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Strand Displacement by DNA Polymerase III Occurs through a τ-ψ-χ Link to Single-stranded DNA-binding Protein Coating the Lagging Strand Template*

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In addition to the well characterized processive replication reaction catalyzed by the DNA polymerase III holoenzyme on single-stranded DNA templates, the enzyme possesses an intrinsic strand displacement activity on flapped templates. The strand displacement activity is distinguished from the single-stranded DNA-templated reaction by a high dependence upon single-stranded DNA binding protein and an inability of γ-complex to support the reaction in the absence of τ. However, if γ-complex is present to load β_2, a truncated τ protein containing only domains III–V will suffice. This truncated protein is sufficient to bind both the α subunit of DNA polymerase (Pol) III and χψ. This is reminiscent of the minimal requirements for Pol III to replicate short single-stranded DNA-binding protein (SSB)-coated templates where τ is only required to serve as a scaffold to hold Pol III and χ in the same complex (Glover, B., and McHenry, C. (1998) *J. Biol. Chem.* 273, 23476–23484). We propose a model in which strand displacement by DNA polymerase III holoenzyme depends upon a Pol III-τ-ψ-χ-SSB binding network, where SSB is bound to the displaced strand, stabilizing the Pol III-template interaction. The same interaction network is probably important for stabilizing the leading strand polymerase interactions with authentic replication forks. The specificity constant (k_{cat}/K_m) for the strand displacement reaction is ~300-fold less favorable than reactions on single-stranded templates and proceeds with a slower rate (150 nucleotides/s) and only moderate processivity (~300 nucleotides). PriA, the initiator of replication restart on collapsed or misassembled replication forks, blocks the strand displacement reaction, even if added to an ongoing reaction.

All cellular replicases are tripartite assemblies, consisting of a replicative polymerase (Pol[2] III in bacteria, Pol δ or ε in eukaryotes), a sliding clamp processivity factor (β_2 in bacteria, proliferating cell nuclear antigen in eukaryotes), and a clamp loader composed of a five-protein core of AAA$^+$-like proteins that assembles the sliding clamp around DNA (DnaX complex in bacteria, RFC in eukaryotes) (1–3). The *Escherichia coli* DnaX complex comprises three copies of DnaX and one each of δ, δ′, and χψ (4–6). *E. coli* and many other bacteria contain two forms of DnaX, the full-length τ translation product and a shorter protein, γ, that results from translational frameshifting (7–9). Both τ and γ contain the three domains that are required for ATP-dependent β_2 loading onto DNA (6). The third domain of τ and γ is responsible for binding other DnaX subunits as well as δ, δ′, and χψ (5, 10, 11). τ contains two additional domains that interact with the DnaB replicative helicase (domain IV) and Pol III (domain V) (12, 13).

The primary determinant of processivity of the *E. coli* replicase is the interaction of Pol III with β_2 (14, 15). However, other interactions stabilize the interaction of Pol III with the replication fork. Two τ protomers bind the DnaB helicase, further stabilizing the replicase at the fork (16, 17). Pol III alone is unable to replicate single-stranded DNA (ssDNA) coated by SSB. To accomplish this feat, τ, ψ, and χ must be present if β_2 is absent (18). τ does not serve its prototypical role as the clamp loader in this minimal system but apparently only serves as a bridge, tethering χψ in the same complex with Pol III, enabling an otherwise weak Pol III-template interaction to be stabilized by a χ-SSB contact (18).

During processive replication of long single-stranded templates, the Pol III HE typically stops synthesis upon encountering a duplex (19). The elongation reaction is very rapid (400–700 nt/s) and exhibits processivity that may enable replication of the entire *E. coli* chromosome without dissociation (1). However, a more feeble strand displacement activity of the DNA polymerase III holoenzyme has been observed under a variety of conditions (20–23).

Canceill and Ehrlich (20) observed that Pol III HE could replicate through a 30-nt stem. A mechanism was proposed where the enzyme could non-processively elongate a few nucleotides when base pairing is transiently disrupted. Stephens and McMacken (21) observed more extensive strand displacement synthesis on flapped templates using native Pol III HE in a reaction that was dependent upon SSB. Xu and Marians (22), in studies of replicative resolution of recombination intermediates, observed a background strand displacement reaction in the absence of helicase. O'Donnell and co-workers have also observed strand displacement of oligonucleotides containing large internal secondary structures (23).

Observations of the strand displacement activity of Pol III HE have occurred peripheral to studies conducted for other pur-

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[2] The abbreviations used are: Pol, DNA polymerase; Pol III HE, DNA polymerase III holoenzyme; Pol III, DNA polymerase III; ssDNA, single-stranded DNA; SSB, single-stranded DNA-binding protein; nt, nucleotide(s); Pol III*, Pol III$_3$τ$_3$δδ′χψ.

poses. No investigation has been made aimed at understanding the reaction, its protein requirements, and how it might differ from the well characterized reaction that occurs on single-stranded templates. This study was directed toward remedying these deficiencies in knowledge. We found that an interaction of the leading strand polymerase with the lagging strand template, mediated by a Pol III-τ-ψ-χ-SSB bridge, was essential for efficient strand displacement. This interaction network probably stabilizes the replicase at the fork in addition to the characterized Pol III-β_2 and τ-DnaB interactions.

EXPERIMENTAL PROCEDURES

Proteins—*E. coli* DNA Pol III HE protein subunits were purified as previously described: SSB (24), β (25), DnaG (24), Pol III* (Pol III$_3\tau_3\delta\delta'\chi\psi$) (4), Pol III (26), τ (27), $\chi\psi$ (28), τ-complex (29), γ-complex (29), τ_{III-V} (5), and τ_{IV-V} (12). Complexes of τ derivatives with $\chi\psi$ (τ-ψ-χ, τ_{III-V}-ψ-χ, and τ_{IV-V}+ψ-χ) were made by the incubation of equimolar τ, τ_{III-V}, or τ_{IV-V} with $\chi\psi$ for 20 min at 30 °C. SSB-cΔ42 (30) and SSB-cΔ8 (31) were obtained from the laboratories of Tim Lohman (Washington University) and Mike Cox (University of Wisconsin), respectively.

Oligonucleotides—The sequence of the 32-mer primer was 5′-T-dU(5-biotin)-GAACGGTGTACAGATCACGCGCATAGGCTG-3′. The sequence of the 50-mer flap was 5′-TTTTTTTTTTTTTTTTTTTTTTTCTGTGTTTTGTTGTCTTGTTCCCTG-3′. The sequence of the 67-mer primer was 5′-T-dU(5-biotin)-GAACGGTGTACAGATCACGCGCATAGGCTGCCTTTTCTCGTGTTGGCCTGGGGTCCGCTGTCTCT-3′. The sequence of the 91-mer template was 5′-biotin linked by a phosphodiester and a 6-carbon spacer to CAGGGAACAAGACAACAAAACACAAGAGACAGCGGACCCCAGGCCAACACGAGAAAAGGCAGCCTATGCGCGTGATCTGTACACCGTTCAA-3′.

DNA Templates—M13Gori ssDNA, M13Gori template with a 30-nt primer, and activated calf thymus DNA were prepared as described previously (25, 32, 33). pUCNICK tail DNA was generated from plasmid pUCNICK (2,716 bp) (34) and purified by Qiagen plasmid Maxi prep. Purified plasmid DNA (30 μg, 1 μg/μl) was nicked at the single recognition site with Nb.BbvCI nicking enzyme (300 units; New England Biolabs) at 37 °C for 4 h. Nb.BbvCI was thermally inactivated (80 °C, 20 min). A 1% agarose gel showed that the nicking reaction was >90% complete. The nicked plasmid was precipitated by the addition of 0.5 volumes of 5 M ammonium acetate and 1.5 volumes of isopropyl alcohol, and the pellet was washed with 70% ethanol and dissolved in 10 mM Tris-HCl buffer (pH 8) to a 0.2 μg/μl final concentration. Glycerol was added to a 13% final concentration. This solution was incubated with 200 μM dATP, dGTP, and dTTP and Klenow fragment (exo$^+$; 2 units/μg of DNA) at 37 °C for 30 min, so that the 61-nt-long cytosineless DNA flap was formed. EDTA was added to a final concentration of 20 mM. Tailed pUCNICK (2,777 nt) was precipitated with isopropyl alcohol and dissolved in 10 mM Tris-HCl (pH 8) at a concentration of 1 μg/μl. Tailing efficiency, determined by PvuII digestion and 1.8% agarose gel electrophoresis, was >90% complete.

[^{32}P]dTTP-labeled pUCNICK tail template was prepared with the same protocol used for preparation of unlabeled pUCNICK tail, except that 0.5 Ci/liter [α-^{32}P]dTTP was added with the other dNTPs. Unincorporated [α-^{32}P]dTTP was removed by the tandem use of a Microspin G-25 spin column and a NAP-5 column (GE Healthcare). The pooled solution was precipitated with 95% ethanol, the pellet was washed with 70% ethanol, and the DNA was dissolved in 10 mM Tris-HCl (pH 8). The 32- and 67-mer were 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Unincorporated [γ-^{32}P]ATP was removed by a Microspin G-25 spin column. The 32-mer and 91-mer were mixed in 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 200 mM NaCl in a 1:1 ratio for the template 32/91, heated to 95 °C for 5 min, incubated at 65 °C for 1 h, slowly cooled to 25 °C over 2 h, and incubated at 25 °C for 30 min. The 32-mer or 67-mer primer, 50-mer, and 91-mer were incubated at a 1:2:1 ratio to ensure that all templates contained a blocking, flapped oligonucleotide. Native polyacrylamide gel electrophoresis confirmed that no 32/91 or 67/91 existed as contaminants in 32/50/91 or 67/50/91. A 10-fold excess of streptavidin was incubated with 32/91, 32/50/91, and 67/50/91 in 10 mM Tris-HCl (pH 8) at room temperature to form bumpers to prevent loaded β_2 from sliding off.

Single-stranded Replication Assays—Single-stranded replication assays contained 2.3 nM M13Gori DNA, 0.6 μM SSB_4, 6 nM β_2, 40 nM DnaG, 10 nM Pol III, and 4 nM τ-complex. The reaction mixture was incubated with 0.2 mM UTP, CTP, and GTP, 0.3 mM ATP, 18 μM [^{3}H]dTTP (~100 cpm/pmol total nucleotide), 48 μM dATP, 48 μM dGTP, and 48 μM dCTP for 5 min at 30 °C in 25 μl. All replication reactions were quenched by 100 μl of 0.2 M sodium pyrophosphate and 500 μl of 10% trichloroacetic acid unless stated otherwise. Precipitated product was quantified by scintillation counting. All reactions with M13Gori templates were conducted with the single-stranded replication buffer: 10 mM magnesium acetate, 50 mM Hepes (pH 7.5), 100 mM potassium glutamate, 20% glycerol, 200 μg/ml bovine serum albumin, 0.02% Nonidet P-40, and 10 mM dithiothreitol.

Strand Displacement Assays—4 nM pUCNICK tail, 100 μM ATP, 0.75 μM SSB_4, 25 nM β_2, 51 nM Pol III, 17 nM τ-complex, 300 μM dNTPs, and ~130 cpm/pmol [^{3}H]TTP were incubated for 5 min at 30 °C in 25 μl. The strand displacement buffer was the same as the single-stranded replication buffer, except it contained 20 mM NaCl. Incorporation was expressed in terms of total nucleotide by multiplying the total dTTP incorporated by 4 (pUCNICK contains 24.7% T).

Determination of the Rate of Strand Displacement—Initiation complexes between Pol III HE and flapped templates were formed by incubating 5 nM pUCNICK tail, 125 μM ATP, 0.94 μM SSB_4, 31 nM β_2, and 25 nM Pol III* at 30 °C for 1 min. Then 375 μM dNTPs and 5 μCi/reaction [α-^{32}P]dTTP were added to initiate strand displacement. Aliquots of the 20-μl reaction were quenched by 100 mM EDTA (final concentration) at various time points. The samples were loaded along with alkaline agarose gel loading buffer (30 mM NaOH, 2 mM EDTA, 1% glycerol, and 0.02% bromphenol blue) to 0.5% alkaline agarose gels in a running buffer of 30 mM NaOH and 2 mM EDTA. Gels were run at 22 V for 18.5 h, fixed in 5% trichloroacetic acid, dried, and scanned by a PhosphorImager. The molecular weights of the longest reaction products were determined by

measuring their relative mobilities (compared with ^{32}P- labeled DNA size marker) on the alkaline agarose gel.

Determination of the Processivity of Pol III HE on a Short-flapped Template—Challenge assays were performed to determine processivity. In these assays, a large excess of activated calf thymus DNA (challenge) was added to elongating complexes to trap dissociated polymerase so that products represented a single processive association-elongation-dissociation event. Solutions containing strand displacement buffer (125 μM ATP, 0.31 μM SSB_4, 31 nM β_2, and 10 nM Pol III*) were prewarmed and mixed with 1.5 nM γ-^{32}P-labeled 32/91, 32/50/91, or 67/50/91 at 30 °C for 15 s to form the initiation complexes. To block ϵ-catalyzed primer degradation, 10 μM dGTP was added for 32/91 and 32/50/91 templates, and 10 μM dTTP and dCTP was added for 67/50/91 during incubation. Then 375 μM dNTPs (final concentration) and 20 μg of activated calf thymus DNA were added to allow the reaction to occur. After 10 s, 55% formamide, 50 mM EDTA, 0.0083% bromphenol blue, and 0.0083% xylene cyanol (final concentrations) were added to quench the 20-μl reaction. The solution was fractionated on 12% polyacrylamide gel with 8.2 M urea for 3 h at 95 watts. The gel was scanned with a PhosphorImager and quantified with ImageQuant version 5.2 software (Amersham Biosciences). Controls to show that the challenge was effective followed the same procedure, except activated calf thymus DNA was added with the templates before the addition of Pol III HE.

Determination of the Processivity of Pol III HE on a Rolling Circle Template—In this assay, prewarmed 1 nM pUCNICK tail, 100 μM ATP, 0.75 μM SSB_4, 25 nM β_2, and 10 nM Pol III* were assembled to form holoenzyme initiation complexes for 20 s at 30 °C. Then 300 μM dNTPs (final concentration) and 5 μg of activated calf thymus DNA were added to initiate the reaction. This 25-μl reaction was carried out at 30 °C for various time periods and stopped by 83 mM EDTA (final concentration). Each reaction sample was precipitated by the addition of 0.5 volume of 5 M ammonium acetate, 20 μg of glycogen, and then 2.5 volumes of 95% ethanol. The pellet was washed by 70% ethanol and dissolved in 20 μl of 10 mM Tris-HCl buffer (pH 8). DNA was then digested by 75 units of EcoRI at 37 °C for 18 h. Unchallenged strand displacement assays followed the same procedure, except activated calf thymus DNA was added just prior to EcoRI digestion to ensure uniformity in sample workup. After digestion, all samples were treated at 95 °C for 5 min with alkaline agarose gel loading buffer and then loaded onto 1.5% alkaline agarose gels in a running buffer of 30 mM NaOH and 2 mM EDTA. Gels were run at 100 V for 2 h and then fixed in 5% trichloroacetic acid, dried, and scanned by a PhosphorImager. The product bands were quantified by ImageQuant 5.2 software (Amersham Biosciences). Because the reaction products were diffuse, the center of the product in each lane (r_{avg}) was defined as the average length of the product. The whole product was sliced into pieces about every 100 nucleotides. $r_{avg} = \Sigma(r_i \times P_i)/\Sigma P_i$, where r_i represents the average length of product slice i, and P_i is the pixel of product slice i. The processivity of Pol III* was calculated by subtracting the primer length, 67 nt, from r_{avg}.

PriA Inhibition Assay—To test whether PriA could inhibit strand displacement before the initiation complex formed, 10 nM pUCNICK tail, 0.94 μM SSB_4, 31 nM β_2, 125 μM ATP, 375 μM dNTPs, and 100 cpm/pmol [^{3}H]TTP were mixed first and then incubated with 10 nM Pol III* and varying amounts of PriA for 5 min at 30 °C in 20 μl. To test whether PriA could inhibit strand displacement after initiation complex formation, the same procedure was followed, except that protein components were first incubated with pUCNICK tail in the presence of ATP at 30 °C for 2 min, and then dNTPs were added with PriA. For assays on ssDNA templates, PriA was added following the same sequence as described above either before or after the initiation complex formed. Every other component contained the same concentration as described under "Single-stranded Replication Assay," except that 10 nM Pol III* replaced Pol III and τ-complex. To examine whether PriA could inhibit ongoing strand displacement, 8 nM pUCNICK tail was incubated with 100 μM ATP, 0.75 μM SSB_4, 25 nM β_2, and 8 nM Pol III* at 30 °C for 1 min to form initiation complexes. 300 μM dNTPs and [^{3}H]TTP were then added to start strand displacement. At 45 s after the reaction started, 20, 60, or 0 nM PriA was added. At different time points, aliquots of the 25-μl reaction solution were quenched by 20 mM EDTA (final concentration).

RESULTS

An initial characterization of the strand displacement activity of Pol III HE was performed on a circular template with a 61-nt flap. Protein components were titrated and compared with the requirement for standard processive assays on long single-stranded templates (Fig. 1). Approximately twice as much Pol III was required for the strand displacement reaction, probably a consequence of the decreased processivity of Pol III HE in the strand displacement reaction (see below). Both τ-complex and β_2 were required to support the strand displacement reaction at protein levels approximately the same as required on single-stranded templates. Strikingly, the γ form of DnaX could not be substituted for τ in the strand displacement reaction, in contrast to reactions on single-stranded DNA templates. Another significant difference was observed in the SSB requirement. As is typically observed, SSB stimulated the Pol III HE marginally (~2-fold) on preprimed single-stranded templates; the dependence was nearly absolute for strand displacement.

Another profound difference became apparent upon varying dNTP concentrations (Fig. 1*F*). In the ssDNA reaction, a low micromolar K_m was measured. However, nearly 100-fold higher concentrations of dNTPs were required to drive the strand displacement reaction. To obtain a comparison of the specificity differences of the two reactions, we calculated k_{cat}/K_m that revealed a 330-fold preference of Pol III HE for synthesis on an ssDNA template compared with a duplex, during strand displacement (Table 1). A possible explanation for the high K_m for strand displacement could be the need for a rapid second-order nucleotide association reaction being required to trap an intermediate that lies on the elongation pathway and competes with steps that lead to dissociation. It is also possible that association of the polymerase with a displaced strand or loss of ssDNA template contacts might place it into an alternative conformation with a distorted active site that interacts with dNTPs less favorably.

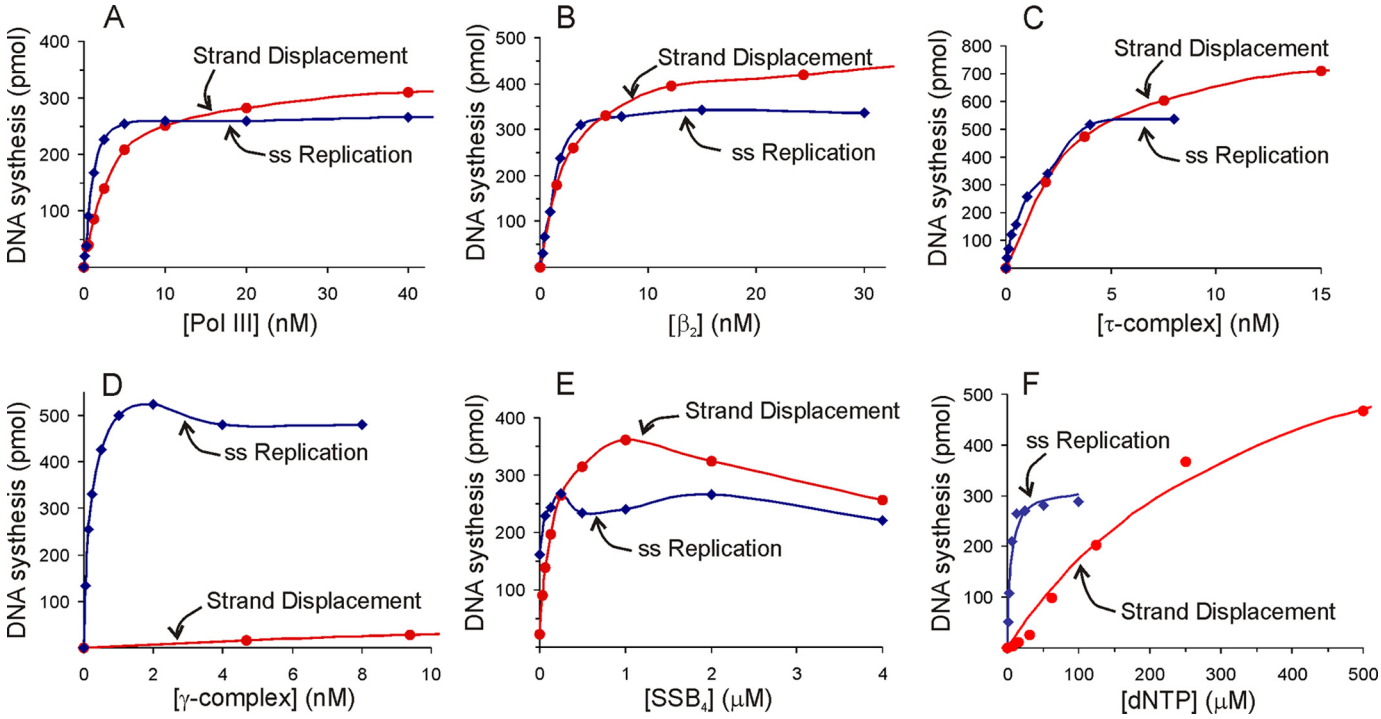

FIGURE 1. **Requirements for Pol III HE components for strand displacement.** Pol III HE protein subunits were titrated in single-stranded (*ss*) replication reactions (♦) and strand displacement reactions (●) in the presence of other protein subunits at optimal saturating levels as described under "Experimental Procedures." *A*, titration with Pol III. *B*, titration with β_2. *C*, titration with τ-complex. *D*, titration with γ-complex. *E*, titration with SSB_4. In this single experiment, an M13Gori template primed by annealing a 30-nt primer was used instead of DnaG primase-primed template, so the elongation requirement of SSB could be determined separately from the absolute requirement for DnaG-catalyzed primer formation. *F*, titration with dNTPs.

TABLE 1

Steady state kinetic parameters for Pol III HE in single-stranded replication and strand displacement

The dNTP titration curves were fit to the Michaelis-Menton kinetic function, $1/v = (K_m/v_{max}[S]) + (1/v_{max})$, by nonlinear least-squares regression to determine both v_{max} and K_m. [S] was the concentration of dNTPs, and v was the rate of dNTP incorporation (pmol/min). The value for k_{cat} at 30 °C for the strand displacement reaction was taken from the data reported in Fig. 4; that for the ssDNA reaction was taken from Ref. 43.

	Single-stranded replication	Strand displacement
K_m (μM)	4.5	380
k_{cat} (s^{-1})	570	150
k_{cat}/K_m ($s^{-1}\cdot\mu M^{-1}$)	130	0.39

Next, we asked whether the τ requirement was a manifestation of a unique role for τ in loading β_2 onto DNA or whether τ performed another function, separate from β_2 loading. To address this issue, we loaded β_2 onto the DNA templates with γ-complex and determined the contribution of various forms of τ. Adding τ alone had little effect, but adding a complex of τ-ψ-χ, which alone is inactive in β_2 loading, stimulated the strand displacement reaction significantly (Fig. 2*A*). τ and γ, in the absence of other proteins, exchange very slowly. The presence of δ, δ', and $\chi\psi$ blocks exchange, eliminating the possibility that τ, added briefly to reactions, exchanges into the γ-complex (35). Furthermore, if the result obtained was due to such an exchange reaction, $\chi\psi$ would not be required, since it is already present in the γ-complex.

In earlier work, we observed an effect of τ in enabling Pol III to replicate ssDNA coated with SSB, which required only its function of binding $\chi\psi$. In this example, τ held χ in the same complex with Pol III, enabling it to bind template-bound SSB and stabilizing an otherwise weak interaction (18). Thus, we added a protein that comprises domains III–V of DnaX, τ_{III-V}. DnaX domain III binds $\chi\psi$, and domain V binds the α subunit of Pol III (5, 12). If the only function of τ is to link Pol III and χ, this truncated protein should suffice to stimulate strand displacement when γ-complex is present to load β_2. Indeed, τ_{III-V} stimulated strand displacement the same amount as full-length τ bound to $\chi\psi$, although higher concentrations were required (Fig. 2*B*). The addition of an equimolar mixture of τ_{IV-V} and $\chi\psi$ did not stimulate the reaction. τ_{IV-V} binds the α subunit of Pol III but not $\chi\psi$.

The above results are consistent with a critical χ-SSB contact required to stabilize the strand displacement reaction. χ interacts with the C-terminal tail of SSB (36, 37). To further test the existence and importance of this interaction in the strand displacement reaction, we replaced wild-type SSB with two C-terminal SSB proteins that had 8 and 42 amino acids deleted from their C termini (30, 31). We observed that neither supported strand displacement (Fig. 3*A*), consistent with our hypothesis. As a control experiment, we tested the effect of both on the Pol III HE single-strand templated reaction and observed an inhibition, although there is not much of a requirement for SSB in the normal reaction (Fig. 3*B*). Thus, deletion of the C terminus of SSB creates a gain of an inhibitory function for SSB, presumably because the protein-interacting tail is not available for modulation of binding state (30) or displacement from the template.

We also determined the rate of elongation for the strand displacement reaction by determining the length of the longest products visible on a denaturing gel starting with labeled primer (Fig. 4). We observed a rate of 150 nt/s, slower than the

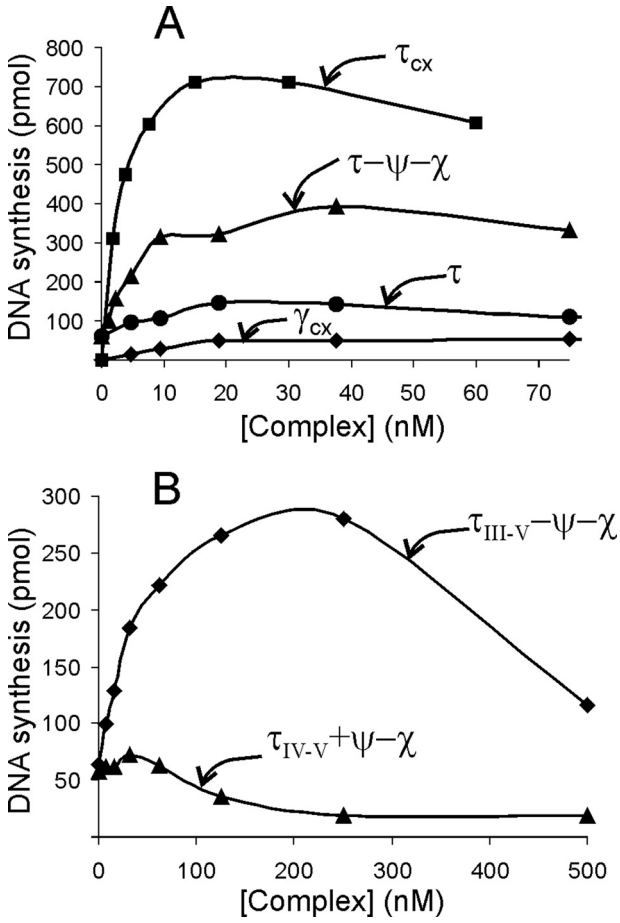

FIGURE 2. **A Pol III-τ-ψ-χ complex is required for strand displacement.** Strand displacement reactions were conducted using 4 nM pUCNICK tail, 0.75 μM SSB_4, 25 nM β_2, 50 nM γ-complex, 100 μM ATP, 300 μM dNTPs, and [^{3}H]TTP. Reactions were assembled on ice with τ-containing complexes of the specified composition. Pol III (final concentration, 51 nM) was added last (25 μl final volume). Reactions were carried out at 30 °C for 5 min. The form of τ-containing complexes in the various complexes was as follows: τ-complex ($\tau_3\delta\delta'\chi\psi$) (■), γ-complex ($\gamma_3\delta\delta'\chi\psi$) (◆), τ (●), and τ-ψ-χ (▲) (*A*); τ_{III-V}-ψ-χ (◆) and τ_{IV-V} + ψ-χ (▲) (*B*).

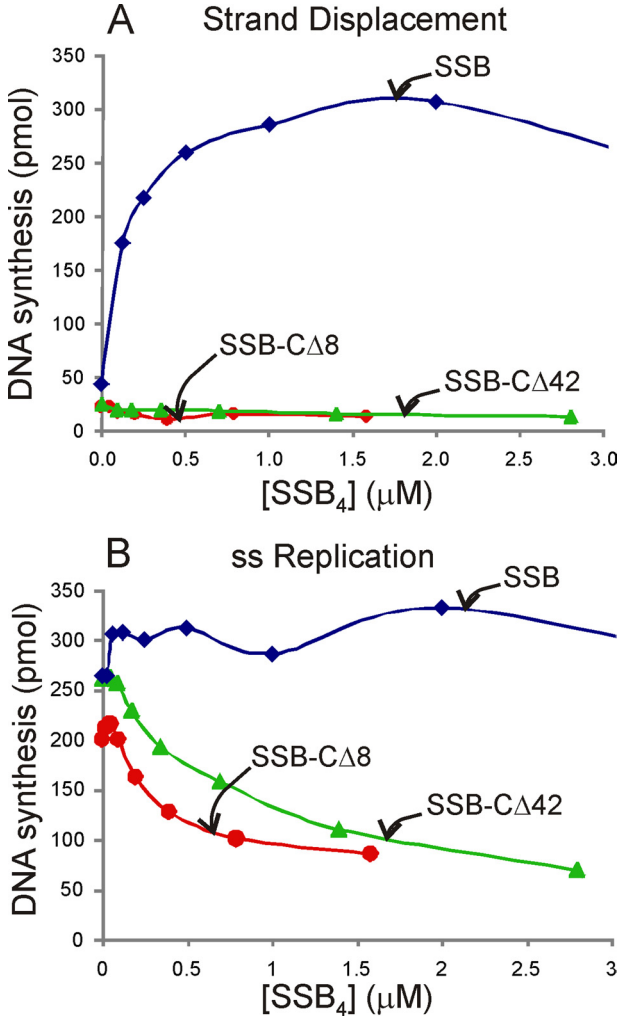

FIGURE 3. **The C-terminal protein interaction sequence of SSB is required to support strand displacement.** SSB_4 titrations in the strand displacement reaction (*A*) and single-stranded (*ss*) templated reaction (*B*) were conducted as described for Fig. 1*E* except the SSB-cΔ42 (▲) and SSB-cΔ8 (●) proteins were substituted for wild-type SSB (◆) in the designated reactions.

400–700 nt/s typically observed for the elongation reaction catalyzed by Pol III HE on ssDNA.

Based on an initial expectation of low processivity, we made a series of synthetic templates with a common 91-nt segment (Fig. 5). One was simply primed at the 3′-end of the template with no other oligonucleotides annealed (template a). Two contained flapped blocking oligonucleotides, one with a 35-nt gap between the primer terminus and the flap (template b) and the other with the primer terminus abutting the flap junction (template c). Processivity was determined by first forming initiation complexes and then adding an excess of challenge DNA to capture any polymerase that dissociated during elongation. The efficacy of the challenge template was demonstrated by complete inhibition if added with the template before the addition of enzymes (Fig. 5, *lanes 4–6*). In the absence of a challenge, all templates were nearly completely elongated to the expected full-length 91-nt product (*lanes 7–9*). If initiation complexes were formed prior to the addition of the challenge template concomitant with dNTPs, again most of the primer was elongated to full-length product (*lanes 10–12*), indicating a processivity greater than 24 for strand displacement. However, 30% of the elongated product on template b terminated when the Pol III HE encountered the flap (*lane 11*), although little product of a length intermediate between 67-mer and 91-mer was detected (<2%).

Because the processivity was too high to estimate on short, linear templates, we turned to longer flapped templates. Initial experiments failed to resolve the product of processive synthesis from the 2,777 nt starting material, indicating limited processivity. To permit resolution, we labeled the primer for the template during creation of the flap, limiting the position of radioactive nucleotides to the 3′-terminal 61 nt. After elongation, this permitted cleavage with restriction endonuclease EcoRI, generating a product of 67 nt plus the number of nt added during the elongation event. This permitted better product resolution. Using this longer template, a challenge experiment was conducted, similar to the one performed on the short, synthetic template above. The presence of a challenge template mixed with the labeled template prior to enzyme addition inhibited the elongation reaction (Fig. 6, *lanes 8* and *9*). Preformation of initiation complexes followed by the addition of the

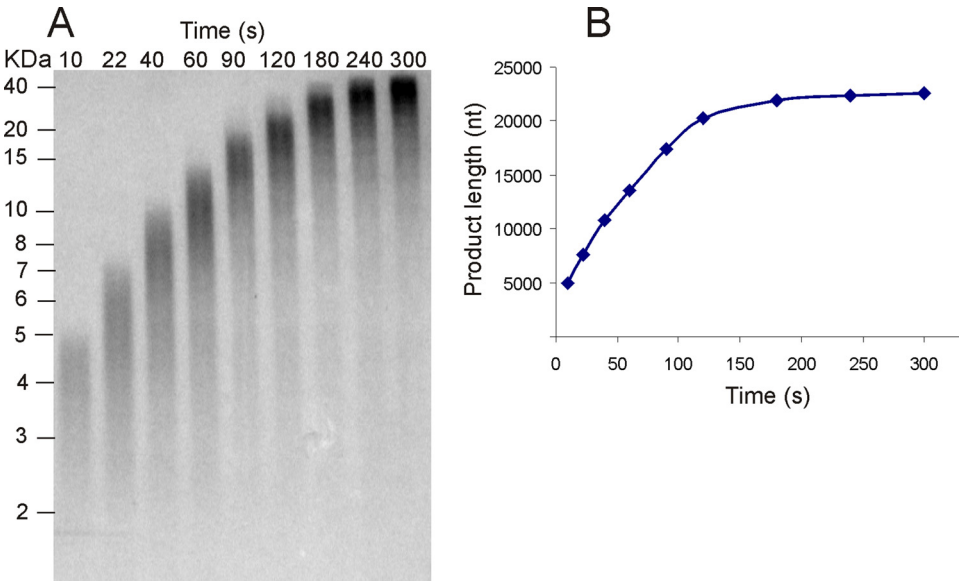

FIGURE 4. **The rate of strand displacement by Pol III HE is 150 nt/s.** *A*, ^{32}P-labeled products of strand displacement using labeled primer were monitored on a 0.5% alkaline agarose gel. *B*, the longest product at each time point was plotted as a function of time, and the rate was determined by the slope of a line intersecting the first five points.

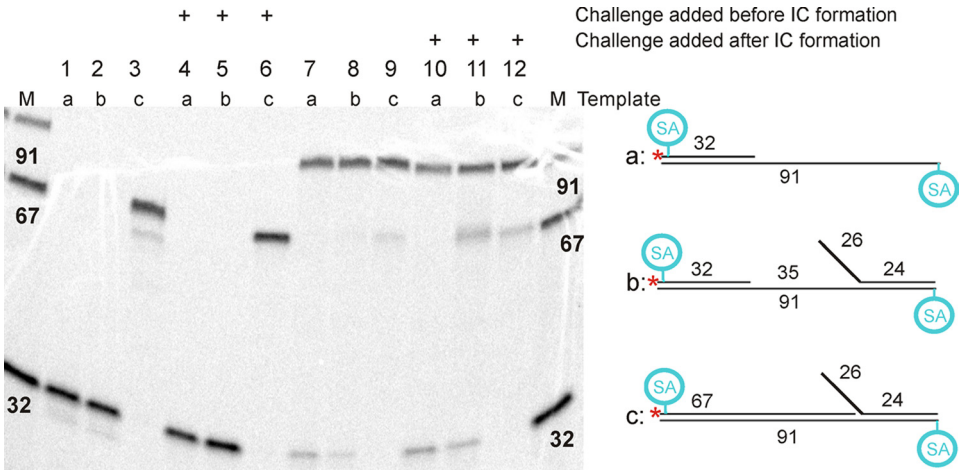

FIGURE 5. **Approximately one-third of the elongating Pol III HE dissociates upon encountering a flap and then displaces greater than 24 nucleotides processively.** *Left*, a 12% polyacrylamide denaturing gel showing the products of processivity determination experiments. *Lanes 1–3*, untreated templates showing the positions of unextended labeled primers; *lanes 4–6*, challenge DNA was added before initiation complex (*IC*) formation; *lanes 7–9*, DNA replication assays conducted without challenge DNA; *lanes 10–12*, challenge DNA was added after the initiation complex formation. *M*, the markers of the 32-, 67-, and 91-mer. The percentages of 32-mer (relative to other bands in the same lane) were 20, 8.3, 34, and 14% for *lanes 7*, *8*, *10*, and *11*, respectively; percentages of 67-mer were 0.2, 7.6, 26, and 19% for *lanes 8*, *9*, *11*, and *12*, respectively; and percentages of 91-mer were 80, 92, 92, 66, 61, and 81% for *lanes 7–12*, respectively. *Right*, the three DNA templates (*a–c*) with ^{32}P-labeled primers (indicated by an *asterisk*) made as described under "Experimental Procedures." *SA*, positions of streptavidin attachment.

challenge template with dNTPs limited synthesis (Fig. 6, *lanes 10–13*) relative to the unchallenged controls (*lanes 4–7*), indicating limited processivity. Since processivity is an intrinsic property of an enzyme, the length of a processive product should not be affected by incubation time. We observed that the product length remained unchanged beyond the initial 5 s time point. Thus, the 10–20 s products from the elongation experiment (Fig. 6, *lanes 11–13*) were used to calculate processivity. The population of products as a function of length was quantified, and an average processivity of 280 nt was calculated.

Xu and Marians (22) have observed helicase-independent strand displacement by Pol III HE in complex reactions where recombination intermediates are resolved replicatively. We investigated whether PriA could block the strand displacement reaction by Pol III HE in the simpler system we use, where SSB is the only other protein present. We observed that PriA blocked the strand displacement reaction whether or not an initiation complex was formed between the Pol III HE and DNA prior to the PriA addition. In contrast, PriA had no effect on the Pol III HE-catalyzed reaction on ssDNA templates (Fig. 7*A*). We also investigated whether PriA could halt an ongoing elongation reaction (Fig. 7*B*). Initiation complexes were formed on flapped templates, and 45 s after initiation, PriA was added, resulting in an immediate block in the presence of 56 nM PriA.

DISCUSSION

We observed that the Pol III HE has an intrinsic strand displacement reaction that has properties markedly different from those of the well studied synthesis reaction catalyzed on ssDNA templates. Both reactions require Pol III, β_2, and a clamp loader. However, unlike the ssDNA-templated reaction, SSB is nearly absolutely required for strand displacement. It only modestly stimulates reactions on ssDNA templates when the complete Pol III HE is present. Even more striking is the observation that γ-complex alone is not effective; τ must be present for strand displacement to occur. However, the unique requirement for τ is not a consequence of its β_2 clamp loading activity. If γ-complex is provided to load β_2, a truncated τ protein that lacks the critical domains required for ATP binding and hydrolysis (domains I and II) will serve to drive strand displacement. The truncated τ must contain domain III, the $\chi\psi$ binding domain (5). The C-terminal tail of SSB that is involved in a variety of protein interactions (38) is required.

These observations are reminiscent of the minimal Pol III that is required for modest replication on SSB-coated ssDNA templates, Pol III-τ-ψ-χ, where τ only serves as a tether to hold χ and Pol III in the same complex (18). The explanation of activity in this system was that χ contacted SSB when bound to

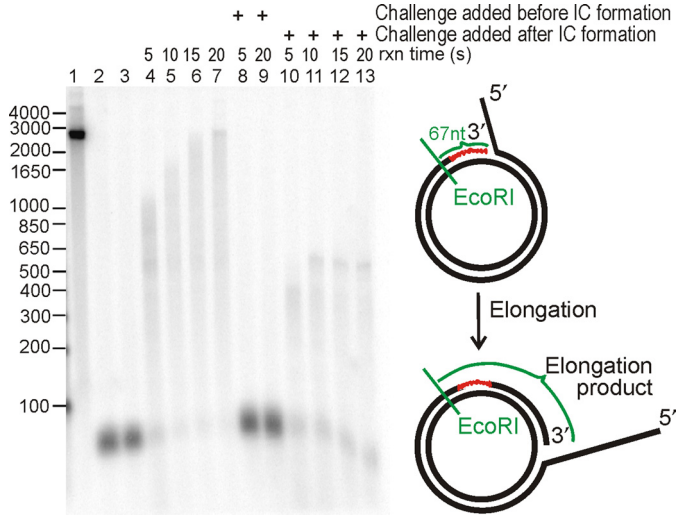

FIGURE 6. **Pol III HE adds 280 nucleotides processively during strand displacement.** Challenge experiments were performed to determine the processivity of Pol III* on the pUCNICK tail as described under "Experimental Procedures." *Left*, *lane 1*, ^{32}P-labeled pUCNICK tail without EcoRI digestion; *lane 2*, ^{32}P-labeled pUCNICK tail and unlabeled activated calf thymus DNA with EcoRI digestion; *lane 3*, ^{32}P-labeled pUCNICK tail, unlabeled activated calf thymus DNA, EDTA, Pol III HE mixture, and dNTPs were mixed sequentially, and then DNA was precipitated with ethanol and digested with EcoRI; *lanes 4–7*, strand displacement reactions of designated times; *lanes 8* and *9*, reactions where the challenge DNA was added before initiation complex formation; *lanes 10–13*, reactions where the challenge DNA was added after initiation complex formation. *Right*, pUCNICK tail strand displacement and EcoRI digestion. The *red segment* indicates the positions of ^{32}P-labeling. Two *brackets* illustrate the unextended ^{32}P-labeled 67-nt primer and the elongation product after EcoRI digestion.

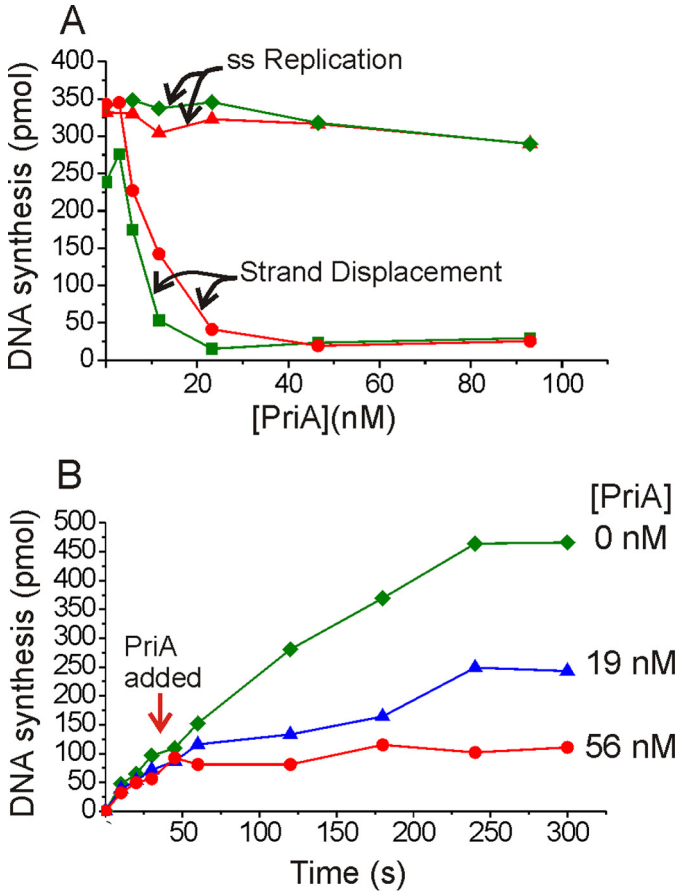

FIGURE 7. **PriA inhibits the strand displacement reaction.** *A*, PriA was titrated before (*red*) the holoenzyme initiation complex formed with pUCNICK tail (●) and M13Gori (▲) and after (*green*) the initiation complex formed with pUCNICK tail (■) and M13Gori (♦) in the presence of the optimal amounts of Pol III HE components as described under "Experimental Procedures." *B*, time course of PriA inhibition of ongoing strand displacement. 0 nM PriA (♦), 19 nM PriA (▲), or 56 nM PriA (●) was added at 45 s after the strand displacement reaction initiated. *ss*, single-stranded.

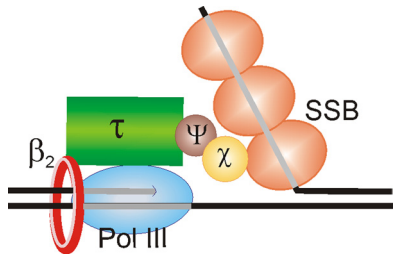

FIGURE 8. **Model for the strand displacement reaction with Pol III HE subunits.** The flap is covered by SSB, and the primer is bound with polymerase. χ connects polymerase to SSB via a τ-ψ link. Thus, SSB-χ-ψ-τ forms a bridge to stabilize interaction of Pol III with the strand displacement template and, presumably, natural replication forks where the flap would represent the lagging strand template and the polymerase shown, the leading strand half of the replicase.

ssDNA, increasing binding of the polymerase. The molecular interactions behind this protein network are well understood. Pol III binds τ in an interaction between the C terminus of Pol III and domain V of τ (12, 39, 40). One ψ protomer binds a trimeric assembly of DnaX proteins through their domain III (5, 11). ψ binds χ (41), and χ binds to SSB through its C-terminal domain (18, 36, 37).

In the strand displacement reaction, however, the only ssDNA available for SSB binding is the displaced strand. Thus, we propose the model depicted in Fig. 8, where a Pol III-τ-ψ-χ-SSB interaction stabilizes the interaction of Pol III with the template sufficiently to permit moderately processive strand displacement. It is interesting that the β_2-Pol III interaction alone is inadequate to stabilize the Pol III-template interaction sufficiently to enable strand displacement. Perhaps limited ssDNA template-Pol III contacts make additional stabilizing interactions necessary. The interaction network shown in Fig. 8 could be important for stabilizing interaction of the leading strand polymerase at the replication fork, through an interaction of the DnaX complex with the lagging strand template. Normally, a τ_2-DnaB$_6$ interaction will further stabilize the replisome, but there may be situations (*e.g.* when difficult structures are encountered during mismatch repair) where the Pol III-τ-ψ-χ-SSB interaction network becomes critical for function. In unusual cases, such as blockage of the leading strand polymerase by a lesion or other obstruction when the helicase continues to progress, single-stranded DNA would be created on the leading strand template and bound by SSB, enabling stabilization of Pol III HE interactions, perhaps providing a tether to localize Pol III HE during polymerase switching.

The Pol III HE strand displacement reaction exhibits only modest (~300 nt) processivity as compared with the proposed megabase processivity of the Pol III HE on ssDNA and the >100,0000-base processivity observed on reconstituted replication forks. Thus, even a combination of the Pol III-β_2 interaction and the τ-mediated χ-SSB contact cannot provide

sufficient stability for the highest levels of processivity. It is interesting that approximately one-third of Pol III HE dissociates upon encountering a flap while actively polymerizing yet appears to dissociate at the very low frequency required for 300-nt processivity at other positions. The enzyme presumably encounters the same structure prior to the addition of each nucleotide. It is possible that formation of the χ-SSB contact with the lagging strand template (displaced strand in Fig. 8) is on the same order as the rate of nucleotide addition and that, upon encountering a flap, a portion of the enzymes fails to form the contact in adequate time and dissociates due to weak interactions.

As first pointed out by Xu and Marians (22), a Pol III HE-catalyzed DnaB-independent strand displacement reaction could present problems for the cell and, under some circumstances, is negatively regulated by PriA. They proposed that PriA could act by binding to the 3′-end of a primer juxtaposed to a fork and block binding by the Pol III HE. Interestingly, gp59, a T4 bacteriophage-encoded protein that has multiple functions, some of which overlap with PriA, can block the action of T4 DNA polymerase by forming a ternary complex with it on DNA, where gp59 site-specifically contacts the polymerase and locks it into a conformation where exonuclease and polymerase activities are inhibited (42). Our studies cannot yet resolve which of these two mechanisms are used by *E. coli* PriA in blocking the Pol III HE strand displacement reaction.

Our studies have an additional practical benefit. Rolling circle DNA replication systems that mimic the action of the replisome at an *in vivo* replication fork typically employ the PriA protein to initiate a series of interactions that result in the biologically relevant assembly of an active DnaB helicase at a replication fork. However, fork systems are sometimes assembled that lack the natural helicase loaders, and researchers instead add large excesses of DnaB to drive self-assembly. Such systems, lacking the PriA checkpoint protein, could be complicated by the strand displacement activities of Pol III HE, especially if conducted in the presence of τ and high Pol III and dNTP concentrations. The understanding of the properties of the intrinsic Pol III HE strand displacement reaction will permit artifacts, driven by its action, to be avoided in such systems in the future.

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REFERENCES

1. McHenry, C. S. (2003) *Mol. Microbiol.* **49,** 1157–1165
2. Kelman, Z., and O'Donnell, M. E. (1995) *Annu. Rev. Biochem.* **64,** 171–200
3. Burgers, P. M. (2009) *J. Biol. Chem.* **284,** 4041–4045
4. Pritchard, A. E., Dallmann, H. G., Glover, B. P., and McHenry, C. S. (2000) *EMBO J.* **19,** 6536–6545
5. Gao, D., and McHenry, C. S. (2001) *J. Biol. Chem.* **276,** 4447–4453
6. Jeruzalmi, D., O'Donnell, M., and Kuriyan, J. (2001) *Cell* **106,** 429–441
7. Flower, A. M., and McHenry, C. S. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 3713–3717
8. Blinkowa, A. L., and Walker, J. R. (1990) *Nucleic Acids Res.* **18,** 1725–1729
9. Tsuchihashi, Z., and Kornberg, A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2516–2520
10. Glover, B. P., Pritchard, A. E., and McHenry, C. S. (2001) *J. Biol. Chem.* **276,** 35842–35846
11. Simonetta, K. R., Kazmirski, S. L., Goedken, E. R., Cantor, A. J., Kelch, B. A., McNally, R., Seyedin, S. N., Makino, D. L., O'Donnell, M., and Kuriyan, J. (2009) *Cell* **137,** 659–671
12. Gao, D., and McHenry, C. S. (2001) *J. Biol. Chem.* **276,** 4433–4440
13. Gao, D., and McHenry, C. S. (2001) *J. Biol. Chem.* **276,** 4441–4446
14. LaDuca, R. J., Crute, J. J., McHenry, C. S., and Bambara, R. A. (1986) *J. Biol. Chem.* **261,** 7550–7557
15. Kong, X. P., Onrust, R., O'Donnell, M., and Kuriyan, J. (1992) *Cell* **69,** 425–437
16. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) *Cell* **84,** 643–650
17. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) *J. Biol. Chem.* **271,** 21406–21412
18. Glover, B. P., and McHenry, C. S. (1998) *J. Biol. Chem.* **273,** 23476–23484
19. O'Donnell, M. E., and Kornberg, A. (1985) *J. Biol. Chem.* **260,** 12884–12889
20. Canceill, D., and Ehrlich, S. D. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93,** 6647–6652
21. Stephens, K. M., and McMacken, R. (1997) *J. Biol. Chem.* **272,** 28800–28813
22. Xu, L., and Marians, K. J. (2003) *Mol. Cell* **11,** 817–826
23. Yao, N., Hurwitz, J., and O'Donnell, M. E. (2000) *J. Biol. Chem.* **275,** 1421–1432
24. Griep, M. A., and McHenry, C. S. (1989) *J. Biol. Chem.* **264,** 11294–11301
25. Johanson, K. O., Haynes, T. E., and McHenry, C. S. (1986) *J. Biol. Chem.* **261,** 11460–11465
26. McHenry, C. S., and Crow, W. (1979) *J. Biol. Chem.* **254,** 1748–1753
27. Dallmann, H. G., and McHenry, C. S. (1995) *J. Biol. Chem.* **270,** 29563–29569
28. Olson, M. W., Dallmann, H. G., and McHenry, C. S. (1995) *J. Biol. Chem.* **270,** 29570–29577
29. Glover, B. P., and McHenry, C. S. (2000) *J. Biol. Chem.* **275,** 3017–3020
30. Roy, R., Kozlov, A. G., Lohman, T. M., and Ha, T. (2007) *J. Mol. Biol.* **369,** 1244–1257
31. Hobbs, M. D., Sakai, A., and Cox, M. M. (2007) *J. Biol. Chem.* **282,** 11058–11067
32. Glover, B. P., and McHenry, C. S. (2001) *Cell* **105,** 925–934
33. Kim, D. R., and McHenry, C. S. (1996) *J. Biol. Chem.* **271,** 20681–20689
34. Jones, C. E., Green, E. M., Stephens, J. A., Mueser, T. C., and Nossal, N. G. (2004) *J. Biol. Chem.* **279,** 25721–25728
35. Pritchard, A. E., and McHenry, C. S. (2001) *J. Biol. Chem.* **276,** 35217–35222
36. Kelman, Z., Yuzhakov, A., Andjelkovic, J., and O'Donnell, M. E. (1998) *EMBO J.* **17,** 2436–2449
37. Witte, G., Urbanke, C., and Curth, U. (2003) *Nucleic Acids Res.* **31,** 4434–4440
38. Shereda, R. D., Kozlov, A. G., Lohman, T. M., Cox, M. M., and Keck, J. L. (2008) *Crit. Rev. Biochem. Mol. Biol.* **43,** 289–318
39. Kim, D. R., and McHenry, C. S. (1996) *J. Biol. Chem.* **271,** 20690–20698
40. Dohrmann, P. R., and McHenry, C. S. (2005) *J. Mol. Biol.* **350,** 228–239
41. Gulbis, J. M., Kazmirski, S. L., Finkelstein, J., Kelman, Z., O'Donnell, M., and Kuriyan, J. (2004) *Eur. J. Biochem.* **271,** 439–449
42. Xi, J., Zhuang, Z., Zhang, Z., Selzer, T., Spiering, M. M., Hammes, G. G., and Benkovic, S. J. (2005) *Biochemistry* **44,** 2305–2318
43. Johanson, K. O., and McHenry, C. S. (1982) *J. Biol. Chem.* **257,** 12310–12315