Molecular Cell, Volume *34*

Supplemental Data

Chaperoning of a Replicative Polymerase onto a Newly-Assembled DNA-Bound Sliding Clamp by the Clamp Loader

Christopher D. Downey and Charles S. McHenry*

SUPPLEMENTAL FIGURES AND LEGENDS

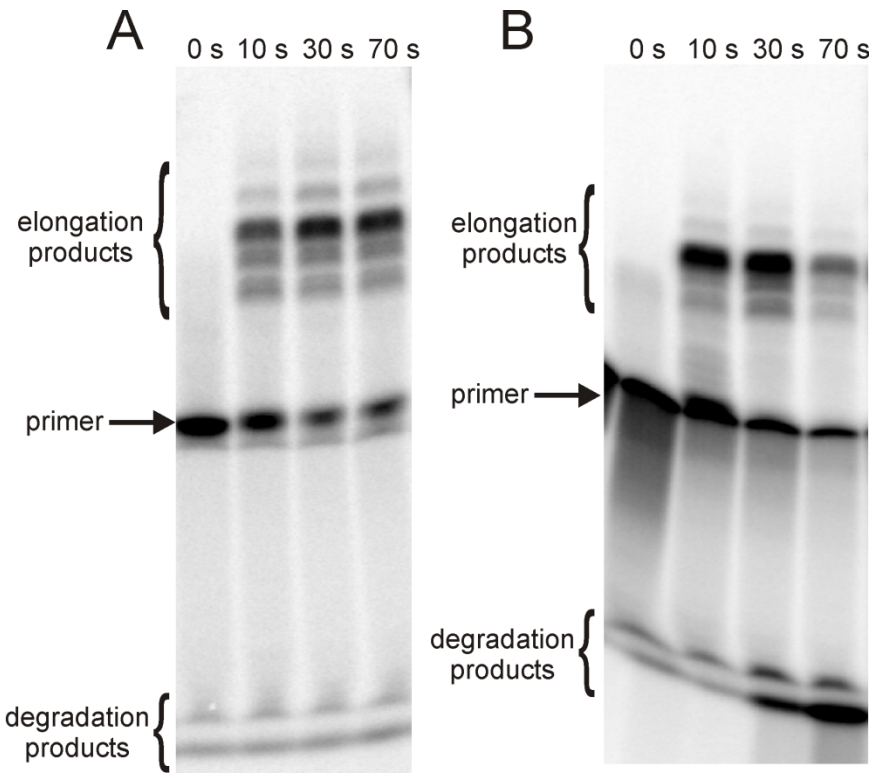

Figure S1. An RNA primer resists degradation by the ε subunit of Pol III.

A) Initiation reaction products with our RNA primer on the M13Gori DNA template after 0, 10, 30, and 70 s initiation. B) Initiation reaction products with a DNA primer of analogous sequence on the M13Gori DNA template after 0, 10, 30, and 70 s initiation (See also Figure 1).

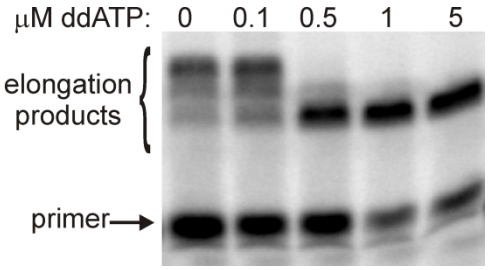

Figure S2. A single primer extension product of well-defined length is produced with ≥1 μM ddATP in the extension reaction.

A PAGE analysis is shown for 30 s τ-complex-catalyzed initiation reactions followed by 10 s extension reactions including the stated concentrations of ddATP to halt extension of the 30 nt primer at a 53 nt product (See also Figure 1).

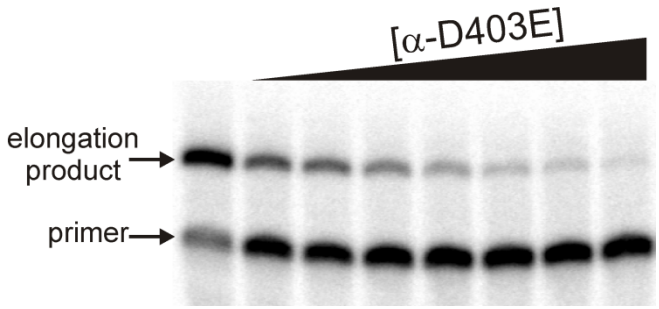

Figure S3. Example PAGE analysis of the primer extension assay.

τ-complex-catalyzed initiation complex formation at increasing concentrations of α-D403E, with the τ-complex exposed to wild-type Pol III and α-D403E simultaneously. This is the gel used to generate the red curve in Figure 1A (see Figure 1A for α-D403E concentrations). The primer fraction extended for each lane was quantified as the counts for the extended product divided by the sum of the counts for the primer and the extended product (See also Figure 2).

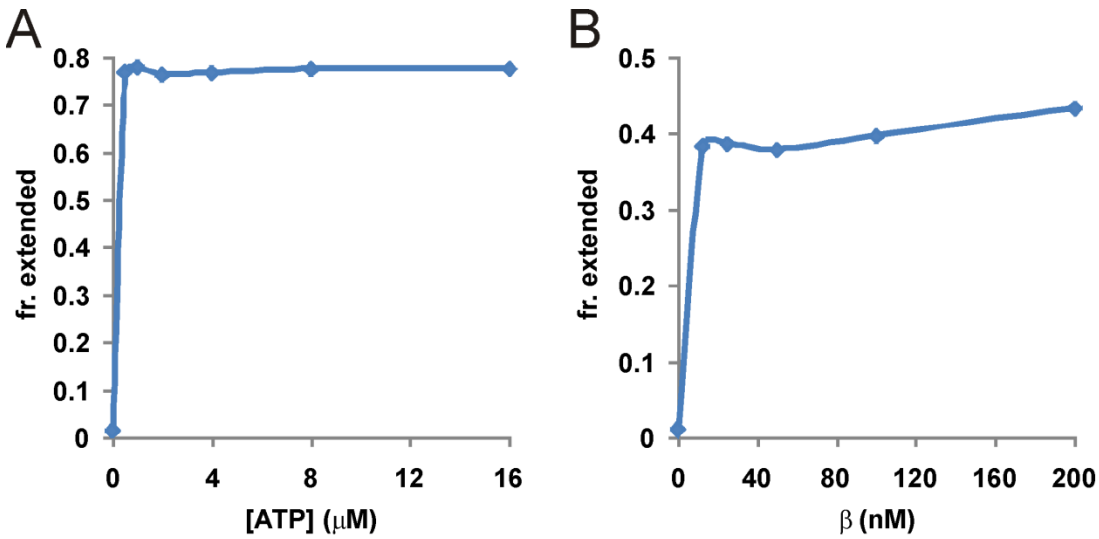

Figure S4. The primer extension assay detects full initiation complexes.

A) ATP dependence for τ-complex catalyzed initiation complex formation. B) β_2 dependence for τ-complex catalyzed initiation complex formation (See also Figure 2).

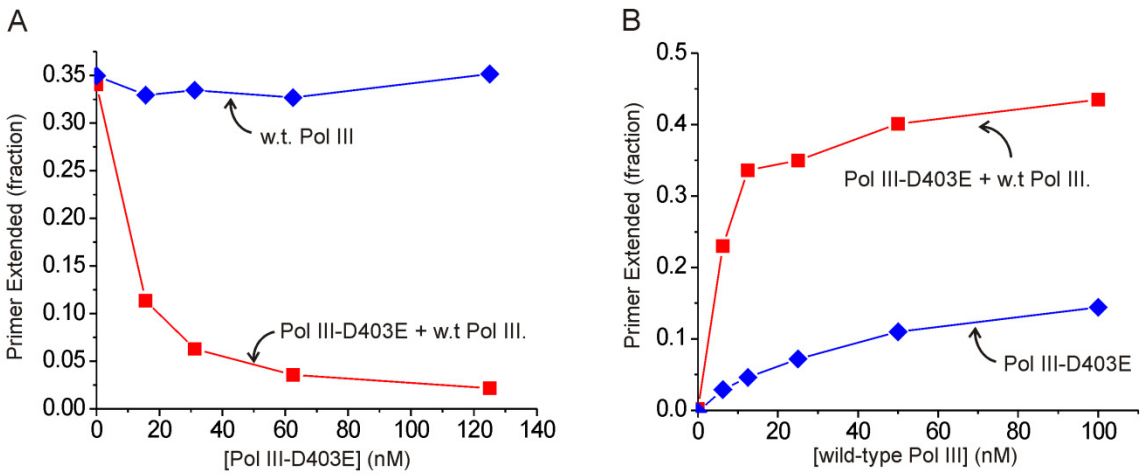

Figure S5. Pol III-D403E behaves similarly to α-D403E in challenge experiments.

A) Initiation complex formation at various Pol III-D403E concentrations with τ-complex pre-incubated with wild-type Pol III (blue) or exposed to Pol III-D403E and wild-type Pol III simultaneously (red). B) Initiation complex formation at various wild-type Pol III concentrations with τ-complex pre-incubated with Pol III-D403E (blue) or exposed to Pol III-D403E and wild-type Pol III simultaneously (red) (See also Figure 3).

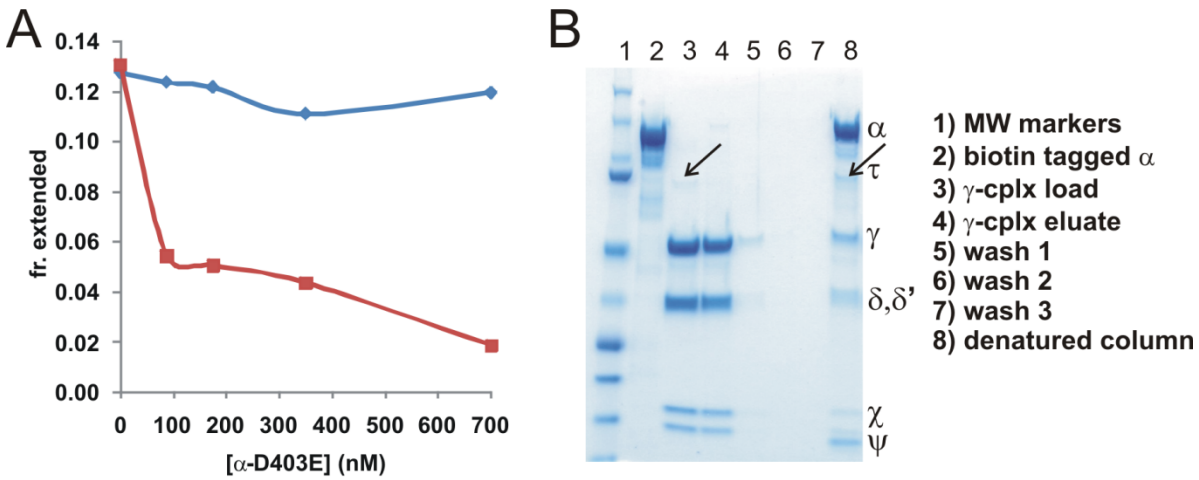

Figure S6. Removal of trace τ contamination from reconstituted γ-complex.

A) τ-like activity of γ-complex prior to α-affinity purification. Initiation complex formation was performed with γ-complex pre-incubated with wild-type Pol III (blue) or exposed to α-D403E and wild-type Pol III simultaneously (red). These reactions contained 0.25 μM SSB_4. B) PAGE analysis of trace τ removal by affinity to biotin tagged α immobilized on streptavidin/agarose beads. Lane (1) molecular weight markers, (2) biotin tagged α alone, (3) γ-complex before incubation with α-bound strepavidin/agarose beads, (4) τ-depleted γ-complex initially eluted from the beads, (5-7) sequential washes of the beads after initial elution, (8) material remaining bound to the streptavidin/agarose beads after washing. Arrows indicate trace τ in the γ-complex input (3) and τ retained on the beads (8) (See also Figure 3).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES AND RESULTS

Proteins and Nucleic Acids

DNA polymerase III replication proteins were expressed and purified as previously described: Pol III (McHenry and Crow, 1979; Kim and McHenry, 1996b); α-D403E (Pritchard and McHenry, 1999); ε (Scheuermann and Echols, 1984); θ (Carter *et al.*, 1993); β (Johanson *et al.*, 1986); τ, γ, δ, δ', χ–ψ, and reconstituted τ-complex and γ-complex (Glover and McHenry, 2000); and SSB (Griep and McHenry, 1989). SSB-cΔ42 (Roy *et al.*, 2007) and SSB-cΔ8 (Hobbs *et al.*, 2007) were obtained from the laboratories of Tim Lohman (Washington University) and Mike Cox (University of Wisconsin), respectively.

To remove any possibility for trace contaminating τ, γ-complex was subjected to an additional purification step based on the affinity of τ for the α subunit. All steps were performed at ambient temperature using the following buffer: 25 mM HEPES (pH 7.5), 200 mM NaCl, 2% glycerol. We pipetted 50 μL of a slurry of streptavidin/agarose beads (Novagen) onto a Spin-X centrifuge tube filter (Corning), spun out the bead storage buffer, and washed 3 times with 200 μL of our buffer. Ninety μg (0.7 nmol) biotin-tagged α subunit (Kim and McHenry, 1996a) in 100 μL buffer were added to the beads and incubated for 10 min. Any unbound α was spun out and the beads were washed 5 times with 200 μL buffer. Eighty-five μg (0.34 nmol) γ-complex in 50 μL buffer were incubated with the α-loaded beads for 15 min to bind any τ, and the unbound τ-depleted γ-complex was spun out and collected. The beads were washed with an additional 50 μL buffer, and this wash was collected and combined with the initial 50 μL γ-complex eluted from

the beads, yielding 66 μg purified γ-complex (78% yield, determined by a Bradford assay using bovine serum albumin as a protein standard). The specific activity of the purified γ-complex was unchanged from the original γ-complex in our standard holoenzyme reconstitution assay described elsewhere (McHenry and Crow, 1979; Pritchard and McHenry, 1999), demonstrating the purification process did not damage the sample.

M13Gori single-stranded DNA was prepared as described (Johanson *et al.*, 1986). A 30 nt RNA oligonucleotide primer was purchased from Thermo Scientific/Dharmacon with the sequence: 5′UGAGCUCGGGGAAUGCGGCGGCGAGAUAGU. This sequence is complementary to positions 7722-7751 of the M13Gori genome. The oligonucleotide was 5′-^{32}P end-labeled using [γ-^{32}P]-ATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs). The labeled primer and M13Gori template were annealed by heating a 100 nM mixture of each strand to 90 °C for 5 min and cooling 1 °C per min to 20 °C in a buffer of 10 mM HEPES (pH 7.5) 50 mM NaCl, and 1 mM EDTA. Calf thymus DNA “activated” by partial digestion with DNase I to produce free 3′ termini was prepared as previously described (Kim and McHenry, 1996b).

Primer Extension Assay for Initiation Complex Formation

Initiation complex formation was initiated by combining 12.5 μL of a solution of Pol III, DnaX complex, β_2, and 0.20 mM ATP with 12.5 μL ^{32}P-labeled primer/template solution (SSB coated where applicable). All reactions were conducted under single turnover conditions, with all Pol III holoenzyme components present in at least 4-fold molar excess over the primer/template substrate (see sections below for specific component

concentrations). After the reaction times described in the following sections, initiation complex formation was stopped by addition of 0.5 (τ-complex reactions) or 0.1 mg/mL (γ-complex reactions) activated calf thymus DNA. Simultaneously, primer extension was initiated by addition of dTTP, dCTP, and dGTP (40 μM each), and ddATP (2 μM). All concentrations are the final values after adding 5.0 μL of a 6X quench/extension solution. The primer elongates 23 nt before terminating upon incorporating the ddA nucleotide. After a 10 s primer extension period, the reaction was stopped by adding an equal volume of a 96% formamide/25 mM EDTA solution. The short (10 s) extension period was used to prevent non-processive extension by Pol III alone, ensuring that only fully assembled initiation complexes were probed. With the single exception described below, all reactions herein were conducted with initiation complex formation and primer extension as separate steps. Reaction products were separated with 16% (w/v) polyacrylamide gel electrophoresis with 8 M urea denaturant. The radioactivity in the primer and product bands was quantified by phosphorimaging using a Typhoon 9400 variable mode imager and ImageQuant 5.2 software (Amersham Biosciences). The fraction of primer elongated was calculated for each reaction as the counts for the product band divided by the sum of the primer and product bands. All reactions were conducted at room temperature with the following buffer: 50 mM HEPES (pH 7.5), 100 mM potassium glutamate, 10 mM magnesium acetate, 0.20 mg/mL bovine serum albumin, 10 mM dithiothreitol, 2.5% (v/v) glycerol, and 0.02% (v/v) Nonidet-P40 detergent.

Pol III Concentration Dependence of Initiation Complex Formation

Pol III concentration dependence was probed under conditions of 0.1 nM ^{32}P-labeled primer/template, 50 nM β_2, and 1.0 nM DnaX complex. No SSB was present in these reactions. Varying concentrations of Pol III were added to the β_2/DnaX/ATP mixture prior to conducting initiation complex formation. Initiation complex formation was performed for 2 min for all samples. The data were fit using SigmaPlot 9.0 to the standard binding isotherm equation $f = f_{max} * [\text{Pol III}] / (K_{1/2} + [\text{Pol III}])$, where f is the fraction of primers elongated, f_{max} is the maximum fraction of elongated primer, and $K_{1/2}$ is the Pol III concentration at half-maximum. The term $K_{1/2}$ is used since the value is clearly not a true K_D, as this is a functional assay of a multicomponent complex association coupled to ATP hydrolysis.

Kinetics of Initiation Complex Formation

The primer extension assay was performed with reaction concentrations of 0.1 nM ^{32}P-labeled primer/template, 50 nM β_2, 1.0 nM DnaX complex, and 5.0 nM Pol III. No SSB was present in these reactions. Each time point was manually sampled as separate 25 μL reactions. The data for the γ-complex reaction were fit to a single exponential rise to maximum using SigmaPlot 9 software: $f = f_0 + \Delta f_{max} * \{1 - \exp(-k_{obs}t)\}$, where f is the fraction of primers elongated, Δf_{max} is the maximum change in fraction of primers elongated, k_{obs} is the observed 1st order rate constant, and t is the reaction time elapsed.

Resistance of an RNA Primer to Degradation by the ε Subunit of Pol III

The ε subunit of Pol III is a 3′ to 5′ proofreading DNA exonuclease, which could digest a DNA primer in an initiation complex prior to elongation. To test whether our RNA primer was affected by this nuclease, we conducted experiments similar to the primer extension assay described in the main text with the following changes. Here, the 10 s primer elongation step was conducted in the absence of ddATP or a trap of unlabeled activated calf thymus DNA. In experiments labeled 0 s, Pol III HE components were already denatured with the 96% formamide/25 mM EDTA solution when added to the primer/template. Thus, these 0 s data points represent negative controls showing the level of primer degradation in the absence any nuclease activity associated with the holoenzyme. The reactions were conducted at room temperature with 10 nM Pol III, 10 nM τ-complex, 50 nM β_2, 0.20 mM ATP, 2 nM primer/template, and 0.25 μM SSB_4. Analyzing the samples by polyacrylamide gel electrophoresis (PAGE) showed that the level of degradation products for the RNA primer did not increase after 70 s exposure to Pol III HE components (Figure S1A), whereas significant degradation products were observed for the DNA primer (Figure S1B). This degradation resulted in a large qualitative decrease in the yield for primer extension.

Addition of ddATP to the Primer Extension Reaction

As evident in Figure S1, primer elongation in the absence of dATP results in products of varying length. This effect likely arises from the incorporation of mispaired nucleotides in the place of dATP. We tested whether an elongation product of well-defined length could be obtained by including 2′,3′ dideoxy-ATP (ddATP) in the elongation mixture, stopping DNA synthesis after incorporation of the first dA nucleotide. The experimental

procedure was identical to that described for our primer extension assay, except that the ddATP concentration in the 10 s elongation reaction was varied. The initiation reactions prior to the elongation reactions were conducted for 30 s at 15 nM Pol III, 15 nM τ-complex, 100 nM β_2, 0.20 mM ATP, 2 nM primer/template, and 0.25 μM SSB_4. The experimental results showed that a single elongation product of well defined length is obtained at ddATP concentrations above 1 μM (Figure S2). A PAGE analysis of a typical experiment for this study employing the primer extension assay with ddATP in the elongation reaction is shown in Figure S3.

ATP and β_2 Dependence of the Primer Extension Assay

To test that our primer extension assay detected *bona fide* initiation complexes of Pol III bound to β_2 loaded onto the DNA substrate, we measured the ATP and β_2 dependence of primer extension. The experimental procedures were the same as those described for the primer extension assay. For the ATP dependence experiments, 30 s initiation complex formation reactions were conducted at varying ATP concentrations with 4.0 nM Pol III, 4 nM τ-complex, 50 nM β_2, 1 nM RNA primer/template, and 0.25 μM SSB_4. For the β_2 dependence, 10 s initiation reactions were conducted at varying β_2 concentrations with 32 nM Pol III, 2 nM τ-complex, 0.20 mM ATP, 1 nM RNA primer/template, and 0.25 μM SSB_4. The results showed no primer extension without ATP to drive the β_2 loading reaction (Figure S4A) or without β_2 (Figure S4B), demonstrating that the assay detects only complete initiation complexes. Both ATP and β_2 reached saturating levels at concentrations much lower than the ATP and β_2

concentrations used throughout this study, showing that neither component was a limiting reagent in any of our experiments.

Pol III-D403E and α-D403E inhibit initiation complex formation similarly

In the challenge experiments reported in the primary manuscript, we competed with αD403E rather than the Pol III form (αD403E-ε-θ) as a precaution to avoid artifacts resulting from the burden of large excesses of the ε nuclease. To verify that the α-D403E subunit behaves analogously to Pol III in our competition assays, we reconstituted an inactive mutant Pol III by incubating α-D403E with 1.5-fold molar excesses of the ε and θ subunits for 10 min at room temperature. This reconstituted Pol III-D403E was then used to challenge τ-complex-catalyzed initiation complex formation in an experiment with similar conditions to that in Figure 1A. The results were similar to those with α-D403E alone, with pre-formed wild type Pol III/τ-complex assemblies completely resisting the challenge from Pol III-D403E and with excess Pol III-D403E inhibiting the reaction when τ-complex was exposed simultaneously to Pol III-D403E and wild-type Pol III (Figure S5A).

We also conducted an experiment where Pol III-D403E was pre-assembled with τ-complex before adding varying concentrations of wild-type Pol III. This experiment, which is the reverse of the experiment in Figure S5A, tests whether the mutant Pol III can exclude excess wild-type Pol III from forming active initiation complexes. ATP-driven initiation complex formation experiments were performed with 0.5 nM ^{32}P-labeled primer/template, 0.25 μM SSB_4, 50 nM β_2, 2.0 nM DnaX complex, and 10 nM Pol III-D403E. 10 nM Pol III-D403E is sufficient to saturate the τ subunits of the τ-complex. As

shown in Figure S5B, preassembling Pol III-D403E with τ-complex greatly inhibits the ability of the system to form active initiation complexes. A 10-fold excess of exogenous wild-type Pol III (the highest [Pol III] value in the blue curve) competing with preassembled Pol III-D403E formed fewer active initiation complexes than did Pol III present at sub-stiochiometric levels of Pol III-D403E when the two species were exposed to τ-complex simultaneously (1st non-zero Pol III value in the red curve). This result is consistent with our model that the polymerase bound to the τ subunits is preferentially delivered to the initiation complex over exogenous polymerase in solution. Increasing activity was observed with increasing exogenous wild-type Pol III levels, indicating that the wild-type Pol III was not completely excluded from forming initiation complexes (Figure S5B, blue curve). The simplest explanation for this result is that initiation complexes formed by the mutant polymerase are less stable than those formed by the wild-type Pol III. Thus, initiation complexes formed with the mutant are not "dead end" complexes and can dissociate on the timescale of the experiment, creating an opportunity for the exogenous wild-type Pol III to attack the loaded β_2 and form a stable active initiation complex. Regardless, it is clear from these experiments and those in Figure 1 that initiation complex formation is much more efficient when the polymerase is bound to the clamp loader via the τ subunit.

Removal of Trace τ from the γ-Complex Sample

If a single τ subunit at any of the three τ/γ positions of the DnaX complex is sufficient for chaperoning of Pol III to the initiation complex, then even trace contamination of τ in the sample from endogenous expression of chromosomal *dnaX* could significantly affect the

Pol III chaperoning behavior of the sample. For example, a τ contamination of 2% of the total DnaX protein concentration would result in ~6% of the DnaX complexes containing at least one τ subunit. Since our assays were conducted with DnaX complex in molar excess over the primer/template, the ratio of this population of τ-containing complexes to the primer/template would be significant. Thus, under conditions where the all-γ DnaX complex population is ineffective for catalyzing the initiation reaction, the τ-containing complexes could still be sufficient to extend a detectable fraction of the primer/template. This τ-complex activity would contribute a significant background signal to experiments with the γ-complex. An example of this phenomenon is shown in Figure S6A. This experiment was identical to the α-D403E challenge experiments described in the main text, performed with γ-complex that had not undergone the α affinity procedure described above to remove trace τ. The assay was conducted at 4 nM Pol III, 2 nM γ-complex, 50 nM β_2, 0.20 mM ATP, 1 nM primer/template, and 0.25 μM SSB_4, with 90 s initiation times. In contrast to the fully purified γ-complex, which was inhibited by α-D403E under all conditions (Figure 1B) and by SSB (Figure 2A), the γ-complex purified only by standard methods showed detectable initiation complex formation (~12% primer elongation) in the presence of saturating concentrations of both α-D403E and SSB when the complex was pre-associated with wild-type Pol III (Figure S6A). This result is indicative of a τ contamination of 1-2% of the total DnaX subunit concentration.

This trace τ population (indicated by an arrow in lane 3, Figure S6B), was effectively removed by binding the τ-containing complexes to biotin-tagged α immobilized on streptavidin/agarose beads. This procedure (see Proteins and Nucleic

Acids section above) depleted τ from the purified γ-complex (lane 4, Figure S6B) and showed τ-enriched DnaX complex retained on the beads (lane 8, Figure S6B). The affinity purified γ-complex shows no sign of τ-like activity, with no resistance to a α-D403E challenge (Figure 1B). The purification had no effect on the specific activity of the γ-complex in our standard Pol III holoenzyme reconstitution assay described elsewhere (McHenry and Crow, 1979; Pritchard and McHenry, 1999), with a value of 2 x 10^7 pmol mg^{-1} min^{-1} observed both before and after exposure to the beads. γ-complex purified by a previously described SP-Sepharose chromatography procedure that separates DnaX complexes of various τ/γ stoichiometries (Pritchard *et al.*, 2000) showed identical effects as γ-complex purified by the α affinity beads with α-D403E and with SSB (data not shown). These results demonstrate that the affinity bead purification procedure does not damage the sample. The α affinity bead method is a simple and rapid method for eliminating the possibility of any τ contaminations that could cause spurious effects like those in Figure S6A, and all γ-complex used throughout this study was subjected to the α affinity bead procedure.

SUPPLEMENTAL REFERENCES

Carter,J.R., Franden,M.A., Aebersold,R., Kim,D.R., and McHenry,C.S. (1993) Isolation, Sequencing and Overexpression of the Gene Encoding the θ Subunit of DNA Polymerase III Holoenzyme Nucleic Acids Res 21: 3281-3286.

Glover,B.P., McHenry,C.S. (2000) The DnaX-binding Subunits δ' and ψ are bound to γ and not τ in the DNA Polymerase III Holoenzyme J Biol Chem 275: 3017-3020.

Griep,M.A., McHenry,C.S. (1989) Glutamate Overcomes the Salt Inhibition of DNA Polymerase III Holoenzyme J Biol Chem 264: 11294-11301.

Hobbs,M.D., Sakai,A., and Cox,M.M. (2007) SSB protein limits RecOR binding onto single-stranded DNA J Biol Chem 282: 11058-11067.

Johanson,K.O., Haynes,T.E., and McHenry,C.S. (1986) Chemical Characterization and Purification of the β Subunit of the DNA Polymerase III Holoenzyme from an Overproducing Strain J Biol Chem 261: 11460-11465.

Kim,D.R., McHenry,C.S. (1996a) Biotin Tagging Deletion Analysis of Domain Limits Involved in Protein-Macromolecular Interactions: Mapping the τ Binding Domain of the DNA Polymerase III α Subunit J Biol Chem 271: 20690-20698.

Kim,D.R., McHenry,C.S. (1996b) *In Vivo* Assembly of Overproduced DNA Polymerase III: Overproduction, Purification, and Characterization of the α, α–ε, and α–ε–θ Subunits J Biol Chem 271: 20681-20689.

McHenry,C.S., Crow,W. (1979) DNA Polymerase III of *Escherichia coli*: Purification and Identification of Subunits J Biol Chem 254: 1748-1753.

Pritchard,A.E., Dallmann,H.G., Glover,B.P., and McHenry,C.S. (2000) A novel assembly mechanism for the DNA polymerase III holoenzyme DnaX complex: association of δδ' with DnaX(4) forms DnaX(3)δδ' EMBO J 19: 6536-6545.

Pritchard,A.E., McHenry,C.S. (1999) Identification of the Acidic Residues in the Active Site of DNA Polymerase III J Mol Biol 285: 1067-1080.

Roy,R., Kozlov,A.G., Lohman,T.M., and Ha,T. (2007) Dynamic Structural Rearrangements Between DNA Binding Modes of *E. coli* SSB Protein J Mol Biol 369: 1244-1257.

Scheuermann,R.H., Echols,H. (1984) A Separate Editing Exonuclease for DNA Replication: the ε Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme Proc Natl Acad Sci U S A 81: 7747-7751.