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Characterization of a Triple DNA Polymerase Replisome

Peter McInerney,[1,2,3] Aaron Johnson,[1,2,4] Francine Katz,[1,2] and Mike O'Donnell[1,2,*]
[1] Laboratory of DNA Replication
[2] Howard Hughes Medical Institute
The Rockefeller University, 1230 York Avenue, Box 228, New York, NY 10021, USA
[3] Present address: Helicos Biosciences Corp., Cambridge, MA 02139, USA.
[4] Present address: Harvard Medical School, Boston, MA 02115, USA.
*Correspondence: odonnel@mail.rockefeller.edu
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SUMMARY

The replicase of all cells is thought to utilize two DNA polymerases for coordinated synthesis of leading and lagging strands. The DNA polymerases are held to DNA by circular sliding clamps. We demonstrate here that the *E. coli* DNA polymerase III holoenzyme assembles into a particle that contains three DNA polymerases. The three polymerases appear capable of simultaneous activity. Furthermore, the trimeric replicase is fully functional at a replication fork with helicase, primase, and sliding clamps; it produces slightly shorter Okazaki fragments than replisomes containing two DNA polymerases. We propose that two polymerases can function on the lagging strand and that the third DNA polymerase can act as a reserve enzyme to overcome certain types of obstacles to the replication fork.

INTRODUCTION

Multiprotein replisome complexes consist minimally of two DNA polymerases, one for each strand of duplex DNA, and a clamp loader complex that loads circular sliding clamps onto DNA for the two DNA polymerases (Benkovic et al., 2001; Johnson and O'Donnell, 2005; McHenry, 2003; Nossal, 1994). Replisomes also contain a helicase that separates the parental duplex and a primase that synthesizes short RNA oligomers that initiate lagging strand Okazaki fragments (Kornberg and Baker, 1992). In *E. coli*, the clamp loader organizes these replisome components by forming tight connections to the two DNA polymerase III (Pol III) core heterotrimers ($\alpha\varepsilon\theta$ subunits) and to DnaB helicase. The clamp loader is composed of several different subunits, but only the τ subunit of the clamp loader connects to Pol III core and DnaB helicase (Gao and McHenry, 2001a; Kim et al., 1996; Studwell-Vaughan and O'Donnell, 1991). Therefore the clamp loader must contain at least two τ subunits in order to bind two Pol III cores for coordinated replication of leading and lagging strands.

The τ subunit (71 kDa) is encoded by *dnaX*, which also encodes the γ subunit (47 kDa) (see Figure 1A). The τ subunit is the full-length product of *dnaX*, whereas γ is truncated by a translational frameshift that almost immediately results in a stop codon. Of the five domains that comprise τ, the N-terminal three domains are shared with γ. The two domains unique to τ bind DnaB (domain IV) and Pol III core (domain V) (Gao and McHenry, 2001a; Gao and McHenry, 2001b). Domains I and II of γ and τ are homologous to the AAA+ ATP binding motif present in a large family of ATPases that typically function to remodel other proteins (Erzberger and Berger, 2006; Jeruzalmi et al., 2001). The *E. coli* clamp loader also requires one copy each of δ and δ', which are AAA+ proteins but do not bind ATP. Domain III of γ/τ, δ, and δ' mediate protein-protein contacts to form a $(\tau/\gamma)_3\delta\delta'$ circular oligomer (Jeruzalmi et al., 2001). The remaining clamp loading subunits, χ and ψ, are not required for clamp loader activity, but they stabilize the clamp loader complex and interact with SSB (Bloom, 2006; Glover and McHenry, 1998; Kelman et al., 1998; Olson et al., 1995). The γ and τ subunits are both capable of assembling with the δ, δ', χ, and ψ subunits to form active clamp loader complexes with stoichiometries of γ_3-$\delta\delta'\chi\psi$ and τ_3-$\delta\delta'\chi\psi$ (Onrust et al., 1995b; Pritchard et al., 2000).

The *E. coli* chromosomal replicase, DNA Pol III holoenzyme, is thought to contain one clamp loader of stoichiometry $\tau_2\gamma_1$-$\delta\delta'\chi\psi$, because two τ subunits must be present to bind two molecules of Pol III core for leading- and lagging-strand synthesis (McHenry, 2003; O'Donnell et al., 2001). However, it is also conceivable that a clamp loader with three τ subunits (τ_3-$\delta\delta'\chi\psi$) functions at a replication fork, as it should bind at least two Pol III cores and may even bind three polymerases. Indeed, cells in which *dnaX* is mutated to prevent production of γ are viable (Blinkova et al., 1993) and presumably produce a replicase having a clamp loader with three τ subunits. Further, many bacterial *dnaX* genes produce only τ. Therefore we wished to examine whether an *E. coli* Pol III replicase with three τ subunits truly contains three Pol III cores and to compare its action at a replication fork to a replicase with two Pol III cores.

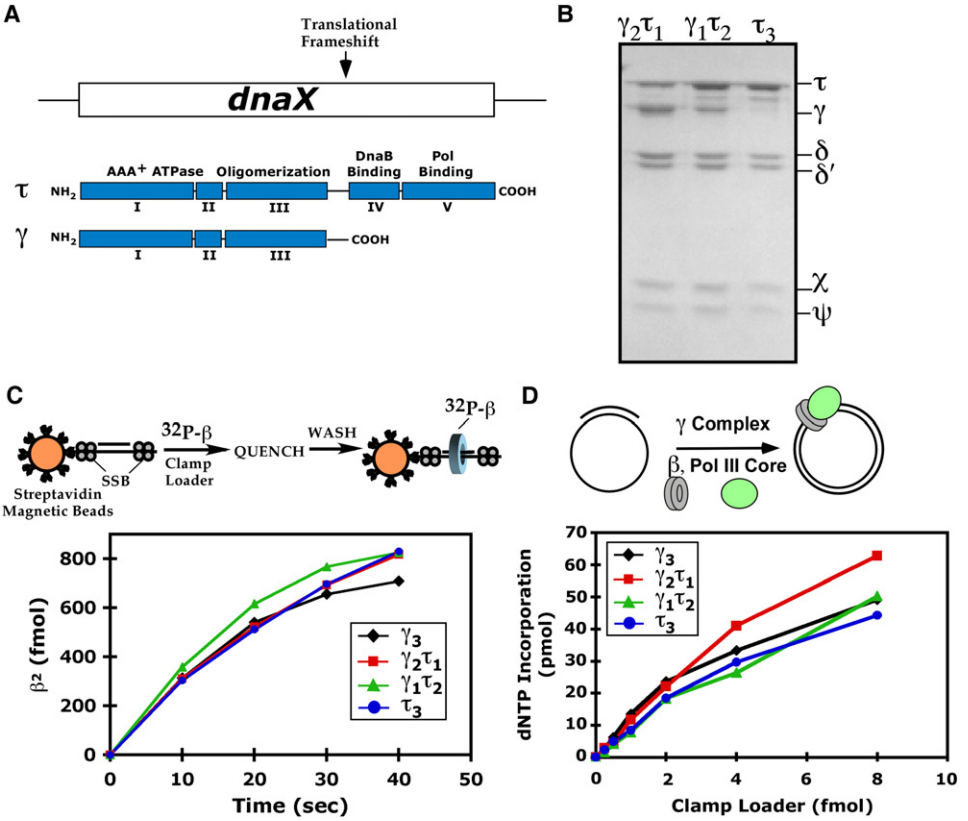


Figure 1. Reconstitution and Activity of Mixed γ/τ Complexes

(A) The τ and γ subunits are both produced by the *dnaX* gene. τ is the full-length product, and γ is produced by a translational frameshift. τ contains five domains: domains I–III are the clamp loader domains (which γ also contains), domain IV binds DnaB helicase, and domain V binds the α subunit of Pol III core.

(B) SDS-PAGE of reconstituted clamp loader complexes (1.5 μg each lane).

(C) Clamp loading rate was determined by using a primed template conjugated to magnetic beads.

(D) Clamp loader complexes were titrated into β-dependent Pol III core replication assays with singly primed M13 mp18 ssDNA.

The main line of evidence that Pol III holoenzyme of *E. coli* contains at least one γ subunit, and therefore only two τ subunits, is the presence of γ in Pol III holoenzyme preparations (McHenry and Kornberg, 1977). However, τ is easily proteolyzed to a form indistinguishable in size to γ and may account for the observed γ-like protein in Pol III holoenzyme preparations (Pritchard et al., 1996). In fact, preparations of τ suffer from proteolysis even in *ompT* protease-deficient cells (M. Skangalis and M.O.D., unpublished data). Although a form of Pol III holoenzyme that contains both γ and τ can be reconstituted in vitro, elaborate subunit order of addition schemes must be followed (Onrust et al., 1995b; Pritchard and McHenry, 2001). This is because γ and τ are homo-oligomers that do not stably exchange subunits as evidenced by ability to purify homo-oligomers of γ and τ from cells that coexpress γ and τ simultaneously, but inability to purify stabile γ/τ hetero-oligomers (Onrust et al., 1995b; Pritchard et al., 2000). Clamp loaders that appear to contain both γ and τ are formed by high-level coexpression of all five genes (Pritchard et al., 2000), but proteolysis of τ could still provide the source of γ in these complexes, especially because no τ_3-δδ′χψ complex is obtained, consistent with conversion of τ to γ by proteolysis. Alternatively, cotranslation of γ and τ on the same ribosome during artificially high-level gene expression may result in γ/τ hetero-oligomers that do not form during normal low-level expression of *dnaX*.

In the current study, we reconstitute all four permutations of clamp loaders that contain γ and/or τ (i.e., clamp loaders containing either γ_3-δδ′χψ, $\tau_1\gamma_2$-δδ′χψ, $\tau_2\gamma_1$-δδ′χψ, or τ_3-δδ′χψ). We find that they are all equally active in loading β onto primed DNA. Analysis of the τ_3-δδ′χψ clamp loader shows that it binds a total of three molecules of Pol III core. Moreover, active-site titration analysis indicates that all three Pol III core molecules can function simultaneously within this complex. We also demonstrate that the trimeric Pol III_3-τ_3-δδ′χψ complex assembles into replisomes and is functional with DnaB helicase, primase, and β sliding clamps. Comparisons of dimeric and trimeric DNA polymerase replisomes indicate that leading-strand extension by the trimeric polymerase is at

least as fast as the dimeric polymerase. Analysis of lagging-strand synthesis demonstrates that the trimeric polymerase replisome produces slightly shorter Okazaki fragments compared to the dimeric polymerase replisome, consistent with more efficient use of RNA primers by an additional DNA polymerase. Implications of these findings are explored further in the Discussion.

RESULTS

Characterization of Four Different Clamp Loader Species

The four different *E. coli* clamp loader complexes, γ_3-$\delta\delta'\chi\psi$, $\gamma_2\tau_1$-$\delta\delta'\chi\psi$, $\gamma_1\tau_2$-$\delta\delta'\chi\psi$, and τ_3-$\delta\delta'\chi\psi$, are characterized in the experiments of Figure 1. The γ_3-$\delta\delta'\chi\psi$ and τ_3-$\delta\delta'\chi\psi$ complexes are reconstituted by simply mixing the subunits together, followed by purification of the complex from unassembled subunits by chromatography on Mono Q (Onrust et al., 1995a). The τ_3-$\delta\delta'\chi\psi$ complex preparation is shown in Figure 1B. Both γ and τ are stabile homo-oligomers, and formation of complexes containing both γ and τ ($\gamma_2\tau_1$-$\delta\delta'\chi\psi$ and $\gamma_1\tau_2$-$\delta\delta'\chi\psi$) requires preincubating γ and τ together at a high concentration for over an hour (Onrust et al., 1995b). Then δ, δ', χ, and ψ are added, and the amounts of $\gamma_2\tau_1$-$\delta\delta'\chi\psi$ and $\gamma_1\tau_2$-$\delta\delta'\chi\psi$ complexes that form can be resolved by chromatography on Mono S (McInerney and O'Donnell, 2004; Pritchard et al., 2000). The preparations used here are shown in Figure 1B.

In Figure 1C, we developed a magnetic bead-based assay to accurately compare rates at which the four different clamp loader complexes operate (see the scheme in Figure 1C). In this assay, a synthetic 102-mer DNA oligonucleotide, labeled with biotin at the 3′ terminus, is primed with a 30-mer DNA and then conjugated to streptavidin-coated magnetic beads. The bead-DNA conjugate is then preincubated with *E. coli* SSB to prevent β clamps from sliding off the end of the DNA after they are loaded. To follow β loading, β is ^{32}P labeled using a six residue C-terminal kinase recognition site (Kelman et al., 1995). Clamp loading reactions are initiated upon adding clamp loader complex and then quenched at the indicated times with EDTA, which halts the ATP-dependent clamp loader by removing magnesium. ^{32}P-β that is clamped onto DNA is then quantitated by separating ^{32}P-β on DNA from unassociated ^{32}P-β using a magnetic concentrator. The results show that the four different clamp loaders have very similar rates of β loading activity. Therefore the C-terminal 24 kDa unique to τ has little or no effect on the basic clamp loading reaction.

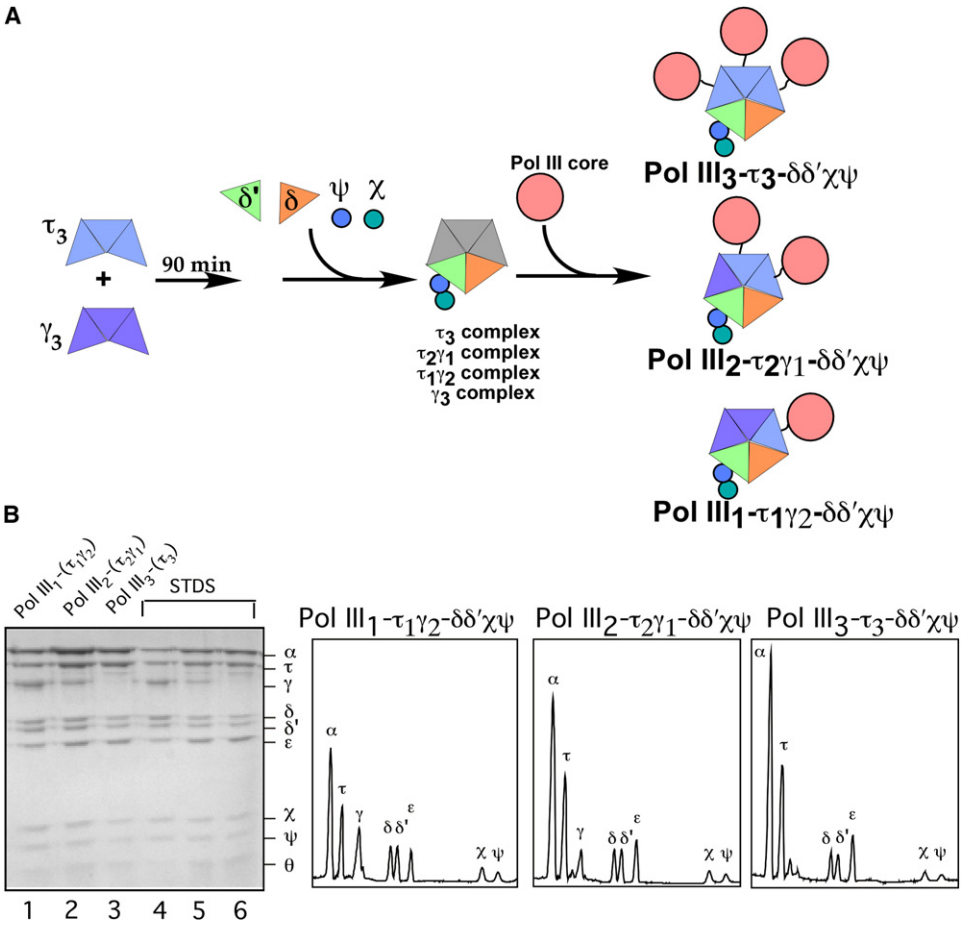
Next we tested the four different clamp loaders in replication assays with Pol III core and a singly primed 7.2 kb M13 mp18 bacteriophage single-strand DNA (ssDNA) substrate. Pol III core absolutely requires β to produce full-length 7.2 kb RFII duplex product (Fay et al., 1981). One may predict that the different clamp loaders should be equally active with both β and Pol III core because they have similar clamp loading rates. As expected, the results show that all four clamp loaders are equally active with β and Pol III core (Figure 1D). The τ_3-$\delta\delta'\chi\psi$ clamp loader has been shown previously to function with ATPγS, whereas γ_3-$\delta\delta'\chi\psi$ does not (Glover and McHenry, 2001). We find here that ATPγS is used equally well by the $\tau_1\gamma_2$-$\delta\delta'\chi\psi$, $\tau_2\gamma_1$-$\delta\delta'\chi\psi$, and τ_3-$\delta\delta'\chi\psi$ clamp loaders in primed M13 mp18-based replication assays with Pol III core; γ_3-$\delta\delta'\chi\psi$ is inactive (see Figure S1 in the Supplemental Data available with this article online). This result indicates that only one τ subunit is needed for activity with ATPγS.

Three Pol III Cores Bind the τ_3-$\delta\delta'\chi\psi$ Clamp Loader Complex

The Pol III replicase only requires two molecules of Pol III core to replicate both strands of duplex DNA (Yuzhakov et al., 1996; Zechner et al., 1992). It is therefore possible that only two Pol III cores bind to the τ_3-$\delta\delta'\chi\psi$ clamp loader, even though three τ subunits are present. For example, binding of a third Pol III core may be sterically occluded by the first two Pol III cores. To determine the number of Pol III core molecules that bind to the τ_3-$\delta\delta'\chi\psi$ clamp loader, a 6-fold molar excess of Pol III core was added to the clamp loader to ensure that all "available" τ subunits are saturated. Pol III-clamp loader complexes were resolved from excess Pol III core by chromatography on Mono Q (see scheme in Figure 2A). As controls, similar reconstitution reactions were performed with the $\tau_1\gamma_2$-$\delta\delta'\chi\psi$ and the $\tau_2\gamma_1$-$\delta\delta'\chi\psi$ complexes. Laser densitometry analysis of the Coomassie blue-stained SDS polyacrylamide gels, using subunits of defined concentrations as standards, revealed that one Pol III core was present per $\tau_1\gamma_2$-$\delta\delta'\chi\psi$ complex, two Pol III cores were bound to the $\tau_2\gamma_1$-$\delta\delta'\chi\psi$ complex, and that three Pol III cores bind to τ_3-$\delta\delta'\chi\psi$ (Figures 2B and 2C).

The Three DNA Polymerases Appear Active in the Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ Complex

Because only two DNA polymerases are needed for concurrent replication of both strands of duplex DNA, it is possible that only two Pol III cores in the Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex may be active at a given time. To test this possibility, we designed an active-site titration assay and examined the number of active Pol III cores present in the Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex. Results were compared to those obtained with monomeric Pol III$_1$-$\tau_1\gamma_2$-$\delta\delta'\chi\psi$ and dimeric Pol III$_2$-$\tau_2\gamma_1$-$\delta\delta'\chi\psi$ complexes. In the initial preincubation with ATP and only two dNTPs, the Pol III cores that are active should assemble with β-DNA complexes (see scheme in Figure 3). A burst of replication is then initiated upon adding the remaining two dNTPs, followed by quenching the reaction after 20 s, sufficient time to produce one 7.2 kb RFII duplex product per active DNA polymerase. If only two Pol III cores in the Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex are active, it should produce a similar amount of RFII product as the Pol III$_2$-$\tau_2\gamma_1$-$\delta\delta'\chi\psi$ complex. However, the results show that the Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex consistently produces about 2.5 molecules of RFII compared to 1.6



Stoichiometry

	α	ε	τ	γ	δδ'	χ	ψ
Pol III_1-($\tau_1\gamma_2$) complex	0.96 ± .16	0.93 ± .26	(1)	1.74 ± .20	0.95 ± .16	1.09 ± .12	1.06 ± .13
Pol III_2-($\tau_2\gamma_1$) complex	1.78 ± .24	1.66 ± .29	(2)	0.78 ± .14	0.99 ± .25	1.32 ± .04	1.06 ± .07
Pol III_3-(τ_3) complex	2.76 ± .44	2.64 ± .83	(3)	N.A.	1.26 ± .11	1.18 ± .24	1.07 ± .28

Figure 2. Reconstitution of a Replicase Containing Three Pol III Cores

(A) Strategy to reconstitute Pol III-clamp loader complexes. First, $\tau_1\gamma_2$-δδ′χψ complex, $\tau_2\gamma_1$-δδ′χψ complex, and τ_3-δδ′χψ complex were reconstituted and purified. Clamp loader complexes were then mixed with a molar excess of Pol III core (2-fold over τ protomers) followed by removing unattached Pol III core by chromatography on a Mono Q column (see the Experimental Procedures for details).

(B) Coomassie blue-stained SDS polyacrylamide gel of purified reconstituted Pol III-clamp loader complexes (left). The first three lanes show the different reconstituted Pol III-clamp loader species (1.5–2 μg/lane). For comparison, lanes 4–6 are mixtures of pure subunits in the molar ratios: $(\alpha\varepsilon\theta)_1$-$\tau_1\gamma_2$-δδ′χψ, $(\alpha\varepsilon\theta)_2$-$\tau_2\gamma_1$-δδ′χψ, and $(\alpha\varepsilon\theta)_3$-$\tau_3$-δδ′χψ, respectively, determined from their known molar extinction coefficients at 280 nm in 6M guanidine hydrochloride. The three panels to the right of the gel are laser densitometer traces of purified Pol III-clamp loader complexes.

(C) Subunit stoichiometries were determined from quantitation of laser densitometer traces from three independent reconstitution experiments for each of the three Pol III-clamp loader complexes. The molar ratio of each subunit was calculated from their known molecular mass and normalized to the number of τ subunits in the clamp loader species used in the reconstitution.

molecules of RFII for the Pol III_2-$\tau_2\gamma_1$-δδ′χψ complex. We also analyzed the single Pol III_1-$\tau_1\gamma_2$-δδ′χψ complex in these experiments. As expected, the dimeric Pol III_2-$\tau_2\gamma_1$-δδ′χψ complex produced about twice the amount of RFII product compared to the monopolymerase Pol III_1-$\tau_1\gamma_2$-δδ′χψ complex. The somewhat lower amounts of RFII molecules produced relative to the total number of DNA polymerases present in each complex may be explained by some inactive polymerase present in the preparations. Overall, these results indicate that the three Pol III cores in the trimeric Pol III_3-τ_3-δδ′χψ complex can extend primed sites at the same time.

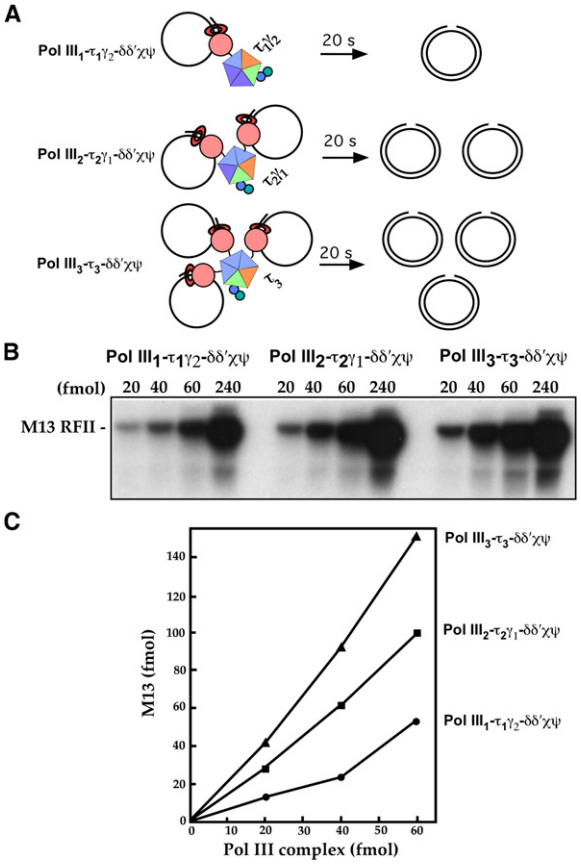


Figure 3. The Three Pol III Cores within the Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ Complex Appear Capable of Simultaneous Activity

(A) Each Pol III-clamp loader species was assembled onto an excess of SSB-coated singly primed M13 mp18 ssDNA (170 fmol as circles) with ATP and only two dNTPs and then allowed to form duplex RFII products in a 20 s burst of synthesis initiated upon adding remaining dNTPs.

(B) Autoradiogram of RFII products formed upon adding increasing amounts of each species of Pol III-clamp loader complex.

(C) Quantitation of duplex circles produced in the first three lanes of each titration. The fourth lane was excluded because substrate was substoichiometric to total Pol III-clamp loader complex.

The Triple Polymerase Functions at a Replication Fork

To address the functionality of a three-polymerase complex at a replication fork, we reconstituted replisomes on synthetic minicircle tailed duplex substrates with DnaB-helicase, DnaG-primase, and either the dimeric Pol III$_2$-$\tau_2\gamma_1$-$\delta\delta'\chi\psi$ complex or the trimeric Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex (see Figure 4). The synthetic minicircle substrate lacks dA residues on one strand and lacks dT residues on the other. Hence, leading and lagging strands can be specifically labeled in separate reactions with either α-^{32}P-dATP or α-^{32}P-dTTP. The results show that both dimeric Pol III$_2$-$\tau_2\gamma_1$-$\delta\delta'\chi\psi$ and trimeric Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ replisomes are equally functional on both strands of a replication fork (Figure 4A). Interestingly, the trimeric polymerase replisome produces slightly smaller Okazaki fragments (average of 1.9 Kb) than the dimeric polymerase replisome (average of 2.3 Kb). The smaller Okazaki fragments may be the result of more efficient use of RNA primed sites by the triple-polymerase replisome compared to the dimeric replicase. Alternatively, primase may target the triple-polymerase replisome more frequently than the dimeric replisome. Another possibility is that the third polymerase removes a degree of freedom form the second polymerase, which enables it to find the primer more efficiently. A third possibility is that the τ_3-$\delta\delta'\chi\psi$ clamp loader is more efficient at loading the polymerase/clamp onto the RNA primer compared to the $\tau_2\gamma_1$-$\delta\delta'\chi\psi$ clamp loader within the context of the replisome.

In the experiment of Figure 4B, we compared replication fork speeds of the dimeric and trimeric polymerase replisomes. Replisomes were assembled on the minicircle TF II DNA in a preincubation reaction in the absence of two dNTPs, and then replication was initiated upon adding the remaining two dNTPs and rapid timed aliquots were collected and analyzed in an alkaline agarose gel. The results clearly show that the rate of leading-strand extension is essentially the same for the dimeric and trimeric polymerase replisomes. Comparison of the leading edge of the DNA products to size standards in the gel yields maximal fork movement rates of ~600 nt/s.

We also examined the leading-strand rate by using an equivalent amount (100 fmol) of the monopolymerase Pol III$_1$-$\tau_1\gamma_2$-$\delta\delta'\chi\psi$ complex at a replication fork (Figure 4B). Interestingly, the results show that the monopolymerase replisome drives replication fork progression at essentially the same rate as the dimeric and trimeric DNA polymerase replisomes. This result indicates that only one τ subunit is needed for productive coupling with DnaB helicase for rapid DNA unwinding. However, we note that the monopolymerase gives a weaker signal than observed with the di- and tripolymerase complexes. This presumably results from less efficient DNA template utilization, perhaps due to a weaker interaction between the DnaB and the single τ subunit within the monopolymerase Pol III$_1$-$\tau_1\gamma_2$-$\delta\delta'\chi\psi$ complex.

The Three DNA Polymerases Remain Attached upon Assembly into the Replisome

To address whether all three Pol III cores remain attached to the replisome, or if one Pol III core dissociates, we reconstituted a doubly labeled ^{32}P/^{3}H-(Pol III)$_3$-$\tau_3\delta\delta'\chi\psi$ containing ^{32}P-Pol III core and ^{3}H-$\tau_3\delta\delta'\chi\psi$ of known molar-specific activities. A ~3-fold molar excess of ^{32}P/^{3}H-(Pol III)$_3$-$\tau_3\delta\delta'\chi\psi$ was mixed with β, DnaB, DnaG, SSB, and a 7.2 Kb M13 mp18 TF II DNA substrate and allowed to enter rolling circle synthesis for 5 min before analysis by gel filtration on BioGel A15M (Figure 5). This large pore resin excludes the large DNA (fractions 10–15), and protein bound to it, from unassociated protein (fractions 16–30). The result shows that the molar ratio of ^{32}P-Pol III core to ^{3}H-$\tau_3\delta\delta'\chi\psi$ in the fractions containing DNA is ~3:1 (3.08:1.0 when averaged over fractions 10–15); the

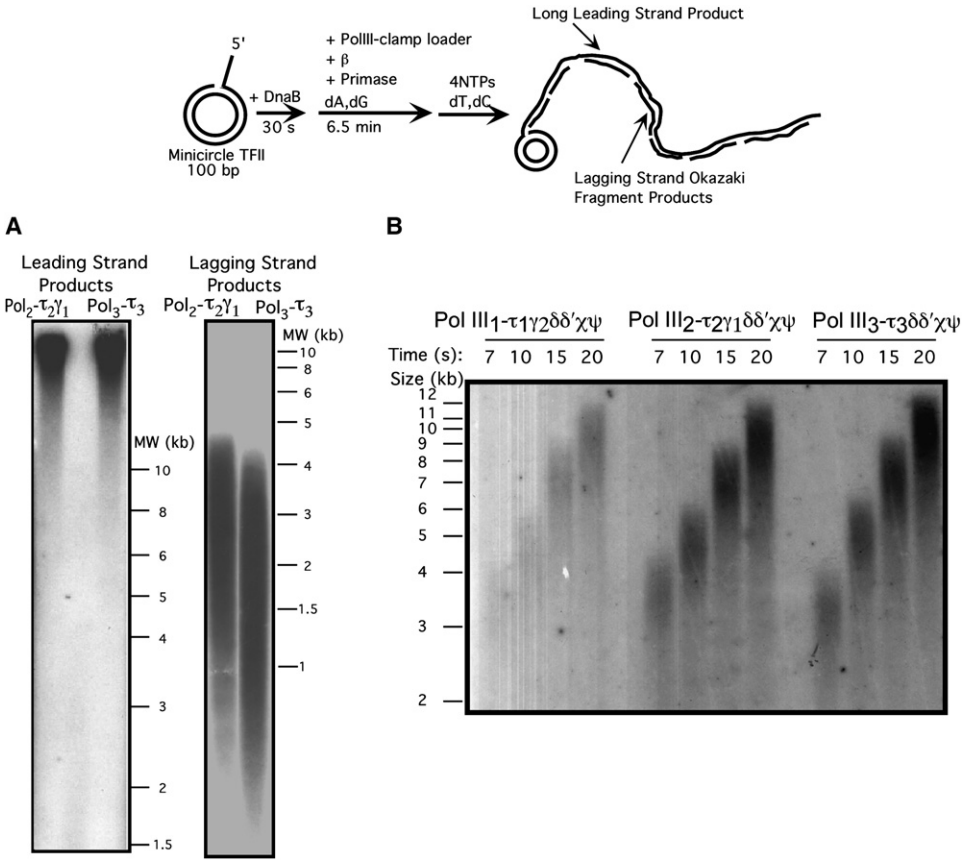


Figure 4. The Triple Pol III Replisome Is Active at a Replication Fork

The scheme at the top summarizes the assembly reaction with the minicircle tailed-form II (TF II) DNA template. TF II DNA consists of a 100-mer duplex circle and 40-mer 5′ tail comprised of only three of the dNTPs, which allows exclusive labeling of either the leading or lagging strand, depending on the ^{32}P-dNTP added to the reaction.

(A) The autoradiograms are 0.6% alkaline agarose gels of reaction products using either Pol III$_2$-$\tau_2\gamma_1$-$\delta\delta'\chi\psi$ or Pol III$_3$-τ_3-$\delta\delta'\chi$. The gel at the left shows leading-strand products, and the gel at the right shows lagging-strand products. Reactions that monitor the lagging strand contained 120 pmol DnaG.

(B) Replication forks driven by replisomes containing either one, two, or three DNA polymerases move at similar speeds. Rolling circle reactions were performed as in (A), except only leading strands were labeled and reactions were terminated at the indicated times.

In both (A) and (B), 100 fmol of Pol III-clamp loader species was used in each reaction, and each Pol III-clamp loader species is indicated above the autoradiograms.

amount of ^{32}P/^{3}H-(Pol III)$_3$-$\tau_3\delta\delta'\chi\psi$ recovered from the column is 70% the molar value of input DNA.

The Triple Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ Complex Assembles in Preference to Mono- and Dipolymerase Forms

Our earlier studies, and the work of others, indicated that γ does not enter the polymerase-clamp loader complex without staged addition of the subunits (Onrust et al., 1995b; Pritchard and McHenry, 2001). In Figure 6, we simply mixed γ and τ together on ice and then rapidly added α (the polymerase), ε, θ, δ, δ', χ, and ψ and incubated at 15°C for 1 hr. The assembly reaction was then passed over a sizing column, and fractions were analyzed in a SDS polyacrylamide gel. The early fractions contain the heavy Pol III core-clamp loader complexes, in which the τ_3-$\delta\delta'\chi\psi$ complex resides, but not γ_3-$\delta\delta'\chi\psi$ complex. The γ_3-$\delta\delta'\chi\psi$ complex elutes later, along with some excess Pol III core. Thus assembly of the triple Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex is highly preferred over forms that have only one or two Pol III cores.

DISCUSSION

The τ and γ Subunits Are Fully Interchangeable in Clamp Loading Function

The *E. coli* clamp loader contains seven subunits, one each of δ, δ', χ, and ψ and three protomers of the *dnaX* gene product. The *dnaX* gene produces both γ and τ; τ (71 kDa) is the full-length product and γ (47 kDa) is truncated by a translational frameshift. The C-terminal 24 kDa unique to τ consists of two domains that bind Pol III α subunit and DnaB helicase (Gao and McHenry, 2001a, 2001b;

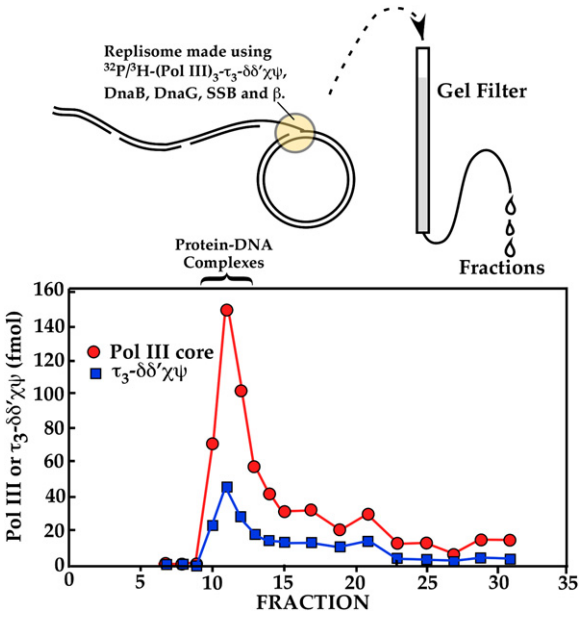


Figure 5. The Triple Polymerase Remains Intact within the Replisome

^{32}P-(Pol III)$_3$-^{3}H-(τ_3-$\delta\delta'\chi\psi$) was assembled onto a 7.2 kb TF II DNA, followed by DNA synthesis for 5 min (see scheme). Gel-filtration analysis of the reaction is shown in the plot. Moles of Pol III core and τ_3-$\delta\delta'\chi\psi$ contained in the replisome in column fractions were calculated from their known molar-specific radioactivity.

Kim et al., 1996; Studwell-Vaughan and O'Donnell, 1991). This report demonstrates that the four permutations of clamp loaders that contain either γ_3, $\tau_1\gamma_2$, $\tau_2\gamma_1$, or τ_3 are equally active in loading β onto DNA. Therefore the extra C-terminal 24 kDa of τ confers no significant effect on clamp loading activity.

The crystal structure of γ reveals a three-domain architecture (Jeruzalmi et al., 2001). The two N-terminal domains constitute the region of homology to AAA+ proteins and interact with ATP. The C-terminal α-helical domain mediates most of the oligomeric contacts. The N-terminal region of the τ subunit shares these sequences and thus is organized similarly. The C-terminal 46 residues of γ contain many hydrophilic residues and 15 prolines, plus it is susceptible to proteolysis, suggesting that this region is unstructured (Leu et al., 2003; O'Donnell et al., 2001; Pritchard et al., 1996). Thus, the C-terminal domains of τ that bind to DnaB and Pol III core appear to be connected by a flexible linker to the N-terminal domains that integrate into the clamp loader structure.

The Three Polymerase Replicase Is the Dominant Assembly Form

The current report demonstrates that a complex containing three Pol III cores can be assembled and that mixing all the Pol III subunits at once results in a trimeric Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex. Moreover, all three Pol III cores within the trimeric polymerase replicase are active and can synthesize DNA simultaneously. The difficulty with which γ and τ assemble together into the same clamp loading assembly is consistent with our previous studies, and those of others, showing that the τ and γ oligomers do not easily exchange subunits (Onrust et al., 1995b; Pritchard and McHenry, 2001). To obtain mixed γ and τ complexes, γ and τ must be incubated together for a long time (e.g., ~1–2 hr) in the absence of δ and δ'. Once δ and δ' associate with γ or τ, the γ_3-$\delta\delta'$ and τ_3-$\delta\delta'$ pentamers become "locked" and subunit exchange between the complexes is prevented (Onrust et al., 1995b). Given that preincubation of γ and τ in the absence of other subunits is not a physiological constraint, complexes that assemble in the cell likely contain exclusively τ (i.e., Pol III$_3$-τ_3-$\delta\delta'\chi\psi$) or γ (i.e., γ_3-$\delta\delta'\chi\psi$). Of course, it remains possible that another factor facilitates γ and τ subunit exchange inside the cell.

The structure of Pol III holoenzyme is thought to be a polymerase dimer assembled onto a clamp loader scaffold that contains two τ subunits and one γ subunit (O'Donnell et al., 2001; Pritchard and McHenry, 2001). This expectation is partly based on the fact that *E. coli* produces both γ and τ and also the fact that Pol III holoenzyme preparations contain both τ and γ. However, we show here that the trimeric Pol III holoenzyme is the form that assembles spontaneously and that it is at least as efficient as the dimeric Pol III holoenzyme in replisome function at a replication fork. The small amount of γ in Pol III preparations may be explained by proteolysis of one τ subunit during purification, because τ is sensitive to proteolysis at a position only two residues from the normal C terminus of γ (Pritchard et al., 1996). It is also possible that the γ subunit in Pol III holoenzyme preparations derives from a small amount of γ complex that contaminates the preparation, because γ complex is difficult to resolve from Pol III containing assemblies.

Why would *E. coli* produce a γ subunit if it were not incorporated into the replicase for chromosome replication? In fact, genetic studies have shown that γ is not essential to cell viability (Blinkova et al., 1993). In these studies the translational frameshift signal needed to produce γ was mutated so that only τ is produced from *dnaX*. Hence, in these genetically modified *E. coli* strains, the clamp loader contains three τ subunits. The current study shows that the τ_3-$\delta\delta'\chi\psi$ clamp loader binds three Pol III cores. Together, these two lines of information indicate that a trimeric Pol III$_3$-τ_3-$\delta\delta'\chi\psi$ complex is functional at replication forks in vivo.

Although *E. coli* does not need to make γ for cell viability, it may be presumed that γ confers a selective advantage to the cell. Production of γ may allow formation of a clamp loader species ($\gamma_3\delta\delta'\chi\psi$) that is not dedicated to the replication fork and therefore is free to load β onto DNA for use by other DNA metabolic enzymes. For example, β interacts with quite a diverse set of proteins involved in repair (Dalrymple et al., 2001; Lopez de Saro and O'Donnell, 2001).

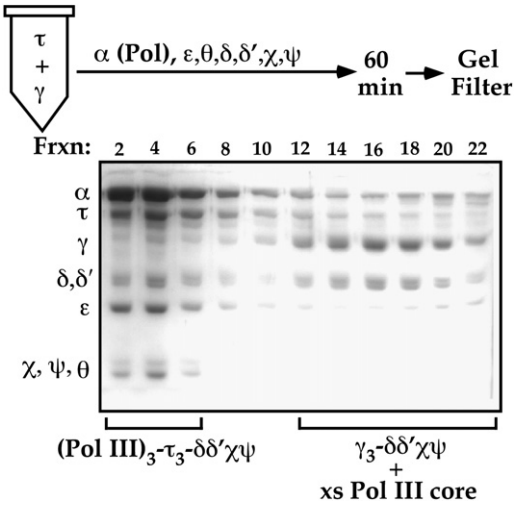


Figure 6. The Triple Pol III Replicase Assembles in Exclusion to Other Forms

The γ and τ subunits were added together and then all other subunits were added and incubated for 1 hr at 15°C. The reactions were analyzed on a gel-filtration sizing column. Column fractions that elute early (fractions 2–6) contain the Pol III core-clamp loader complex. Later fractions contain the γ complex and the excess Pol III core (fractions 12–22).

Why Three DNA Polymerases at a Replication Fork?

A plausible model by which three DNA polymerases may function simultaneously at one replication fork is shown in Figure 7. As one DNA polymerase extends the leading strand, the other two DNA polymerases can function on two lagging-strand fragments. This model is consistent with recent electron microscopic studies in the T4 system that have identified a subpopulation of forks with three DNA polymerases, two of which appear to simultaneously extend different Okazaki fragments (Nossal et al., 2007).

Replication of the lagging strand is significantly more complicated than the leading strand, and there are several possible reasons that two (or more) polymerases may coordinate their action on the lagging strand. Certain features unique to the lagging strand may slow it down relative to the leading strand, and a third DNA polymerase may help it catch up to the leading strand. For example, the lagging-strand polymerase must dissociate from DNA upon terminating a Okazaki fragment in order to start extension of the next fragment. Yet the lagging-strand polymerase is tethered to the finished Okazaki fragment by the β clamp. A mechanism exists to disengage the lagging polymerase from β within about 1 s, but this is still sufficient time for the leading polymerase to advance several hundred nucleotides (Leu et al., 2003; Stukenberg et al., 1994). If a second lagging-strand polymerase were present in the replisome, it could begin extension of the next RNA primer before or during the time required for the other lagging-strand polymerase to disengage from β. Alternatively, the second lagging-strand polymerase may finish an Okazaki fragment while the original lagging-strand polymerase continually recycles to the new RNA primer.

A second reason lagging-strand synthesis may be slower than the leading strand is the exclusive presence of SSB on the lagging strand. SSB is a tight ssDNA binding protein and almost certainly slows the intrinsic rate of polymerase progression. Despite this, estimates of polymerase extension on SSB-coated ssDNA are within the observed range of replication fork movement in vivo (~650 nt/s) (Breier et al., 2005). But even small differences in the rates of leading- and lagging-strand extension could eventually produce a significant disparity and require a mechanism to even them out. Two polymerases on the lagging strand may be one such mechanism.

Another mechanism by which the lagging strand can "catch up" to a leading strand is by premature release of the lagging polymerase from β prior to completing an Okazaki fragment. Premature release of a lagging polymerase from an unfinished Okazaki fragment will leave a gap of ssDNA in the wake of a moving fork, but the ssDNA gap can presumably be filled in later. Premature release is observed in the *E. coli* system when the leading-strand polymerase is given a long head start (Li and Marians, 2000) or when extension of a lagging-strand fragment is blocked (McInerney and O'Donnell, 2004). Premature release is also observed in the T4 replication system, especially under conditions of polymerase starvation (Yang et al., 2006). A third polymerase in the replisome may take over lagging-strand synthesis when the original polymerase stalls. It is also possible that the third polymerase finishes fragments that are repeatedly started by the original lagging-strand polymerase.

A three DNA polymerase replisome may also hold one DNA polymerase in "reserve" for unusual circumstances. For example, the lagging strand is repeatedly primed by primase, whereas the leading strand only needs to be primed once or a few times. A delay in primase action may result in a leading strand that proceeds too far ahead of the lagging strand for normal replication fork function. In the T7 replisome, primer synthesis induces a transient pause in replication fork progression (Lee et al., 2006), further revealing the tight coordination among all replisome components, and underscores the utility of having backup mechanisms in place. In this particular instance, a third DNA polymerase could facilitate close coordination of the leading and lagging strands by extending the next RNA primer while the initial lagging-strand polymerase is busy finishing the unusually long Okazaki fragment.

Another circumstance that may be rescued by a third DNA polymerase is a situation in which one polymerase becomes stalled by a damaged template nucleotide. In the case of a lagging-strand block, normal primase action on the lagging strand will soon generate a new primed site at which the third DNA polymerase could associate. Recently it was shown that primase can also synthesize an RNA primer ahead of a block (Heller and Marians, 2006). Thus, a third DNA polymerase that is held in reserve could

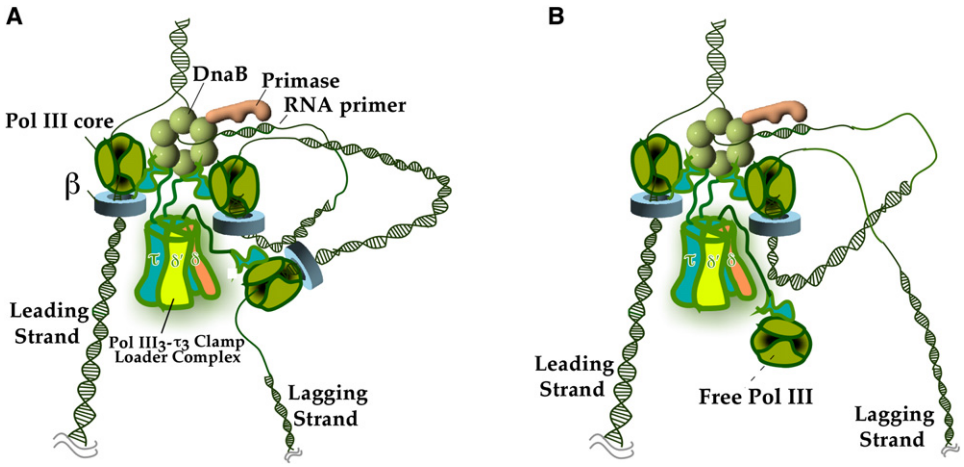


Figure 7. A Trimeric Replicase at the *E. coli* Replication Fork

The illustrations depict a replication fork containing a triple polymerase Pol III_3-τ_3-$\delta\delta'\chi\psi$ complex. In (A), two of the Pol III cores are proposed to function on the lagging strand. The τ subunits (blue) of the clamp loader are shown with a flexible linker that connects the N-terminal clamp loading domains (I–III) to the C-terminal domains (IV and V) of τ that bind DnaB helicase and Pol III core (see also Figure 1A). Two τ subunit C-terminal extensions are illustrated with connections to DnaB helicase, although work in this report shows that only one τ-DnaB connection is needed for rapid fork progression. The two lagging-strand Pol III cores are depicted as extending two Okazaki fragments at the same time, producing two lagging-strand loops. (B) illustrates the third Pol III core off DNA. This may occur when only one Pol III core is needed on the lagging strand. The third Pol III core may be held in reserve and only be called into action when the lagging strand has trouble keeping pace with the leading strand or when either the leading- or lagging-strand polymerase becomes stalled or inactivated.

come in handy to restart a replication fork at newly primed sites ahead of template blocking lesions.

Finally, a third DNA polymerase would rescue a fork in which one τ within a Pol III_3-τ_3-$\delta\delta'\chi\psi$ complex becomes proteolyzed or one Pol III core becomes inactivated. In these cases, the two Pol III cores that remain would be capable of continuing replication of the chromosome. This built-in redundancy of multiple DNA polymerases in one replisome should enable it to overcome a certain number of obstacles. Evolution of a highly robust replication apparatus may in fact be expected, considering the vital and central role of the replisome in cell duplication and, therefore, in species propagation.

This report demonstrates that replisomes that contain either two or three Pol III cores are capable of performing the task of concurrent leading- and lagging-strand replication. Overall, these studies demonstrate that a three polymerase replicase can efficiently assemble and function at replication forks. Further studies will be needed to more fully characterize the difference between replication forks that contain either two or three DNA polymerases. But the fact that both the two and three polymerase forms of Pol III holoenzyme are functional at replication forks underscores the intrinsic versatility of this crucial multisubunit machine.

EXPERIMENTAL PROCEDURES

Materials

Radioactive nucleotides were from PerkinElmer Life Sciences, Inc. Unlabeled NTPs and dNTPs were from Pharmacia LKB Biotechnology Inc. Unlabeled ATPγS was from Roche Diagnostics. Streptavidin-coated Dynabead M-280 magnetic beads were from Dynal Biotech. Gel-purified DNA oligonucleotides were from Invitrogen-GIBCO or Integrated DNA Technologies (IDT), Inc. *E. coli* replication proteins were purified as described (Yuzhakov et al., 1996). Protein concentrations were determined by Bradford (Bio-Rad) using BSA as a standard.

Buffers

Buffer A is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Reaction buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 4% glycerol (v/v), and 40 μg/ml BSA. Clamp loading buffer is 30 mM HEPES-NaOH (pH 7.5), 7 mM $MgCl_2$, 1 mM DTT, and 1 mM CHAPS.

DNAs

The following oligonucleotides were synthesized and gel purified by Integrated DNA Technologies (IDT): 30-mer, 5′-GCAATAACTGGCCG TCGTTTGAAGATTTCG-3′; 66-mer, 5′-CCATTCTGTAACGCCAGGGTT TTCGCAGTCAACATTCGAAATCTTCAAACGACGGCCAGTTATTGC-3′. A 102-mer 3′ biotinylated oligonucleotide was synthesized and gel purified by the W.M. Keck Facility at Yale University: 5′-CCATTCTG TAACGCCAGGGTTTTCGCAGTCAACATTCGAAATCTTCAAACGACG GCCAGTTATTGCTCTTCTTGAGTTTGATAGCCAAAACGACCATTATA G-3′(Biotin). To form synthetic primed template, the 30-mer primer oligonucleotide was mixed with the 102-mer template strand in a 1:1.2 ratio of primer to template in 50 μl of 5 mM Tris-HCl, 150 mM NaCl, and 15 mM sodium citrate (final pH 8.5) and then placed in a 95°C water bath and cooled to 23°C over a 30 min interval. M13 mp18 phage was purified on two cesium chloride gradients (Turner and O'Donnell, 1995) and then the ssDNA was extracted by using phenol and primed with a DNA 30-mer as described (Studwell and O'Donnell, 1990).

Reconstitution of Clamp Loader Complexes

Clamp loaders containing both γ and τ were reconstituted as follows. A mixture of τ (2.2 mg, 17.9 nmol as trimer) and γ (3.1 mg, 22.3 nmol as trimer) was incubated for 90 min at 15°C, followed by addition of δ (1.75 mg, 46.9 nmol), δ' (1.73 mg, 46.9 nmol), χ (1.32 mg,

93.8 nmol), and ψ (0.88 mg, 62.5 nmol), followed by further incubation for 60 min. Clamp loaders with $\tau_2\gamma_1$-δδ′χψ and $\tau_1\gamma_2$-δδ′χψ compositions were resolved on a 1 ml Mono S column as described (Pritchard and McHenry, 2001) by using a 20 ml gradient of 0–0.5 M NaCl in buffer A. Column fractions of 0.25 ml each were collected and analyzed on a 14% SDS polyacrylamide gel. The γ_3-δδ′χψ and τ_3-δδ′χψ complexes were reconstituted and purified from unassociated subunits on a Mono Q column as described previously (Onrust and O'Donnell, 1993). Fractions containing each clamp loader were pooled, aliquoted, and stored at −70°C.

Reconstitution of Pol III-Clamp Loader Complexes

Pol III-clamp loader complexes were reconstituted as described previously (Onrust et al., 1995b). Briefly, Pol III_1-$\tau_1\gamma_2$-δδ′χψ was formed upon mixing $\tau_1\gamma_2$-δδ′χψ with a 2-fold molar excess of Pol III core, Pol III_2-$\tau_2\gamma_1$-δδ′χψ was formed by mixing $\tau_2\gamma_1$-δδ′χψ with a 4-fold molar excess of Pol III core, and Pol III_3-τ_3-δδ′χψ was reconstituted upon mixing τ_3-δδ′χψ with a 6-fold molar excess of Pol III core. In each case, protein mixtures were incubated for 60 min at 15°C and then resolved from excess core on a Mono Q column as described (Onrust et al., 1995b). Complexes were analyzed in a 14% SDS polyacrylamide gel and stained with Coomassie blue, and individual lanes were scanned with a laser densitometer and quantitated as described (Onrust et al., 1995b). Pol III-clamp loader complexes were pooled, aliquoted, and stored at −70°C.

To assess the extent to which γ assembles with the other subunits of Pol III without preincubating γ and τ, the α (0.5 mg, 3.9 nmol), ε (0.16 mg, 5.8 nmol), θ (0.05 mg, 5.8 nmol), δ (0.13 mg, 3.4 nmol), δ′ (0.13 mg, 3.4 nmol), χ (0.06 mg, 3.4 nmol), and ψ (0.06 mg, 3.4 nmol) (Onrust et al., 1995b) subunits were mixed on ice in one tube. In a second tube, γ (0.12 mg, 2.54 nmol) and τ (0.18 mg, 2.54 nmol) were first mixed together on ice and then this mixture was rapidly transferred to the tube containing the α, ε, θ, δ, δ′, χ, and ψ subunits. The entire protein mixture was then allowed to incubate for 60 min at 15°C and then the reaction was analyzed on a 24 ml Superose 12 column in the cold room. After collecting the void fractions (214 drops), six drop fractions were collected and analyzed in a 12% SDS polyacrylamide gel stained with Coomassie blue. Subunit composition was analyzed as described (Onrust et al., 1995b).

Magnetic Bead Clamp Loading Assays

The assay used to measure the rate of β clamp loading was developed based on a previous design (Johnson et al., 2006). 30-mer/102-mer biotinylated DNA was conjugated to Streptavidin Dynabeads M-280 (Dynal Biotech), and the DNA-bead conjugate was incubated with 5 mg/ml BSA in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl at 23°C with agitation for 30 min, followed by three washes in the same buffer. The typical yield of DNA conjugated to beads was ~100 pmol DNA/mg Dynabeads. Clamp loading reactions utilized β^{PK}, which contains a six residue C-terminal kinase recognition site (Kelman et al., 1995) and was labeled to a specific activity of 10 dpm/fmol with γ^{32}P-ATP using the recombinant catalytic subunit of cAMP-dependent protein kinase (Sigma). Reactions contained 0.5 mM ATP, 170 nM DNA, 510 nM *E. coli* SSB (tetramer), and 700 nM ^{32}P-β^{PK} (dimer) and were incubated for 1 min at 16°C in clamp loading buffer. Clamp loading was initiated upon adding clamp loader complex (100 nM) and was quenched upon adding 21 mM EDTA. DNA beads were isolated with a magnetic concentrator (1 min) at 4°C and then washed twice in clamp loading buffer containing 100 mM NaCl at 4°C. Protein was stripped from the beads by using 1% SDS and counted by liquid scintillation.

Primed M13 mp18 DNA Synthesis Assays

The activity of clamp loader complexes with Pol III core and β was assayed based on the requirement to load β onto primed circular M13 mp18 ssDNA to observe nucleotide incorporation by Pol III core. Reactions contained β_2 (10 nM), SSB (420 nM tetramer), and M13 mp18 ssDNA (1.1 nM) primed with a DNA 30-mer, 60 μM each dATP, dCTP, and dGTP, 20 μM α-^{32}P TTP, 1 mM ATP, 10 mM $MgCl_2$, and Pol III core (5 nM) in 25 μl of reaction buffer. Replication was initiated upon addition of the indicated amount of clamp loader and incubated at 37°C for 5 min. Reactions were quenched upon addition of 25 μl 1% SDS, 40 mM EDTA, then spotted onto DE81 filters, and then washed and quantitated by liquid scintillation as described (Studwell and O'Donnell, 1990). Where indicated, half of the quenched reaction was analyzed in a 0.8% native agarose gel.

Titration of Pol III-clamp loader complexes into reactions containing primed M13 mp18 ssDNA was performed as above with the following modifications. Reactions contained 7 nM (170 fmol) primed M13 mp18 ssDNA (as circles), 22 nM β_2, 3 μM SSB, and 60 μM each dCTP and dGTP in 25 μl reaction buffer. Pol III-clamp loader complexes were added to reactions and incubated for 3 min at 37°C, and then synthesis was initiated upon adding 60 μM dATP and 20 μM ^{32}P-dTTP and complexes were quenched after 20 s with SDS/EDTA. The molar amounts of Pol III-clamp loaders were determined from the calculated molecular mass for each species: Pol III_1-$\tau_1\gamma_2$-δδ′χψ (440 kDa), Pol III_2-$\tau_2\gamma_1$-δδ′χψ (629 kDa), and Pol III_3-τ_3-δδ′χψ (819 kDa). DNA products were analyzed in a 0.8% native agarose gel.

Rolling Circle Replication Reactions

Pol III_2-$\tau_2\gamma_1$-δδ′χψ and Pol III_3-τ_3-δδ′χψ were tested for leading- and lagging-strand replication by using a minicircle TF II DNA template as described previously (McInerney and O'Donnell, 2004). DnaB helicase (4 pmol) was assembled on the minicircle TF II (100 fmol) in 15 μl of reaction buffer for 30 s at 37°C, followed by addition of 60 μM each dGTP and dATP, Pol III-clamp loader (100 fmol), and β (350 fmol), and then incubated a further 6.5 min. Replication was initiated by adding 1 μg SSB, 60 pmol DnaG, 50 μM each of the four rNTPs, and 60 μM each of dTTP and dCTP to a final volume of 25 μl. Leading-strand synthesis was monitored in reactions containing [α-^{32}P]dATP. Lagging-strand synthesis was monitored in reactions containing [α-^{32}P]dTTP. Reactions were quenched after 10 min upon addition of 25 μl 1% SDS/40 mM EDTA. DNA synthesis was analyzed with DE81 filters, and DNA products were analyzed in 0.6% alkaline agarose gels.

In reactions containing ^{32}P/^{3}H-(Pol III)$_3$-τ_3-δδ′χψ, ^{32}P-Pol III core was reconstituted with α subunit with an N-terminal six residue kinase sequence that was labeled with α-^{32}P ATP and protein kinase (55,000 cpm/pmol) as described (Kelman et al., 1995). The τ_3-δδ′χψ complex was reconstituted with ^{3}H-δ′ (40,000 cpm/pmol) labeled by reductive methylation as described (Kelman et al., 1995). The ^{32}P/^{3}H-(Pol III)$_3$-τ_3-δδ′χψ complex was reconstituted as described above; it retains essentially full (i.e., 95%) activity relative to (Pol III)$_3$-τ_3-δδ′χψ complex lacking tags or radioactive subunits in assays using primed M13 mp18 ssDNA as substrate. Reactions were as described as for the minicircle TFII except the reaction volume was 100 μl and contained 700 fmol ^{32}P/^{3}H-(Pol III)$_3$-τ_3-δδ′χψ, 10 pmol DnaB, 1.5 pmol β, 150 pmol DnaG, and 2.5 μg SSB, and the substrate was M13 mp18 TF II DNA (250 fmol, prepared as described in Yuzhakov et al. [1996]). Reactions were analyzed by filtration on BioGel A15M equilibrated in reaction buffer containing 60 μM each dCTP and dGTP. Fractions of 250 μl were collected and counted.

Supplemental Data

Supplemental Data include one figure and can be found with this article online at http://www.molecule.org/cgi/content/full/27/4/527/DC1/.

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