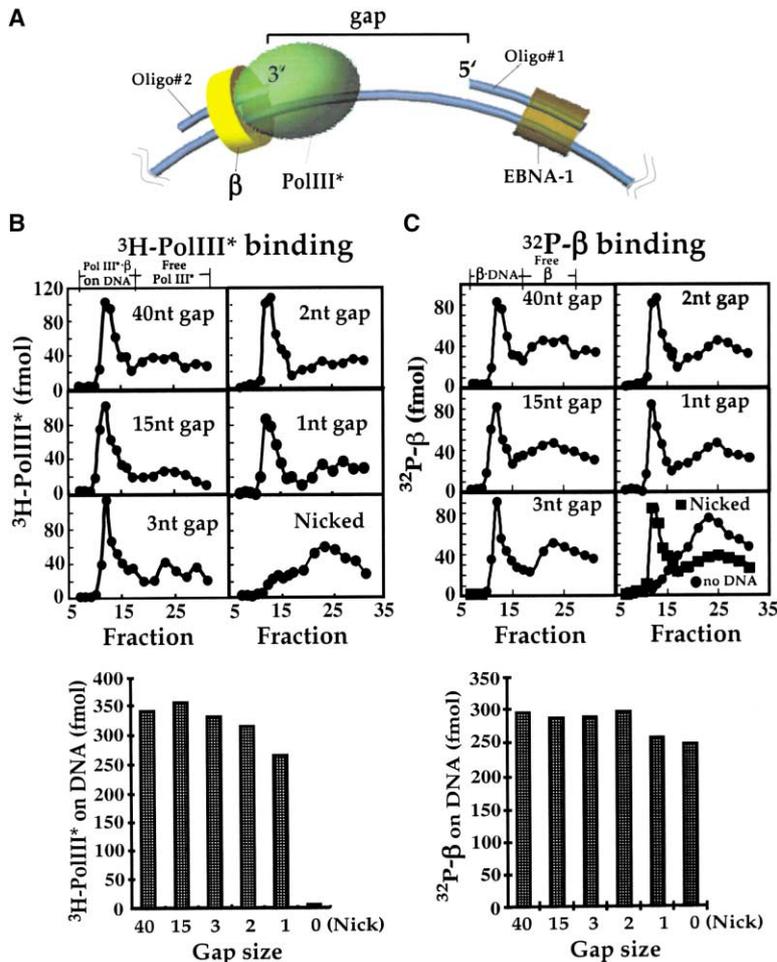
Figure 2. The Processivity Switch Flips at a Nick

Pol III holoenzyme, composed of ^{3}H-Pol III* bound to ^{32}P-β, is assembled on M13 ssDNA to which two DNA oligos are annealed to produce gaps of defined size (A). Oligo #1 contains an EBNA-1 site, and binding of EBNA-1 prevents holoenzyme assembly. Thus, the holoenzyme is directed to Oligo #2. Stability of ^{3}H-Pol III* (B) and ^{32}P-β (C) on DNA is assessed upon analysis by gel filtration, which resolves protein bound to DNA from protein in solution. The bar plots summarize the total protein·DNA complex on each DNA substrate.

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 ^{3}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 a 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. ^{3}H-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 ^{3}H-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 ^{3}H-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. ^{3}H-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 was 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,

Figure 4. The Processivity Switch Is Rapid

The time course for Pol III* dissociation from β upon completing 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.

Figure 4A). The combination of EBNA-1 and biotin-strepavidin traps the β ring on the DNA and confines polymerase dissociation to β at an internal position.

Next, Pol III* and β were passed over the immobilized DNA; the resulting increase in mass indicates holoenzyme assembly onto the primed site. The speed of polymerase dissociation at the gap was examined upon passing buffer containing only dTTP over the chip. As expected, the holoenzyme was quite stable, with a k_{off} of approximately $5.5 \times 10^{-5} s^{-1}$. Next, we added the four dNTPs to allow the polymerase to move forward and fill the gap. The result shows a very rapid release of Pol III*. The release of Pol III* is too rapid for a kinetic analysis, since the instrument only takes one measurement per second, and the first half-life appears to be over at the first data point ($k_{off} \leq 0.5 s^{-1}$). Hence, the processivity switch is rapid and occurs within the time frame required for lagging-strand synthesis.

Subunit Requirements for the Processivity Switch

To examine whether one or more clamp loader subunits participate in the switch, we utilized small linear primed templates that β can thread onto, allowing us to study the switching process in the absence of a functional clamp loader. For these studies, we prepared a primed template having 34 bp of duplex and 28 nucleotides of ssDNA. To trigger the switch, we prepared a 62 bp duplex containing a single internal nick. Each substrate was assembled and gel purified to remove any unannealed oligos. First we examined stability of core polymerase with β on these DNA substrates using a Superose 12 column, which resolves β (80 kDa) from core (165 kDa).

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 DNA, 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 χψ 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·β 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 quantitate 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 (β^og) and used it as a probe of complex formation (Figure 6). The fluorescence intensity of β^og increases upon complex formation with either core or core·τ, and thus provides a signal to measure the K_d
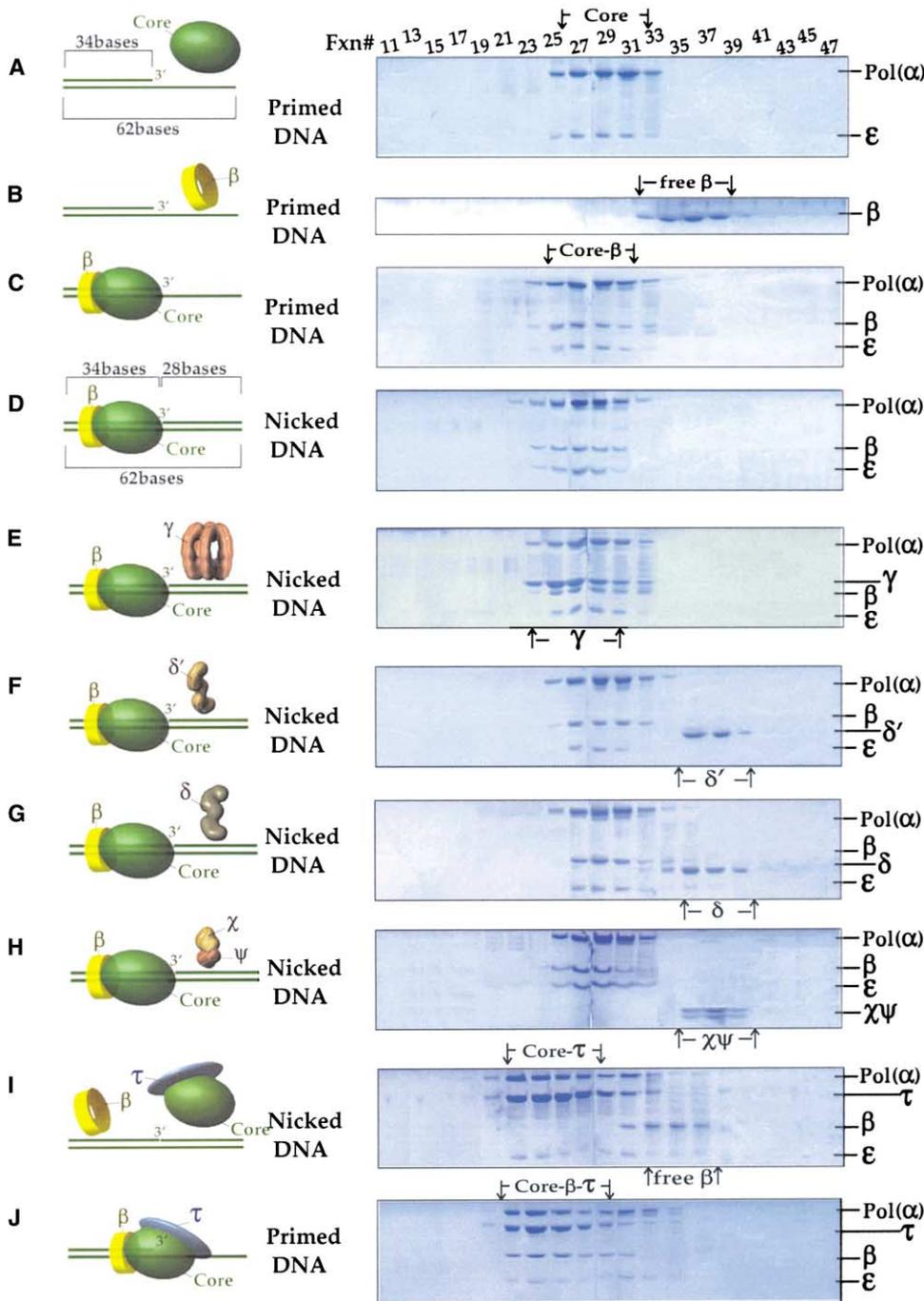
Figure 5. The τ Subunit Is Required to Separate Core from β

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) γ, (F) δ, (G) δ′, (H) χψ, 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 $β^{og}$ 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 $β^{og}$ 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
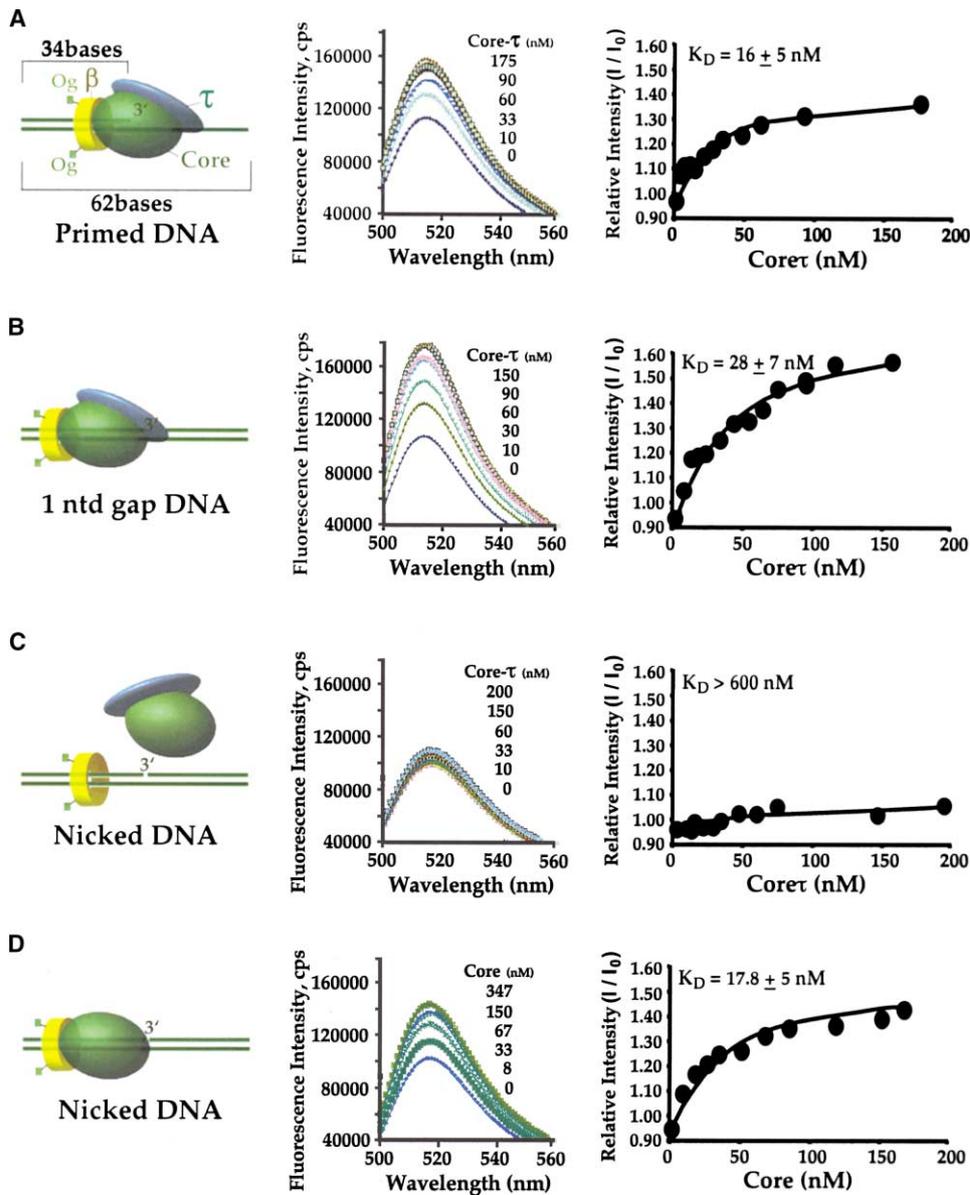
Figure 6. DNA Structure Modulates τ Action in the Processivity Switch

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

nM (Figure 6C). When τ is absent, the K_d value of the β·core interaction is ~18 nM in the presence of nicked DNA (Figure 6D). Thus, the τ subunit is required to decrease the affinity between core and β over 20-fold on a nicked DNA substrate.

The Processivity Switch Requires Only the C-Terminal Section of τ

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

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

In Figure 7A, we used β^{og} to measure its affinity to core·τ_c in the presence of either the synthetic primed template or the duplex containing a single nick. The K_d

A

B

C

D

Linker Sequence: PRMPLPEPEVPRQSFAPVAPTAVMTPTQVPPQPQSAPQQAPTVPLP
Flexible linker in τ
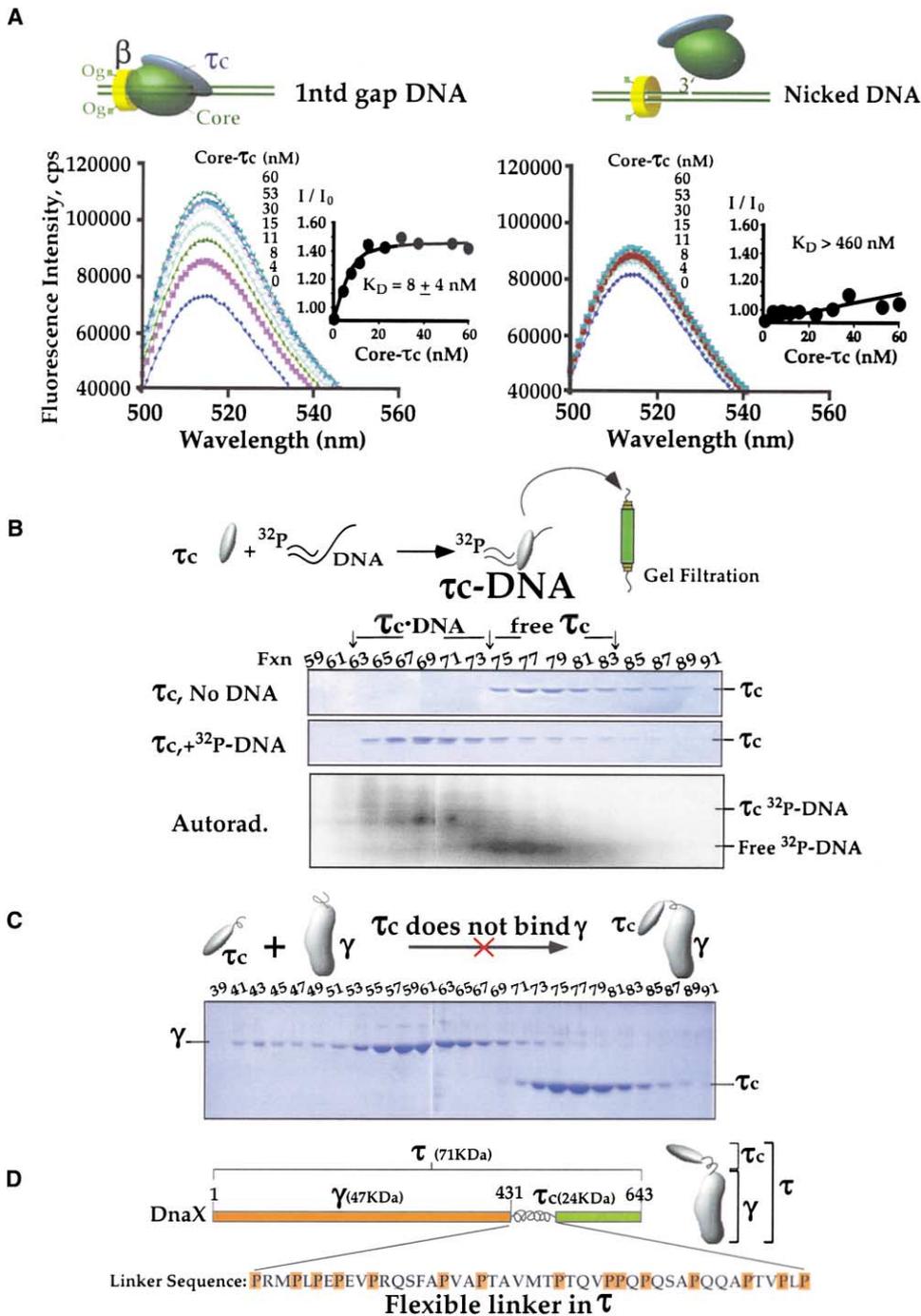
Figure 7. The C-Terminal Section of τ Activates the Processivity Switch

(A) The K_d of core·τ_c to β^{og} in the presence of primed DNA (left side) is much lower than in the presence of nicked DNA (right side).

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

(C) τ_c has no detectable affinity for γ upon analysis by gel filtration (Superose 12) at high concentration (100 μM τ_c 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 τ_c to γ. Flexibility is consistent with the many prolines in this sequence.

value of the core·τ_c interaction with β on primed DNA was 8 nM, similar to using full-length τ. This value rose to greater than 460 nM on the nicked DNA, demonstrating that τ_c contains the necessary information to trigger the processivity switch.

The processivity switch requires DNA recognition. To determine whether τ_c contains DNA binding activity, τ_c was mixed with ^{32}P-labeled primed DNA, and then the mixture was analyzed by gel filtration. Column fractions were analyzed by denaturing SDS-PAGE to identify τ_c,

and in a native gel followed by autoradiography to detect the ^{32}P-DNA (Figure 7B). The result shows that τ_c with DNA elutes earlier than τ_c without DNA, indicating formation of a τ_c·DNA complex. The autoradiogram of the native gel confirms that the ^{32}P-DNA comigrates with τ_c and that the ^{32}P-DNA·τ_c complex is retarded in the gel relative to unbound ^{32}P-DNA (Figure 7B).

Next, we asked whether τ_c binds to γ 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 τ. This experiment has been repeated in the presence of primed DNA and nicked duplex DNA, but γ and τ_c still do not comigrate (data not shown). Shown in Figure 7D is a sequence near the C-terminal region of γ that is disordered in the γ complex crystal structure, implying it may be a disordered peptide that links τ_c to the rest of τ. Consistent with disorder in the γ complex structure, the sequence contains many prolines and relatively few hydrophobic residues. This peptide is located near the C terminus of γ (see scheme in Figure 7D). Taken together with the lack of detectable binding between τ_c and γ, the structural data provide evidence that this peptide may act as a flexible tether that links τ_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 β 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 (~1 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 β 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 β 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 τ subunit of the clamp loader is responsible for modulating the core·β 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, τ does not disengage the β ring from the DNA polymerase. But when no ssDNA remains (i.e., a nick), the τ subunit severs the core·β interaction. Exactly how does τ sense the DNA structure and transduce this to a signal that modulates the affinity between core polymerase and β? 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 τ subunit has DNA binding activity, and we demonstrate here that the activity resides in the C-terminal sequences unique to τ (i.e., missing in γ) and thus is in the same general area as the core polymerase binding sequences of τ. It therefore seems quite possible that τ contains the DNA recognition activity that distinguishes the complete product DNA from primed template. A possible mechanism by which τ functions is illustrated in Figure 1B, in which the DNA binding site of τ is in close proximity to the primed template binding site of the α subunit of core polymerase, perhaps even forming a part of its substrate binding site. In this model, where τ is situated near the DNA binding site of α, 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 β. Alternatively, the DNA binding domain of τ 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 β on DNA, allowing τ to come into position and check whether the template is complete.

Core has considerable affinity for β, even at a nick. Thus one may ask whether core functions in the cell with β but without τ. We have examined this issue and find that core without τ is not as stable with β on DNA compared to core in the presence of τ (Stukenberg and O'Donnell, 1995). This is indicated by low yields of core on β·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_{off} of core from β·DNA relative to core·τ (core could have a more rapid k_{on} for β than core·τ). The decreased stability between core and β in the absence of τ may obviate the need for a processive switch when τ 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 τ, as the C-terminal sequences of τ are essential for cell viability (Blinkova et al., 1993). Therefore, a processive switch is needed to facilitate departure of Pol III* from its β 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_c, 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 holoenyzme, 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 moiety 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 Kornberg, 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; Kaboord 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 stabile 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% (v/v) glycerol, 0.1 mM EDTA, 40 μg/ml bovine serum albumin (BSA), 5 mM DTT, 1 mM ATP, and 8 mM MgCl$_2$. Buffer C is 20 mM Tris-HCl (pH 7.5), 8 mM MgCl$_2$, 4% (v/v) glycerol, 100 mM NaCl, 0.5 mM EDTA, 100 μg/ml BSA, and 2 mM DTT. Buffer D is 20 nM 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, δ′,χ, ψ, and θ (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), ^{3}H-Pol III*, core, and αε (Stukenberg and O'Donnell, 1995) were reconstituted from pure subunits and purified as described, except the ε subunit was mutated (D12A, E14A) to eliminate the 3′-5′ exonuclease activity

(Fijalkowska and Schaaper, 1996). β^{PK} contains a 6 residue C-terminal kinase site and was labeled (150 dpm/pmol) using $[\gamma\text{-}^{32}P]$-ATP and cAMP-dependent protein kinase (a gift from Dr. Susan Taylor, UCSD) (Naktinis et al., 1995). τ_c contains a kinase site (MGRRASVPG) just prior to Ser431 of τ. τ_c was expressed in 12 liters of BL21 (DE3) pET-τ_c cells following IPTG induction for 3 hr at 37°C. Cells were lysed using a French press, and τ_c was purified using an ammonium sulfate 60%–100% cut and ion exchange chromatography on SP-agarose and Fast Flow Q.

DNAs

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 6251–6381). 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 duplex was assembled upon mixing 8 nmol of DNA 62-mer (5′-ccc ATc gTA TAc gAA ggg AgT cgA cTg gcT Agc TAg cTA gcT cTA TAg cTA gcT Agc TAg cT-3′) and 40 nmol each of 28-mer (5′-ccA gTc gAc Tcc cTT cgT ATA cgA Tgg g-3′) and 34-mer (5′-Agc TAg cTA gcT Agc TAT AgA gcT Agc TAg cTA g-3′) in 100 μl 50 mM Tris-Cl (pH 7.5), 10 mM MgCl$_2$. The reaction was heated to 95°C, slowly cooled to 23°C, and then incubated 3 hr at 23°C. The reaction was adjusted to 60 mM Tris-HCl (pH 7.5), 20 mM MgCl$_2$, 50 mM NaCl$_2$, and 1 mM DTT in 110 μl and then purified in a 10% native polyacrylamide gel. The primed template was prepared as described above except only the 62-mer and 34-mer were used. The one-nucleotide gap DNA was prepared using the 62-mer, 28-mer, and a 33-mer that lacks the 3′ dG residue of the 34-mer.

Stability of Pol III* with β on DNA Gaps of Defined Size
Gapped DNA (3 pmol) was mixed with 7.4 pmol EBNA-1 (as dimer) and 22 pmol SSB (as tetramer) in 50 μl buffer B. Reactions were incubated at 37°C for 3 min, and then 1 pmol ^{3}H-Pol III* and 5 pmol ^{32}P-β were added. Reactions were incubated 5 min at 37°C, and then 25 μl was applied to 5 ml columns of Bio-Gel A15m equilibrated in buffer C at 25°C. Fractions of 180 μl were collected, and the amount of ^{3}H-Pol III* and ^{32}P-β was calculated from their known specific activities. The amounts of protein in the six peak fractions were added to obtain the total amount of either Pol III* or β bound to DNA. Recovery of total radioactive protein was typically 80%–95%. Results were not corrected for recoveries or the amount of reaction loaded on the column. Control reactions using Oligo #1 alone demonstrated that EBNA-1 completely blocked assembly of ^{3}H-Pol III* and ^{32}P-β.

To determine the effect of a single dNTP on ^{3}H-Pol III* and β at a one-nucleotide gap, 120 μM of either complementary dCTP or noncomplementary dGTP was added 3 min prior to analysis by gel filtration. To anneal a 15-mer in the reaction containing Pol III*/β at a 15 nucleotide gap, 0.25 U hexokinase and 150 nmol of glucose were added to remove ATP within 1 s as described (Turner et al., 1999). Then 20 pmol of either a matching or noncomplementary DNA 15-mer was added to fill the gap, followed by 3 min at 37°C before analysis by gel filtration. To determine whether ATP is required for the switch, ^{3}H-Pol III* and β were assembled onto the single-nucleotide gapped DNA, and then hexokinase (0.25 units) and glucose (150 nmol) were added to remove ATP. After 3 min at 37°C, either no nucleotide or 120 μM dCTP was added, incubated another 3 min, and then analyzed by gel filtration. To determine whether the last nucleotide must be incorporated for the processivity switch, we used an Oligo #2 terminated with a ddC residue at the 3′ end to produce a dideoxy terminated single-nucleotide gap. After ^{3}H-Pol III* and β were assembled onto the DNA, either no nucleotide or 120 μM dCTP was added, followed by incubation for 3 min at 37°C before analysis by gel filtration.

Gel Filtration Analysis on Short Linear DNA Templates
Proteins and linear DNA substrates were incubated at 15°C in 50 μl of buffer C for 45 min. Reactions contained 350 μM core (αε), 75 pmol β$_2$ (82 kDa), and 3 nmol DNA (either primed or nicked). Where indicated, reactions also contained 600 pmol of either τ$_4$, γ$_4$, δ, δ′, or χψ. Reactions were injected onto a Superose 12 column HR 3.2/30 using an FPLC Smart System preequilibrated at 22°C in buffer C containing 100 mM NaCl. After the first 600 μl, 60 fractions of 30 μl each were collected. Proteins were analyzed in a 12% SDS polyacrylamide gel.

K_d Values Using Fluorescent β^{og}
β can be uniquely labeled at Cys333 using maleimide derivatives (Griep and McHenry, 1988). β (3 mg) was labeled using Oregon Green 488 maleimide (Molecular Probes) in 1 ml of 50 mM potassium phosphate (pH 7.5) and 100 mM NaCl. The Oregon Green maleimide (1 pmol) was dissolved in 100 μl DMSO, and then 80 μl was added to β with gentle stirring at 4°C, followed by overnight incubation at 4°C in the dark. β^{og} was separated from unreacted reagent on a 50 ml column of BioGel 6 developed in buffer D. The β^{og} contained ~0.91 molecules of Oregon Green per β monomer as determined from protein absorbance at 280 nm (ϵ_{280} = 14,890 M^{-1} cm^{-1}) and Oregon Green at 490 nm (ϵ_{491} = 76,000 M^{-1} cm^{-1}).

Titration of core into β^{og} on synthetic gel purified DNAs was performed as follows. Reactions contained 12 nM β$_2$^{og}, 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 × 3 mm cuvette. Excitation was at 490 nm, and emission was monitored from 500−600 nm in a PTI Quantamaster 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·τ interaction with β^{og} was performed as described above with the exception that 0.36 nmol core was incubated with 0.72 nmol τ at 15°C for 15 min before use.

Surface Plasmon Resonance
Duplex DNA (5′-GGG TAG CAT ATG CTT CCC GAA TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA CTG GGA AAA CCC TGG CGT TAC CCA ACT T-3′) containing a 10 nucleotide gap was constructed upon mixing 0.5 nmol of a gel-purified 5′ biotin end-labeled 79-mer, 5 nmol of a 39-mer (5′-AAA ACG ACG GCC AGT GAA TTC GGG AAG CAT ATG CTA CCC-3′) that anneals to the 5′ sequence of the 79-mer, and 5 nmol of a 30-mer (5′-AAG TTG GGT AAC GCC AGG GTT 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. The resulting substrate contains a 10 nucleotide 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 III* (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 β·DNA was determined using the BiaEvaluation 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.

References

Alberts, B.M., Barry, J., Bedinger, P., Formosa, T., Jongeneel, C.V., and Kreuzer, K.N. (1983). Studies on DNA replication in the bacteriophage T4 in vitro system. Cold Spring Harb. Symp. Quant. Biol. *47*, 655–668.

Blinkova, A., Hervas, C., Stukenberg, P.T., Onrust, R., O'Donnell, M.E., and Walker, J.R. (1993). The *Escherichia coli* DNA polymerase III holoenzyme contains both products of the *dnaX* gene, τ and γ, but only τ is essential. J. Bacteriol. *175*, 6018–6027.

Bruck, I., and O'Donnell, M. (2000). Reconstitution of the DNA replication apparatus of *Streptococcus pyogenes*, a gram positive organism. J. Biol. Chem. *275*, 28971–28983.

Bruck, I., Yuzhakov, A., Yurieva, O., Jeruzalmi, D., Skangalis, M., Kuriyan, J., and O'Donnell, M. (2002). Analysis of a multicomponent thermostable DNA polymerase III replicase from an extreme thermophile. J. Biol. Chem. *277*, 17334–17348.

Davey, M., Jeruzalmi, D., Kuriyan, J., and O'Donnell, M. (2002). Motors and switches: AAA+ machines within the replisome. Nat. Rev. Mol. Cell Biol. *3*, 826–835.

Fijalkowska, I.J., and Schaaper, R.M. (1996). Mutants in the Exo I motif of *Escherichia coli dnaQ*: defective proofreading and inviability due to error catastrophe. Proc. Natl. Acad. Sci. USA *93*, 2856–2861.

Fu, T.J., Sanders, G.M., O'Donnell, M., and Geiduschek, E.P. (1996). Dynamics of DNA-tracking by two sliding-clamp proteins. EMBO J. *15*, 4414–4422.

Gao, D., and McHenry, C. (2001a). τ binds and organizes *Escherichia coli* replication proteins through distinct domains: partial proteolysis of terminally tagged τ to determine candidate domains and to assign domain V as the α binding domain. J. Biol. Chem. *276*, 4433–4440.

Gao, D., and McHenry, C. (2001b). τ binds and organizes *Escherichia coli* replication proteins through distinct domains: domain IV located within the unique C terminus of τ, binds the replication fork helicase, DnaB. J. Biol. Chem. *276*, 4441–4446.

Glover, B.P., and McHenry, C.S. (2001). The DNA polymerase III holoenzyme: an asymmetric dimeric replicative complex with leading and lagging strand polymerases. Cell *105*, 925–934.

Griep, A., and McHenry, C. (1988). The dimer of the β subunit of *Escherichia coli* DNA polymerase III holoenzyme is dissociated into monomers upon binding magnesium (II)+. Biochemistry *27*, 5210–5215.

Gulbis, J.M., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996). Structure of the C-terminal region of p21waf1/cip1 complexed 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 each Okazaki fragment. J. Biol. Chem. *269*, 24221–24228.

Herendeen, D.R., Kassavetis, G.A., and Geiduschek, E.P. (1992). A transcriptional enhancer whose function imposes a requirement that proteins track along DNA. Science *256*, 1298–1303.

Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. (2001). Crystal structure of the processivity clamp loader gamma (γ) complex of *E. coli* DNA polymerase III. Cell *106*, 429–441.

Kaboord, B.F., and Benkovic, S.J. (1995). Accessory proteins function as matchmakers in the assembly of the T4 DNA polymerase holoenzyme. Curr. Biol. *5*, 149–157.

Kelman, Z., and O'Donnell, M. (1995). DNA polymerase III holoenzyme: structure and function of a chromosomal replicating machine. Annu. Rev. Biochem. *64*, 171–200.

Kim, S., Dallmann, H.G., McHenry, C.S., and Marians, K.J. (1996). Coupling of a replicative polymerase and helicase: a τ-DnaB interaction mediates rapid replication fork movement. Cell *84*, 643–650.

Kong, X.P., Onrust, R., O'Donnell, M., and Kuriyan, J. (1992). Three-dimensional structure of the β subunit of *E. coli* DNA polymerase III holoenzyme: a sliding DNA clamp. Cell *69*, 425–437.

Kornberg, A., and Baker, T. (1992). DNA Replication, Second Edition (New York: W.H. Freeman and Company).

Krishna, T.S.R., Kong, X.-P., Gary, S., Burgers, P., and Kuriyan, J. (1994). Crystal structure of the eukaryotic DNA polymerase processivity factor PCNA. Cell *79*, 1233–1243.

Kuriyan, J., and O'Donnell, M. (1994). Sliding clamps of DNA polymerases. J. Mol. Biol. *234*, 915–925.

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 polymerase III. Identification of τ as a subunit of the DNA polymerase III holoenzyme. J. Biol. Chem. *257*, 2657–2663.

Munn, M.M., and Alberts, B.M. (1991). The T4 DNA polymerase accessory proteins form an ATP-dependent complex on a primer-template junction. J. Biol. Chem. *266*, 20024–20033.

Naktinis, V., Onrust, R., Fang, L., and O'Donnell, M. (1995). Assembly 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. Biol. Chem. *270*, 13358–13365.

Naktinis, V., Turner, J., and O'Donnell, M. (1996). A molecular switch in a replication machine defined by an internal competition for protein rings. Cell *84*, 137–145.

O'Donnell, M.E. (1987). Accessory proteins bind a primed template and mediate rapid cycling of DNA polymerase III holoenzyme from *Escherichia coli*. J. Biol. Chem. *262*, 16558–16565.

O'Donnell, M., and Kornberg, A. (1985). Dynamics of DNA polymerase III holoenzyme of *Escherichia coli* in replication of a multiprimed template. J. Biol. Chem. *260*, 12875–12883.

Onrust, R., and O'Donnell, M. (1993). DNA polymerase III accessory 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. (1995). Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader, and sliding clamps in one holoenzyme particle. III. Interface between two polymerases and the clamp loader. J. Biol. Chem. *270*, 13366–13377.

Stewart, J. (2001). Dynamics of the DNA pol III holoenzyme of *E. coli*: β clamp opening by the δ subunit and dissociation of the pol from replicated DNA. Ph.D. thesis, Rockefeller University, New York.

Stillman, B. (1994). Smart machines at the DNA replication fork. Cell *78*, 725–728.

Studwell-Vaughan, P.S., and O'Donnell, M. (1991). Constitution of the twin polymerase of DNA polymerase III holoenzyme. J. Biol. Chem. *266*, 19833–19841.

Stukenberg, P.T., and O'Donnell, M. (1995). Assembly of a chromosomal 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.

Stukenberg, P.T., Studwell-Vaughan, P.S., and O'Donnell, M. (1991). Mechanism of the sliding β-clamp of DNA polymerase III holoenzyme. J. Biol. Chem. *266*, 11328–11334.

Stukenberg, P.T., Turner, J., and O'Donnell, M. (1994). An explanation for lagging strand replication: polymerase hopping among DNA sliding clamps. Cell *78*, 877–887.

Tsurimoto, T., and Stillman, B. (1991). Replication factors required for SV40 DNA replication in vitro. I. DNA structure-specific recognition of a primer-template junction by eukaryotic DNA polymerases and their accessory proteins. J. Biol. Chem. *266*, 1950–1960.

Turner, J., Hingorani, M.M., Kelman, Z., and O'Donnell, M. (1999). The internal workings of a DNA polymerase clamp-loading machine. EMBO J. *18*, 771–783.

Waga, S., and Stillman, B. (1994). Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. Nature *369*, 207–212.

Yao, N., Turner, J., Kelman, Z., Stukenberg, P.T., Dean, F., Shechter, D., Pan, Z.Q., Hurwitz, J., and O'Donnell, M. (1996). Clamp loading, unloading and intrinsic stability of the PCNA, β and gp45 sliding clamps of human, *E. coli* and T4 replicases. Genes Cells *1*, 101–113.

Yao, N., Leu, F.P., Anjelkovic, J., Turner, J., and O'Donnell, M. (2000). DNA structure requirements for the *Escherichia coli* γ complex clamp loader and DNA polymerase III holoenzyme. J. Biol. Chem. *275*, 11440–11450.

Yuzhakov, A., Kelman, Z., Hurwitz, J., and O'Donnell, M. (1999). Multiple competition reactions for RPA order the assembly of the DNA polymerase δ holoenzyme. EMBO J. *18*, 6189–6199.