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The Clamp Loader Assembles the β Clamp onto Either a 3′ or 5′ Primer Terminus

*THE UNDERLYING BASIS FAVORING 3′ LOADING**[S]

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Mee Sook Park and Mike O'Donnell[1]

From the Howard Hughes Medical Institute, Rockefeller University, New York, New York 10065

Clamp loaders assemble sliding clamps onto 3′ primed sites for DNA polymerases. Clamp loaders are thought to be specific for a 3′ primed site, and unable to bind a 5′ site. We demonstrate here that the *Escherichia coli* γ complex clamp loader can load the β clamp onto a 5′ primed site, although with at least 20-fold reduced efficiency relative to loading at a 3′ primed site. Preferential clamp loading at a 3′ site does not appear to be due to DNA binding, as the clamp loader forms an avid complex with β at a 5′ site. Preferential loading at a 3′ *versus* a 5′ site occurs at the ATP hydrolysis step, needed to close the ring around DNA. We also address DNA structural features that are recognized for preferential loading at a 3′ site. Although the single-stranded template strand extends in opposite directions from 3′ and 5′ primed sites, thus making it a favorite candidate for distinguishing between 3′ and 5′ sites, the single-strand polarity at a primed template junction does not determine 3′ site selection for clamp loading. Instead, we find that clamp loader recognition of a 3′ site lies in the duplex portion of the primed site, not the single-strand portion. We present evidence that the β clamp facilitates its own loading specificity for a 3′ primed site. Implications to eukaryotic clamp loader complexes are proposed.

Chromosomal replicases are tethered to DNA by a circular ring-shaped clamp (1). The clamp encircles DNA and slides along the duplex behind the polymerase, holding it to the primed site for highly processive DNA synthesis. Sliding clamps are assembled onto primed sites by a clamp loader machine that harnesses the energy of ATP hydrolysis to open and close the sliding clamp around DNA (2). Bacterial sliding clamps are homodimers called β, while eukaryotic and archaeal sliding clamps are homotrimers referred to as PCNA.

Structural studies of clamp loader complexes show that they are circular AAA+ heteropentamers (2–4). The N-terminal domains contain the AAA+ region of homology and are arranged in a spiral to form a central chamber that binds double-stranded (ds) DNA. The C-terminal domains form a closed circular cap above the DNA binding chamber. This results in a screw cap architecture that underlies the structure-specific DNA binding of a primed site. Specifically, DNA cannot penetrate the closed cap, and must make a sharp bend to exit the central chamber through the side of the clamp loader, where there is a gap between the AAA+ domains of two subunits. Duplex DNA is far too stiff to make this sharp bend, but the single-stranded (ss)[2] DNA portion of a primed site has the required flexibility to accommodate this sharp bend.

The minimal γ complex clamp loader of *Escherichia coli* contains three γ subunits and one subunit each of δ and δ′. The complete γ complex contains two additional small subunits, χ and ψ, which are not essential to clamp loading but stimulate the reaction. The chromosomal replicase, Pol III*, contains the heterotrimeric Pol III core (α, DNA polymerase; ε 3′-5′ exonuclease and θ) attached to one clamp loader assembly. Attachment of Pol III core to the clamp loader is mediated by the τ subunit (71.1 kDa), which is encoded by the same *dnaX* gene as γ. The τ subunit is the full-length product of *dnaX* while γ (47.5 kDa) is truncated at the C terminus by a translational frameshift (5). Two to three τ subunits replace γ in the clamp loader within Pol III*, and each τ subunit binds to one molecule of Pol III core via the C-terminal sequence unique to τ (6, 7). In the current report, the term "γ complex" refers to the form of the clamp loader that contains three γ subunits and no τ subunit (*i.e.* $\gamma_3\delta\delta'\chi\psi$).

ATP binding to the γ subunits promotes a conformational change that enables the clamp loader to bind and open the β clamp (8, 9). The opened clamp adopts a spiral "lock washer" shape that complements the spiral arrangement of the clamp-loading subunits (10). DNA binding triggers ATP hydrolysis, which ejects the clamp loader and allows the clamp to close around DNA (9, 11, 12). Ejection of the clamp loader is important because Pol III, attaches to the same site on the clamp to which the clamp loader binds (13, 14).

Clamp-loading function appears specific to 3′ primer template (P/T) junctions (15). It is hypothesized that the clamp loader binds to a P/T junction and recognizes the minor groove to position the template ssDNA of a 3′ P/T junction adjacent to the exit channel from the central chamber (see Fig. 7*A*) (2). In this hypothesis, minor groove recognition is used to distinguish between 3′ and 5′ primed sites since the ssDNA at a 5′ site would be positioned at the back wall of the central chamber, far from the exit channel. This predicts that a 5′ P/T junction will not fit into the central chamber.

Surprisingly, the recent crystal structure of γ complex bound to a primed site reveals that the clamp loader does not recognize

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S8.

[1] To whom correspondence should be addressed: 1230 York Ave., Box 228, NY, NY 10065. Tel.: 212-327-7255; Fax: 212-327-7253; E-mail: odonnel@mail.rockefeller.edu.

[2] The abbreviations used are: ss, single-stranded; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; wt, wild type; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

the minor groove, but instead only forms contacts to the template strand (16). In fact, superimposition of a 5′ P/T junction into the structure indicates that it forms essentially the same contacts to the clamp loader as a 3′ P/T junction (*e.g.* see Fig. 7*A*). Therefore, the basis by which the clamp loader distinguishes between 3′ P/T and 5′ P/T structures is not clear. Considering that the template ssDNA extends outward 3′-5′ at a 3′ P/T junction, and extends 5′-3′ from a 5′ P/T site, we have proposed that the clamp loader may distinguish 3′ P/T and 5′ P/T junctions by reading the direction of the template ssDNA (16).

The current study examines the underlying basis for specificity of clamp loading at a 3′ P/T *versus* a 5′ P/T in the *E. coli* system. We find that the γ complex binds tightly to a 5′ P/T structure in the presence of the β clamp and ATP, and therefore DNA binding does not underlie discrimination of a 3′ *versus* a 5′ primed site. Surprisingly, we even observe clamp loading at a 5′ P/T junction, although the efficiency of this process is at least 20-fold reduced compared with loading at a 3′ P/T junction. The rate-limiting step in 5′ loading appears to be the ATP hydrolysis step, as the clamp loader-clamp complex at a 5′ P/T junction is stabile, yet is very slow to hydrolyze ATP. Thus, preference for 3′ loading is derived from a decreased rate in coupling ATP hydrolysis to clamp closure at a 5′ P/T site.

We also examined the DNA structural basis for preferential loading at a 3′ site. The most obvious difference in the structures of 3′ and 5′ P/T junctions is the polarity of the template ssDNA. However, we find that the polarity of template ssDNA is not a source of specificity in distinguishing a 3′ P/T from a 5′ P/T junction. Therefore, some feature of the duplex portion of a primed site is recognized to discriminate a 3′ P/T from a 5′ P/T junction. Evidence in support of duplex DNA recognition for 3′ *versus* 5′ loading is gained from use of β clamp mutants, which implicate direct contacts between the clamp and DNA in distinguishing 3′ from 5′ sites. We propose that the β clamp itself facilitates the productive orientation of a 3′ P/T junction in the clamp loader.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled nucleoside triphosphates were from GE Healthcare; radioactive nucleoside triphosphates were from PerkinElmer Life Sciences, Inc. *E. coli* proteins α, ε, θ, γ, τ, δ, δ′, χ, ψ, β, and SSB were purified as described (17). Mutant β clamps (18) and β containing an N-terminal kinase tag (19) were prepared as described. The γ complex (20), Pol III* (21) and Pol III* ED containing an inactive ε 3′-5′ exonuclease subunit (22) were reconstituted and purified from unassociated subunits as described. ^{32}P-β was prepared using [γ-^{32}P]ATP and the recombinant catalytic subunit of cAMP-dependent protein kinase produced in *E. coli* (a gift from Dr. Susan Taylor, University of California at San Diego) as described (19). Protein concentrations were determined by Bradford (Bio-Rad) using bovine serum albumin as a standard. Oligonucleotides were synthesized and gel-purified by Integrated DNA Technologies (IDT), by Dharmacon Thermo Scientific (Dharmacon), or by the W. M. Keck Facility at Yale University (Keck) as described in supplemental Table S1. Primed templates were formed by annealing primer (47 μM) and template (37 μM) oligonucleotides in 55 μl of 5 mM Tris-HCl, 150 mM NaCl, 15 mM sodium citrate (final pH 8.5) by bringing the reaction to 95 °C and then cooling to room temperature over a 30-min interval. Primer/template (P/T) pairs are given in supplemental Table S1. Streptavidin-coated magnetic beads (Dynabeads M-280) were purchased from Invitrogen. Clamp-loading buffer is: 30 mM Hepes-NaOH (pH 7.5), 1 mM dithiothreitol, 7 mM $MgCl_2$, 1 mM CHAPS. Protein Dilution buffer is: 20% glycerol, 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM dithiothreitol, and 40 μg/ml bovine serum albumin. Quench/Wash buffer is: 30 mM Hepes-NaOH (pH 7.5), 1 mM dithiothreitol, 1 mM CHAPS, 50 mM EDTA, and 100 mM NaCl. The binding buffer is: 1 M NaCl, 5 mM Tris-HCl, 0.5 mM EDTA.

Construction of a Fused Single-chain β Dimer—Two copies of the *dnaN* gene were cloned in tandem, separated by a linker encoding 14 residues, $(SG)_7$, in a pET11-based vector that incorporates a N-terminal tag containing ten histidines and a protein kinase sequence ($MG(H)_{10}$SSGHIEGRHILRRASVSSGHIEGRH). Mutations in the hydrophobic pocket binding site of β (H175S, F278A, Y323S, substitution of the C-terminal 5 residues to AGGGG) were introduced into subunit A (*i.e.* N-terminal protomer) of the wild-type (wt) single-chain β construct. Expression plasmids encoding the single-chain β and mutant single-chain β were transformed into *E. coli* BL21(DE3) and grown in LB to OD 0.6 at 37 °C, then lowered to 20 °C and induced upon adding 1 mM isopropyl-1-thio-β-D-galactopyranoside followed by incubation at 20 °C for 10 h. Cells were lysed and single-chain β was purified by nickel chelate chromotography.

Bead-based Clamp Loading and Clamp-Clamp Loader DNA Binding Assays—To prepare DNA immobilized to beads, streptavidin-coated magnetic beads were washed four times with binding buffer and then biotin end-labeled DNA was incubated with beads in binding buffer using 150 pmol of DNA/mg beads in a 50-μl volume for 1.5 h at room temperature on a rotator. Afterward, beads were washed three times with binding buffer to remove unbound DNA and any excess oligonucleotide primer used to form the P/T substrate, and then two more times with a buffer for the subsequent reaction. Under these conditions, about 130 pmol of a primer/template of 30-mer/75-mer is immobilized per mg of beads.

Clamp-loading reactions were performed in 30 μl of clamp-loading buffer containing 13 pmol of (430 nM) DNA, 12 pmol of (400 nM) ^{32}P-β_2, 20 pmol of (660 nM) SSB_4, 0.5 mM ATP, and the indicated amount of γ complex (or Pol III* ED, ε 3′-5′ exonuclease mutant). A mixture of β_2, SSB_4, and ATP in 20 μl of clamp-loading buffer was added to DNA-conjugated beads on ice, then 10 μl of the indicated amounts of γ complex and 21 mM $MgCl_2$ (final concentration 7 mM) were added, followed by vigorous pipetting and placing the reaction at 37 °C. At the times indicated, reactions were quenched upon adding 20 volumes of ice-cold Quench/Wash buffer. ^{32}P-β_2 loaded onto DNA was quantitated upon separating the beads on a magnet for 1 min on ice and removing the Quench/Wash buffer. Beads were then resuspended in 0.5% SDS and 20 mM EDTA, and incubated at 95 °C for 5 min to release ^{32}P-β_2 from the DNA-bead conjugate. ^{32}P-β_2 was quantitated from its known specific activity by liquid scintillation counting.

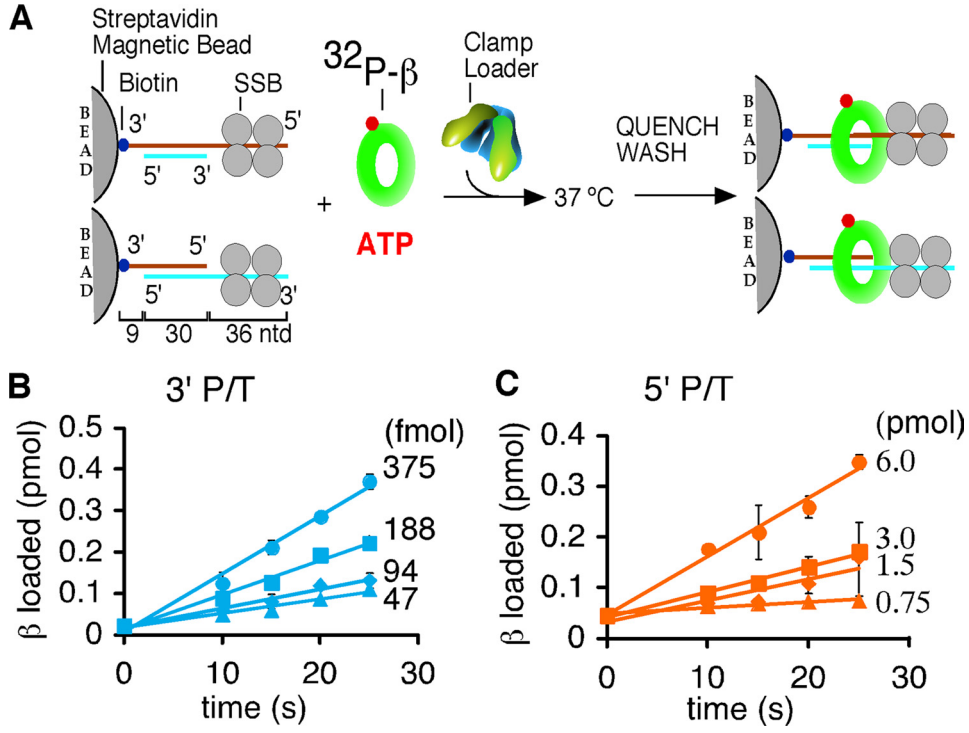

FIGURE 1. **The β clamp can be loaded at a 5′ P/T junction.** *A*, scheme of the synthetic 3′ P/T and 5′ P/T junctions, and the bead-based assay. *B*, clamp loading at a 3′ P/T junction was performed using γ complex at 47 fmol, (*triangles*), 94 fmol (*diamonds*), 188 fmol (*squares*), and 375 fmol (*circles*). *C*, clamp loading at a 5′ P/T junction using γ complex at: 0.75 pmol (*triangles*), 1.5 pmol (*diamonds*), 3 pmol (*squares*), or 6 pmol (*circles*). Reactions (30 μl) were performed in duplicate (*B* and *C*).

Stabile binding of the clamp-clamp loader complex at a 3′ or 5′ P/T junction was performed in a similar fashion as described above, except reactions were quenched after 20 s at 37 °C by dilution upon adding a 25-fold volume of ice-cold clamp-loading buffer. ^{32}P-β_2 bound to DNA was recovered from the magnetic beads and quantitated as described above.

Stability of 5′ Complexes—The stability of γ complex-β at a 5′ P/T junction was determined by a dilution protocol. The initial clamp-loading reaction was performed with 5′ P/T DNA in presence of 0.5 mM ATP or ATPγS for 20 s at 37 °C as described in above for clamp-loading assays. Then, reactions were diluted 25-fold using prewarmed clamp-loading buffer. Bead-DNA conjugates were collected on a magnetic separator at 37 °C, aliquots were removed at the indicated times, and ^{32}P-β_2 bound to bead-DNA conjugates was quantitated by liquid scintillation.

ATPase Assays—ATPase assays contained 13 pmol of (430 nM) DNA, β_2 (as indicated), 20 pmol of SSB_4 (660 nM), 2 pmol of (67 nM) γ complex, 2 nmol of (67 μM) [γ-^{32}P]ATP, and 7 mM $MgCl_2$ in 30 μl of clamp-loading buffer on ice, unless indicated otherwise in the figure legends. After finally adding γ complex, the reaction was shifted to 37 °C. After 1 min, the reaction was quenched by adding 70 μl of 1% SDS and 40 mM EDTA. 1.5 μl of each quenched aliquot was spotted on a polyethyleneimine cellulose TLC sheet and developed in 0.5 M $LiCl_2$ and 0.5 M formic acid. The TLC sheet was dried, and [γ-^{32}P]ATP and $^{32}P_i$ were quantitated using a PhosphorImager (GE Healthcare).

RESULTS

Sliding Clamps Can Be Loaded at a 5′ P/T Junction—To facilitate our studies of *E. coli* γ complex clamp-loading specificity for a 3′ P/T site *versus* a 5′ P/T site, we constructed synthetic 3′ and 5′ primed template (P/T) DNAs and immobilized them to magnetic beads through a streptavidin-biotin linkage (see *schemes* in Fig. 1*A*). Assembly of the clamp onto DNA was followed using ^{32}P-β. To prevent the ^{32}P-β clamp from sliding off the short linear DNA substrates, SSB was added, which binds the ssDNA and traps ^{32}P-β on the linear DNA (see supplemental Fig. S1). Clamp loading is initiated upon adding γ complex to reactions containing immobilized DNA, ^{32}P-β, and ATP. Timed aliquots are quenched with EDTA, which rapidly chelates magnesium and stops the ATP-dependent clamp-loading reaction. The magnetic beads and the associated ^{32}P-β-DNA complex are separated from unbound ^{32}P-β using a magnetic separator. Fig. 1*B* shows the time course of clamp loading on a 3′ P/T at different concentrations of γ complex.

Next, we examined clamp loading onto a 5′ P/T site. Previous studies indicate that γ complex cannot load β onto a 5′ P/T junction (15). Hence, we were surprised to find that β is loaded at a 5′ P/T junction, albeit substantially more γ complex is required to load β onto a 5′ P/T compared with a 3′ P/T junction (Fig. 1*C*). The requirement for more γ complex explains why clamp loading at a 5′ P/T junction was not observed in our previous study. A plot of the rate of β loading at different concentrations of γ complex reveals a 20-fold difference between 3′ and 5′ loading (supplemental Fig. S2).

Characterization of Clamp Loading at a 5′ P/T Junction—Clamp loading at a 3′ P/T junction requires ATP hydrolysis, and thus one may expect that β loading onto a 5′ P/T junction will also require ATP hydrolysis. Experiments performed in the absence of ATP give no β loading (Fig. 2*A*). Nor is β loaded at a 5′ P/T junction in the presence of the non-hydrolyzable ATP analogue, ATPγS. The positive control shows that β is loaded at a 5′ P/T junction in the presence of ATP. Therefore β loading at a 5′ P/T junction requires hydrolyzable ATP, similar to clamp loading at a 3′ P/T junction.

Next we examined the response of γ complex ATPase activity to a 5′ P/T junction. The β clamp is known to stimulate the DNA-dependent ATPase activity of γ complex in the presence of a 3′ P/T junction (23). In Fig. 2*B* we compare the γ complex ATPase activity in the presence or absence of β using either a 3′ P/T or a 5′ P/T. The results show that in the absence of β, a 3′

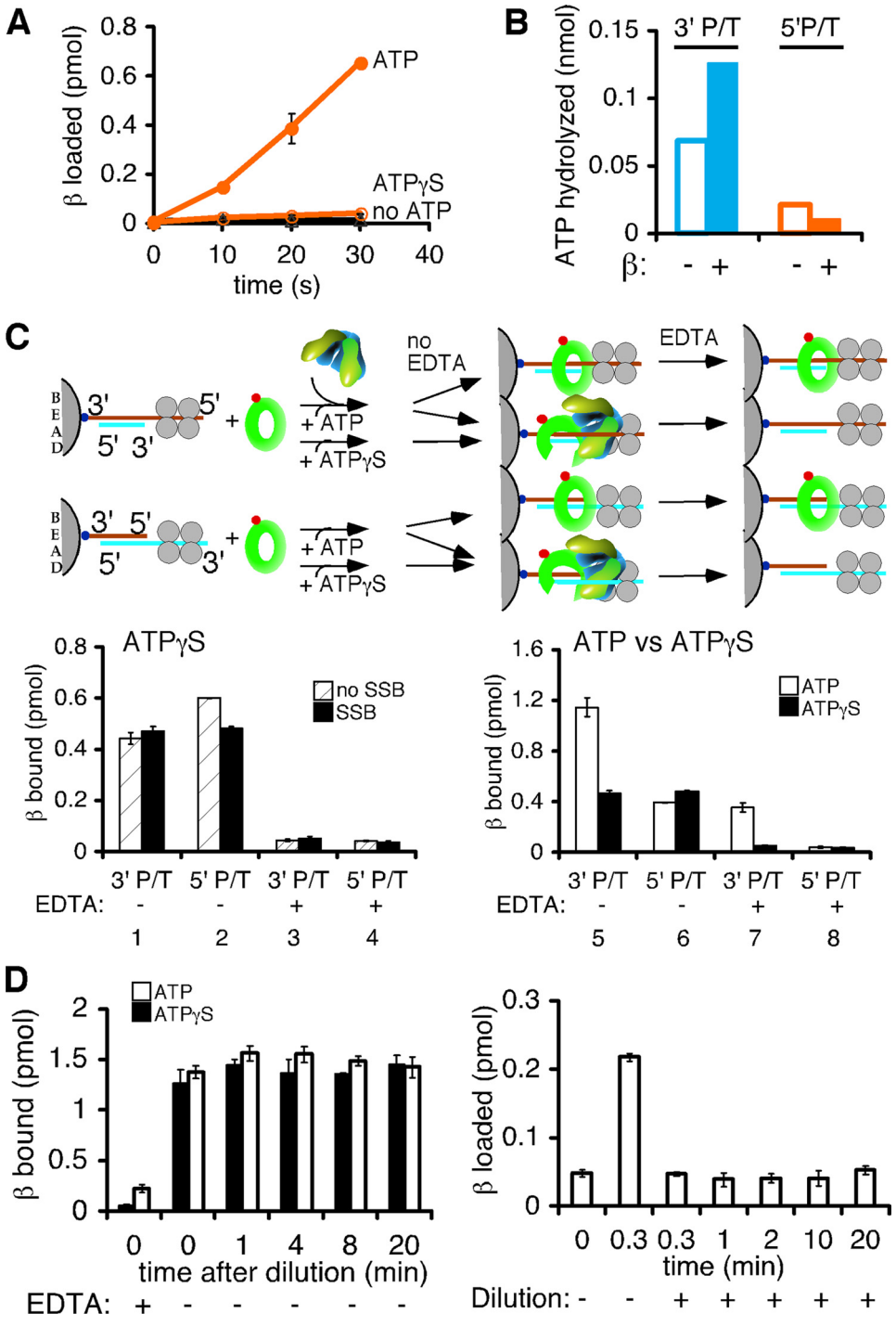

FIGURE 2. **The clamp loader forms a stabile complex at a 5′ P/T junction with β and ATP.** *A*, clamp loading at a 5′ P/T junction was performed in the presence of ATP (*filled circles*), ATPγS (*open circles*), or no ATP (*triangles*). The reaction contained 7 pmol of β and 4 pmol of γ complex. Reactions (30 μl) were performed in duplicate. *B*, ATPase assays (30 μl) contained, when present, 6.4 pmol β (*solid bar*) or no β (*open bar*), and the background (0.069) in the absence of DNA and γ complex is subtracted. *C*, *top*, schemes of DNA-β-γ complex assays are shown (symbols are as in Fig. 1*A*). *Bottom left*, DNA-β-γ complex with ATPγS and either SSB (*solid bars*) or no SSB (*hatched bars*). *Bottom right*, DNA-β-γ complex with ATP (*open bars*) or ATPγS (*solid bars*). Reactions were performed in duplicate. *D*, duplicate reactions were performed in 30 μl with 2 pmol of γ complex. *Left panel*, stability of β-γ complex at a 5′ P/T with ATP (*open bars*) or ATPγS (*solid bars*) upon 20 s of reaction, followed by 25-fold dilution to stop clamp loading. *Right panel*, control showing that dilution stops clamp loading. Reactions were diluted before initiating clamp loading, then clamp loading was initiated with ATP.

P/T induces substantially more ATP hydrolysis compared with a 5′ P/T junction. The presence of β enhances γ complex ATPase activity at the 3′ P/T junction, as expected from previous studies (23). However, β does not stimulate the γ complex ATPase in the presence of the 5′ P/T junction.

The γ Complex Forms a Stabile Complex with β at a 5′ P/T Junction—It is presumed that the specificity of γ complex for a 3′ P/T junction is based in an inability to bind to a 5′ P/T junction. However, the crystal structure of γ complex bound to a 3′ P/T reveals phosphodiester backbone interactions to only one strand, the template strand. Hence, it cannot recognize the minor (or major) groove for orienting the DNA. To assess if the template strand can suffice for sensing direction, a 5′ P/T junction was modeled into γ complex (16). The modeling exercise shows that the template strand phosphates are located in very similar positions for both 5′ and 3′ P/T junctions, regardless of the reverse polarity of the template strand at a 5′ P/T relative to a 3′ P/T junction (16). These findings suggest that γ complex may be capable of binding to a 5′ P/T junction.

To test whether the γ complex can bind a 5′ P/T junction we used the bead-based assay in the experiment of Fig. 2*C*. The experiments in the left panel utilize ATPγS, which is not hydrolyzed by γ complex (24). ATPγS is known to enable γ complex to open β and position it onto DNA, but γ complex remains bound to the open clamp on DNA, since it cannot hydrolyze ATPγS to eject from β (8, 25).

The results show that ATPγS promotes attachment of ^{32}P-β to the 5′ P/T junction (Fig. 2*C*). In fact, a similar amount of ^{32}P-β is retained on the 5′ P/T junction as on the 3′ P/T junction (*solid bars* in *lanes 1* and *2* of the *left panel*, Fig. 2*C*). Hence, the γ complex appears fully capable of binding a 5′ P/T structure. To confirm that the γ complex is present with β on the DNA, we performed similar reactions, but omitted SSB. If the clamp loader is present, it should hold the β clamp on the linear P/T DNA even without the SSB protein block. Experiments performed in

the absence of SSB show that this is in fact the case (*hatched bars* in *lanes 1* and *2*).

It is important to note that the results using ATPγS are only obtained when EDTA is omitted from the wash buffer. When EDTA is present in the wash, ^{32}P-β no longer remains bound to DNA, whether SSB is present or not (*lanes 3* and *4* in the *left panel* of Fig. 2*C*). We presume that EDTA chelates the magnesium and results in reversal of the ATPγS mediated β-γ complex-DNA interaction. This result supports earlier studies indicating that the clamp remains in the open configuration with ATPγS, otherwise it would close around DNA and remain stabile on the magnetic beads during this wash step (*right panel*). To ensure that complexes containing open clamps are not detected in the assays of this report we routinely include EDTA in the wash steps of all magnetic bead assays, unless noted otherwise.

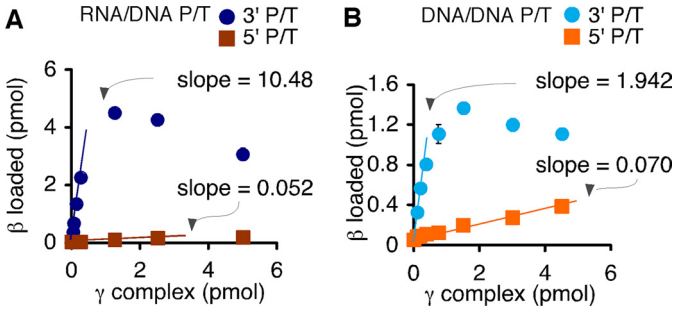

FIGURE 3. **Comparison of clamp loading at a RNA P/T and a DNA P/T site.** Clamp-loading reactions were performed in duplicate (25 s each). The "slope" of β loaded per γ complex added was calculated for data within the linear range ($p < 0.05$) using GraphPad Prism® software. *A*, clamp loading at a 3′ (*circle*) *versus* a 5′ (*square*) RNA/DNA P/T. *B*, clamp loading at a 3′ (*circle*) *versus* a 5′ (*square*) DNA/DNA P/T.

The *right panel* of Fig. 2*C* compares experiments performed in the presence of ATP with those using ATPγS. ATP hydrolysis promotes ejection of the clamp loader from the clamp, allowing β to close around the 3′ P/T junction. Hence, use of ATP results in the stabile attachment of ^{32}P-β on DNA even upon washing with buffer containing EDTA (*lane 7*). The greater amount of ^{32}P-β on a 3′ P/T in the absence of EDTA (*lane 5*) may reflect an additive effect, resulting from two complexes: 1) the open β-γ complex bound to DNA, and 2) the β ring closed around DNA. Interestingly, hydrolyzable ATP promotes retention of ^{32}P-β at the 5′ P/T junction in the absence of EDTA, but not when EDTA is present in the wash (compare *lanes 6* and *8*). This result indicates that ATP is not hydrolyzed, and that ATP binding acts like ATPγS in promoting formation of a stabile β-γ complex at the 5′ P/T junction in the presence of Mg. This interesting result may carry biological significance because both ATP and Mg are present *in vivo*, and therefore this finding implies that the open β-γ complex binds to 5′ P/T junctions in living cells (see "Discussion").

To address the stability of the β-γ complex bound to a 5′ P/T junction, we performed an experiment in which clamp loading was allowed to proceed for 20 s, and then the reaction was diluted 25-fold and aliquots were analyzed to determine the amount of ^{32}P-β-γ complex remaining after different intervals of time at 37 °C (Fig. 2*D*, *left panel*). The ^{32}P-β signal after 20 s of reaction with ATP before dilution shows the amount of ^{32}P-β closed around DNA as "loaded" β and the additional amount of open ^{32}P-β bound to DNA as a β-γ complex that can be washed away by a wash buffer containing EDTA (Fig. 2*D*, *left panel*, compare *lanes 1* and *2*). A control in which the reaction was first diluted, and then ATP was added, shows no significant clamp loading after dilution (Fig. 2*D*, *right panel*; reactions were washed with buffer containing EDTA). The result confirms that the amount of ^{32}P-β retained by the beads should decrease if the clamp-clamp loader complex dissociates from the 5′ P/T junction under the dilute conditions. The time course measurement of ^{32}P-β signal after dilution shows no significant dissociation after 20 min, supporting the conclusion that the β-γ complex forms a stabile complex at a 5′ P/T structure.

Comparison of Clamp Loading at RNA versus DNA P/T Sites—The physiological P/T structure during chromosome replication is an RNA-primed site; DNA-primed sites are predominately associated with repair. The sliding clamp is used in both of these processes. A proposal emanating from the structural studies suggests that the γ complex evolved to bind only to the template strand as means to accommodate both RNA-DNA and DNA-DNA primed sites (the template strand is DNA in both cases). In the experiments of Fig. 3, we compare the rate of clamp loading at RNA- and DNA-primed sites using a 25 s reaction time but different concentrations of γ complex (*panels A* and *B*, respectively). The results clearly demonstrate that the rate of clamp loading at a 3′ RNA-DNA P/T site is about 5-fold more efficient then loading onto a 3′ DNA-DNA P/T site. This rate difference persists upon using another sequence (supplemental Fig. S3). In contrast, the rate of clamp loading at a 5′ P/T junction is essentially the same for RNA-DNA and DNA-DNA P/T sites. The faster rate of clamp loading at a 3′ RNA-DNA P/T junction results in a 3′ *versus* 5′ specificity that is about 5-fold more selective than at DNA-DNA P/T junctions.

The Clamp Loader Does Not Obtain Directional Information from Template ssDNA—The crystal structure of γ complex bound to a primed site shows that template ssDNA contacts several residues in the δ subunit (16). Furthermore, mutation of side chains in δ that bind template ssDNA result in less efficient clamp loading (16). Thus it is hypothesized that these interactions with ssDNA may "read" the polarity of the ssDNA template strand and provide information for discriminating a 3′ from a 5′ P/T junction.

To determine whether the γ complex distinguishes among 3′ and 5′ P/T junctions by recognizing the polarity of the template ssDNA we flipped the polarity of ssDNA by incorporating a reverse polarity link in the template strand at either the 3′ or 5′ P/T junction (Fig. 4). If γ complex reads the polarity of template ssDNA, it should read the 5′ P/T junction of the reverse polarity template strand similar to how it reads a 3′ P/T junction. In this case, the clamp loader will efficiently load ^{32}P-β on the 5′ terminus of the reverse polarity substrate rather than the 3′ terminus. However, the result clearly shows that the clamp loader still prefers to load β at the 3′ terminus, even though the template ssDNA has the same polarity as a 5′ P/T junction. Side-by-side studies of loading onto normal and reverse polarity link junctions show no significant effect of these reverse polarity links on the rates of clamp loading (supplemental Fig. S4). This result also reveals that any asymmetry due to binding of SSB to

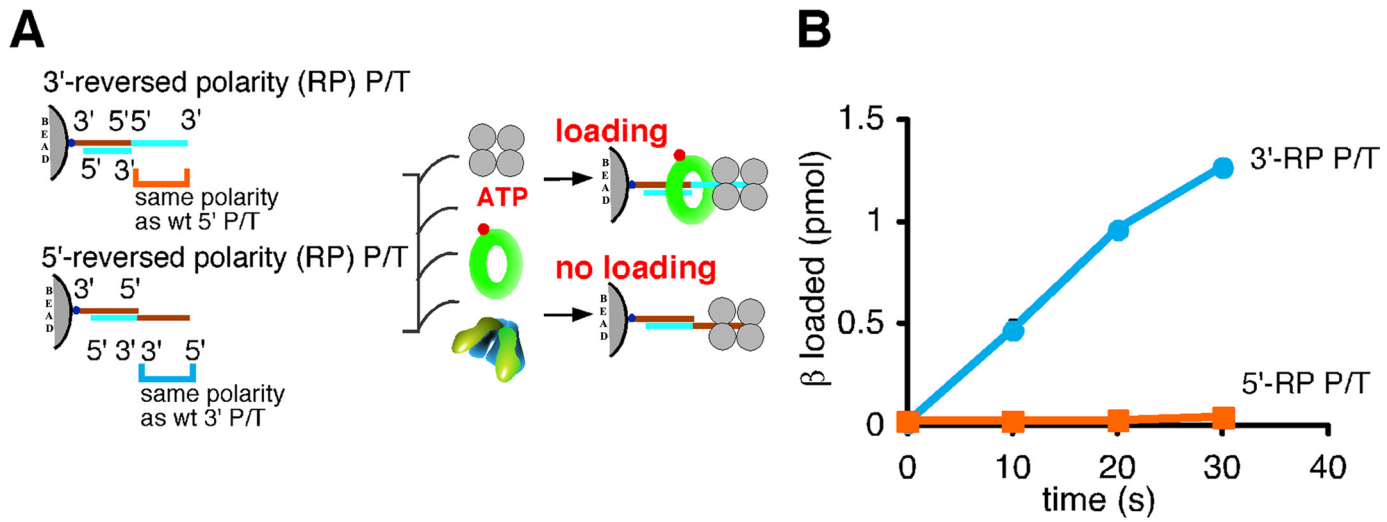

FIGURE 4. **The clamp loader does not obtain directional information from template ssDNA.** *A*, scheme of the reverse polarity DNA substrates used for the clamp-loading reactions. The reversed polarity P/T constructs contain a template strand with a reverse polarity linkage directly across from the P/T junction. *B*, 30-μl reactions contained 7 pmol β, 1 pmol γ complex, and 3′-reversed polarity P/T (*circles*) or 5′-reversed polarity P/T (*squares*). Reactions were performed in duplicate.

ssDNA does not participate in 3′ *versus* 5′ clamp-loading preference because the orientation of SSB will be inverted at a reverse polarity junction, yet clamp loading onto 3′ and 5′ P/T is not affected.

The above results indicate that the clamp loader does not derive directional information from the polarity of the template ssDNA. It is possible that the clamp loader somehow recognizes the 3′ hydroxyl group of the primer strand, unique to a 3′ P/T junction. To test this possibility, we constructed a 3′ dideoxy terminated P/T junction, but the results show a similar rate of ^{32}P-β loading onto 3′ dideoxy and 3′ hydroxyl-terminated primed sites (supplemental Fig. S5).

Polymerase III Provides Exquisite Specificity for a 3′ Flap Junction—DNA polymerases have an intrinsic affinity for a 3′ primer terminus, and therefore one may consider whether Pol III confers additional specificity to the clamp loader for loading β at a 3′ *versus* 5′ P/T junction. We address this possibility in the experiments of Fig. 5 using Pol III* ED, which contains an ϵ 3′-5′ exonuclease mutant subunit. We could not use wt Pol III* containing an active ϵ subunit for clamp loading on a 5′ P/T, because the exonuclease digests the 3′ ssDNA termini and thus destroys the 5′ P/T substrate. The results using Pol III* ED in the clamp-loading assay shows a similar β loading rate to γ complex for both 3′ and 5′ P/T junctions (Fig. 5*A*, compare with Fig. 3*B*). Since the clamp loader within Pol III* ED contains three τ subunits in place of the three γ subunits (*i.e.* $\tau_3\delta\delta'\chi\psi$), the results of Fig. 5*A* demonstrate that a clamp loader that contains τ in place of γ does not give a significantly different result in 3′ *versus* 5′ clamp loading.

There is an instance in which one may expect Pol III* to significantly enhance the specificity of 3′ *versus* 5′ clamp loading. Consider the case in which a 3′ ssDNA flap is produced during recombination or repair. In this situation the ϵ proofreading 3′-5′ exonuclease in Pol III core may excise the 3′ flap to produce a flush 3′ P/T site for efficient clamp loading. The proofreading exonuclease cannot excise 5′ ssDNA (27). Therefore, if ssDNA flaps inhibit clamp loading, Pol III core may specifically excise the 3′ flap and stimulate clamp loading after forming a flush 3′ P/T junction.

The γ complex-DNA structure suggests that the clamp loader will not efficiently accomodate a primed site having a ssDNA flap at either a 3′ or 5′ P/T, consistent with DNA binding studies that examine this issue (16). In Fig. 5*B*, we test the effect of a 9 nucleotide ssDNA flap on clamp loading at either a 3′ or 5′ P/T junction. The result confirms that the flap essentially prevents clamp loading by γ complex at both P/T structures. Use of smaller ssDNA flaps results in detectable, but diminished clamp-loading activity, and correlate with reduced γ complex DNA stimulated ATPase activity (supplemental Fig. S6). An earlier study that detected clamp loading at a forked junction was performed using a gel filtration method that required over 15 min to perform (15). These long incubation times may explain the observed level of clamp loading in the earlier study.

Next, we tested Pol III* for ability to load β onto a 3′ flap. The result demonstrates that the rate of clamp loading onto a 3′ flap approaches over time a similar rate as observed using a flush 3′ P/T junction (Fig. 5*C*, *circles*). Presumably, the 3′-5′ exonuclease within Pol III core efficiently excises the 3′ flap prior to loading. To test whether the 3′-5′ exonuclease activity is required for clamp loading at the 3′ flap, we compared loading at a 3′ flap and a flush 3′ P/T using Pol III* ED. The result shows no observable clamp loading (Fig. 5*C*, *squares*), consistent with a requirement for an active ϵ proofreading subunit that excises the 3′ flap to form a flush 3′ P/T. We were unable to test Pol III* containing an active ϵ subunit for clamp loading on the 5′ flap P/T, because the exonuclease destroys the 5′ P/T substrate as described above. However, we presume that the ϵ subunit of Pol III* will not improve the rate of clamp loading at a 5′ flap junction because the ϵ subunit lacks activity on a 5′ terminus.

In overview, the ϵ 3′-5′ exonuclease activity in Pol III core provides an additional degree of specificity to Pol III* for loading clamps at a 3′ flap junction compared with a 5′ flap junction.

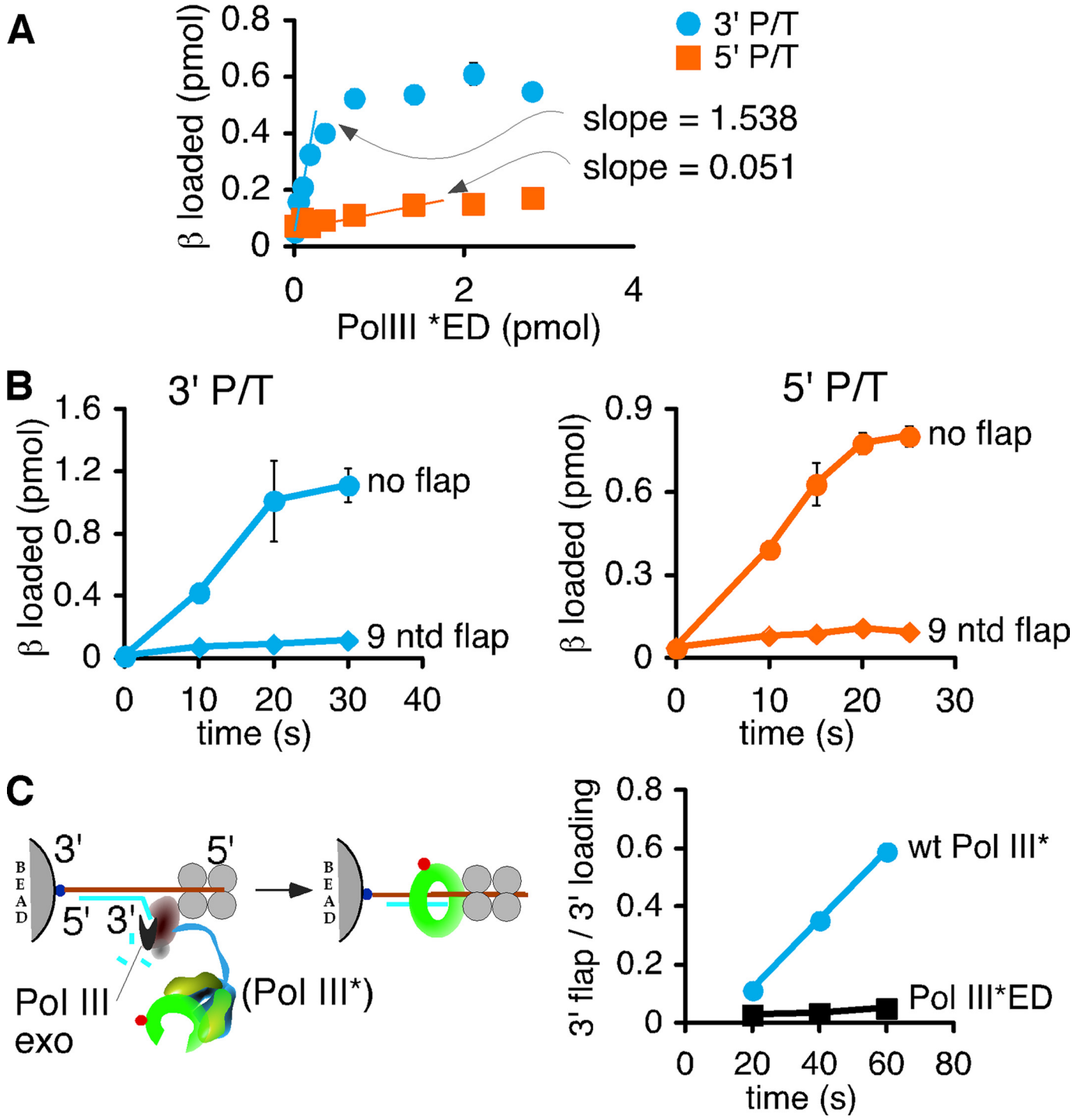

FIGURE 5. **Pol III confers specificity to clamp loading at a flapped 3′ P/T junction.** *A*, clamp loading at a 3′ P/T junction (*circles*) and at a 5′ P/T junction (*squares*) was performed using Pol III* ED (Pol III* reconstituted using an inactive ϵ 3′-5′ exonuclease mutant subunit). Reactions were 25 s, in duplicate. The "slope" was calculated within the linear range ($p < 0.05$) using GraphPad Prism® software. *B*, clamp loading at a P/T with no flap (*circles*) and at a P/T with a 9 nucleotide (*ntd*) flap (*diamonds*). Duplicate reactions (30 μl each) contained 7 pmol of β, and 1 pmol of γ complex for the 3′ P/T (*left panel*) or 6 pmol γ complex for the 5′ P/T (*right panel*). *C*, schematic illustrates Pol III* correcting the 3′ flap using the 3′-5′ exonuclease activity inherent in the ϵ subunit of Pol III core. The ratio of clamp loading at a P/T with a flap and a P/T with no flap is shown in the plot for Pol III* (*circles*) and Pol III*ED (*squares*). The 30-μl reactions contained 7 pmol of β, and 1 pmol of Pol III* or Pol III*ED and 3 dNTPs (dGTP, dCTP, and dTTP).

The β Clamp Participates in Clamp-loading Directionality—It was demonstrated earlier in this report that β stimulates the ATPase activity of γ complex at a 3′ P/T junction, but not at a 5′ P/T junction (Fig. 2*B*). This result indicates that the β clamp may participate in specifying loading at a 3′ P/T junction. Indeed, the β-DNA crystal structure shows interactions between β and DNA at multiple points (18). The β clamp binds both strands of the exiting duplex using residues Arg-24 and Gln-149, which are positioned on exposed loops. Contacts to both stands of DNA may enable the clamp to

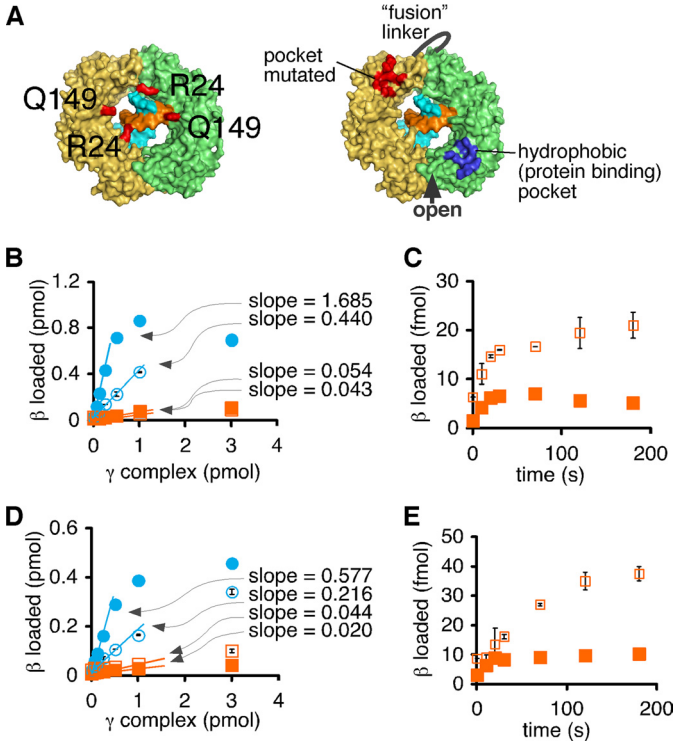

FIGURE 6. **The β clamp participates in clamp loading directionality.** *A*, mutated DNA binding residues of β (*red*), Arg-24 and Gln-149 (in the *left structure*) and mutated residues (*red*) of the binding "pocket" of protomer A in the "fused" single-chain β (to the *right*), are shown in the context of the β-DNA crystal structure (PDB code 3BEP). *B*, clamp loading using wt β (*filled symbols*) or R24A/Q149A mutant β (*open symbols*) at 3′ (*circles*) and 5′ (*squares*) P/T junctions are compared. Reaction times were 25 s each. The "slope" was calculated within the linear range of the data ($p < 0.05$) using GraphPad Prism® software. *C*, clamp loading at a 5′ P/T junction using wt β (*filled squares*) or R24A/Q149A mutant β (*open squares*) are compared. Time courses of clamp-loading reactions were performed using 0.5 pmol (17 nM) of wt or mutant β, and 3 pmol (100 nM) of γ complex. *D* and *E*, experiments and data analyses were performed as described in *B* and *C* except we used the "fused" single-chain β composed of either wt protomers or protomer A with mutated residues in the binding pocket.

correctly align only a 3′ P/T junction, and not a 5′ P/T, for the ssDNA at a 3′ site to exit the central chamber of the clamp loader.

Previous studies of β have shown that replacement of Arg-24 and Gln-149 with alanines results in a clamp mutant (R24A/Q149A) that is inefficiently loaded onto DNA (18). However, the residual activity is sufficient to measure the rates of loading onto 3′ and 5′ P/T junctions. The result shows that mutation of these dsDNA binding residues (Fig. 6*A*, *left structure*) affects the 3′ loading rate considerably, but not the 5′ loading rate (Fig. 6*B*). Hence, the ratio of 3′ *versus* 5′ P/T loading for the mutant β is lowered to a discrimination factor of about 10-fold, as compared with about 30-fold for wild-type β under the same conditions. This result supports the hypothesis that the β clamp participates in clamp-loading polarity.

The β-DNA structure shows that β binds the ssDNA portion of a P/T junction in the same hydrophobic pocket to which the clamp loader and polymerase attach to β (18). We wished to examine the effect of mutations in the hydrophobic pocket of the β clamp. However, mutation of the hydrophobic pocket in both protomers of β inactivates the β clamp. Therefore, to test whether ssDNA binding to β plays a role in loading at a 3′ *versus* 5′ site, we mutated the hydrophobic pocket of only one protomer by constructing a fusion protein, in which the two protomers are connected by an amino acid linker (*i.e.* by cloning two *dnaN* genes in tandem; Fig. 6*A*, *right structure*). The "fused β" retains about 50% the activity of wild-type β. The single-chain fused β clamp provides the opportunity to mutate only one protomer of the β dimer, since a single gene encodes the two protomers. The hydrophobic pocket in protomer B cannot be mutated because it is needed for the δ subunit to bind and open the interface in the fused β clamp (28). Therefore, we mutated residues in the hydrophobic pocket of protomer A and studied the rate of clamp loading at 3′ and 5′ P/T junctions. The result shows that clamp loading at a 3′ P/T junction is compromised by the hydrophobic pocket mutant clamp relative to the wt fused β (Fig. 6*D*). Moreover, clamp loading at a 5′ P/T junction is enhanced by these mutations, lowering the discrimination ratio of 3′ *versus* 5′ loading to a factor of about 5 for the hydrophobic pocket mutant compared with about 30 for wild-type β using similar assay conditions. Comparison of a time course of clamp loading at a 5′ site using mutant β clamps *versus* wt β clearly shows that more mutant β is loaded over time than wt β (Fig. 6, *C* and *E*). A similar effect is seen using an RNA-primed site (supplemental Fig. S7). Consistent with the observed differences in clamp loading rates, the effect of wt β on the ATPase activity of γ complex shows stronger discrimination between 3′ and 5′ P/T junctions than the hydrophobic pocket mutant β (supplemental Fig. S8). In overview, these results support a role of the β clamp in specifying clamp loading at a 3′ *versus* a 5′ P/T junction.

DISCUSSION

This report examines the basis for clamp-loading specificity at a 3′ P/T junction (*i.e.* a primed site) relative to a 5′ P/T junction. Both P/T junctions consist of a duplex region and a ssDNA extension, and both conceivably fit into the γ complex active site chamber. Whereas previous studies indicate there is no loading on a 5′ P/T junction, this report demonstrates that such loading indeed occurs. Nevertheless, we observe at least 20-fold discrimination in clamp loading at a 3′ site compared with a 5′ site.

Basis for Clamp-loading Specificity at a 3′ P/T Junction—The crystal structure of γ complex bound to a 3′ P/T junction shows that the template ssDNA is positioned near the gap between δ and δ′, facilitating exit of the template ssDNA out of the clamp loader (see Fig. 7*A*) (16). We previously proposed minor groove recognition as the basis for specificity in clamp loading at a 3′ P/T junction, by orienting the DNA so that template ssDNA exits the central chamber (2). In this hypothesis, the ssDNA at a 5′ P/T would be positioned deep within the central chamber, preventing the clamp loader from binding a 5′ P/T site. However, the structure of γ complex bound to a 3′ P/T shows that the clamp loader does not recognize the minor groove, but instead binds only the template DNA strand within both the duplex and ssDNA portions of the primed site (16). Furthermore, superimposition of the 5′ → 3′ template strand of a 5′ P/T junction, with the 3′ → 5′ template strand of a 3′ P/T junction shows that the phosphates of the template strand are in very nearly the same position regardless of their opposite

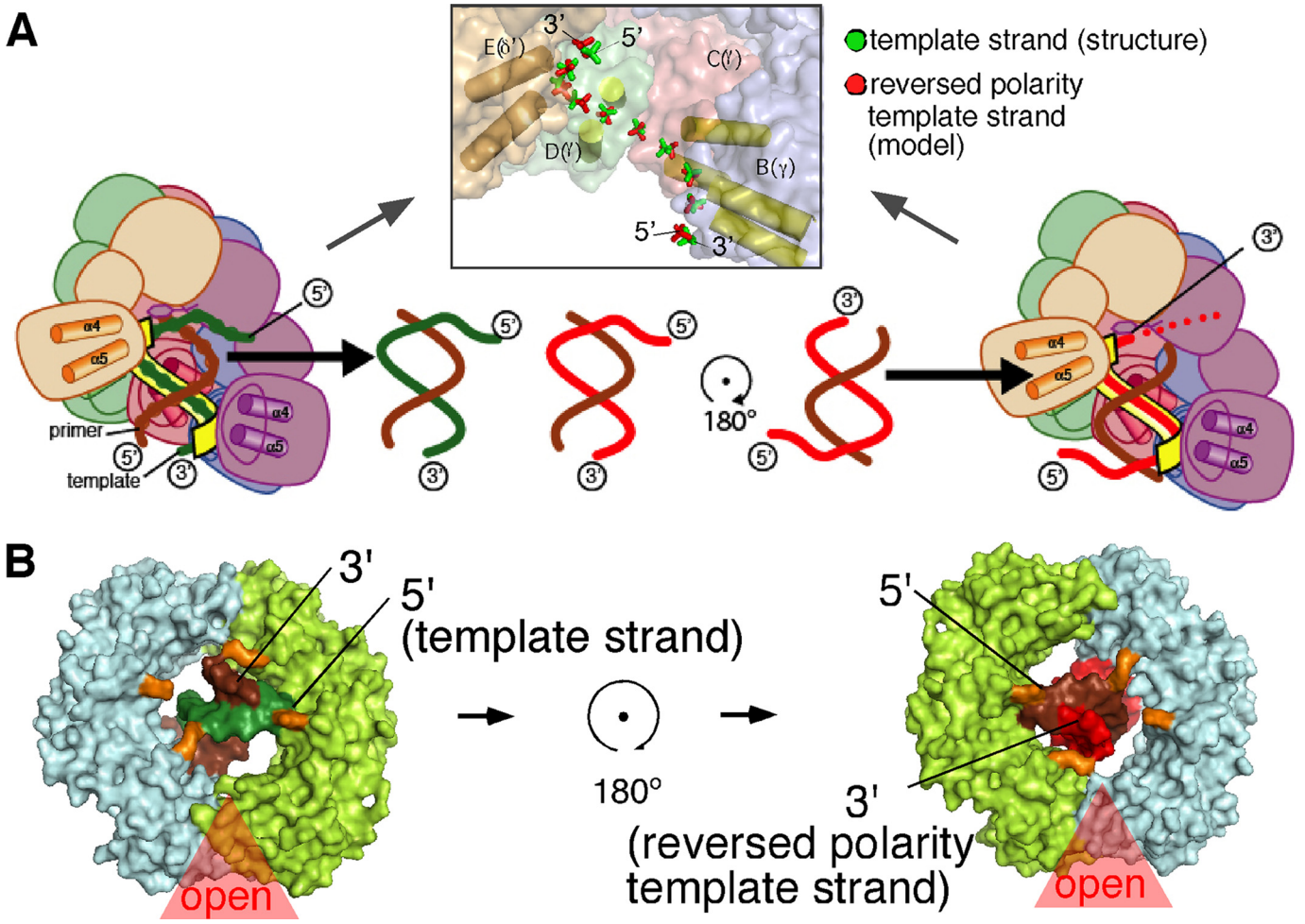

FIGURE 7. **Basis for clamp loading specificity at a 3′ P/T junction.** *A*, cartoon of DNA binding in the central chamber of γ complex, adapted from Fig. S7 of Simonetta *et al.* (16). The diagram to the *left* illustrates binding of a 3′ P/T, and binding to a 5′ P/T is proposed in the diagram to the *right*. The *middle diagram* shows the template strand phosphate atoms of the 3′ P/T in the actual structure (*green*), and the location of phosphates in the template strand of a 5′ P/T superimposed on the 3′ P/T structure (*red*) (PDB code 3GLF). *B*, structure of DNA bound to β (PDB code 3BEP). The two structures illustrate the different conformation of DNA bound to either the *blue* protomer or the *green* protomer, assuming the clamp is positioned on γ complex for opening at the indicated interface.

polarity (*middle illustration* in Fig. 7*A*). Therefore, the structure suggests that the clamp loader may bind either a 3′ or 5′ P/T, and can orient either of them in a way that the template ssDNA extension is adjacent to the gap in the side of the clamp loader (see *left* and *right illustrations* in Fig. 7*A*). Indeed, we find here that γ complex forms a highly stable complex with a 5′ P/T junction in the presence of ATP and β.

Perhaps the clamp loader recognizes the ssDNA extension at a primed site, and distinguishes the 3′ → 5′ direction of ssDNA at a 3′ P/T from the 5′ → 3′ direction of the template strand at a 5′ P/T site. In support of this hypothesis, the structure shows numerous contacts to the phosphodiester backbone of the template ssDNA, but whether this enables it to distinguish the directionality of the ssDNA is unknown. The current study appears to rule out recognition of the ssDNA template strand as a basis of clamp-loading polarity because clamp loading is not diminished by a reversed polarity link that reverses the direction of template ssDNA at a 3′ P/T junction. If the clamp loader were to recognize template ssDNA directionality to discriminate a 3′ from a 5′ P/T junction, the rate of clamp loading at the reversed polarity 3′ P/T junction would be severely diminished. Likewise, reversal of the ssDNA at a 5′ P/T junction does not "fool" the clamp loader into more efficiently loading β onto the reverse polarity 5′ P/T junction relative to an unmodified 5′ P/T junction. Therefore, specificity of clamp loading at a 3′ *versus* a 5′ P/T junction would appear to derive from the duplex portion of a primed site.

We find that the DNA stimulated ATPase activity of the clamp loader is quite low at a 5′ P/T junction compared with a 3′ P/T junction. Furthermore, we demonstrate that the β clamp does not stimulate the γ complex ATPase at a 5′ P/T junction as it does at a 3′ P/T junction. Without ATP hydrolysis the β-γ complex remains bound to the 5′ P/T site. Hence, inefficient 5′ clamp loading derives from inability of a 5′ P/T junction to trigger ATP hydrolysis, despite formation of a tight complex of the clamp loader with β at the 5′ P/T junction.

The β Clamp Facilitates the Specificity of Clamp Loading—In experiments using mutant versions of the β clamp we obtained unexpected evidence for participation of the clamp in specificity of 3′ P/T loading. Binding of the clamp to the clamp loader may alter the conformation of the clamp loader and facilitate recognition of the minor groove of duplex DNA. Alternatively,

the clamp may recognize DNA directly and help position it into the clamp loader for specificity in clamp loading at a 3′ *versus* 5′ P/T junction.

The structure of β bound to DNA shows two residues, Arg-24 and Gln-149, that bind the two strands of duplex DNA in an asymmetric fashion; only one face of DNA interacts with both Arg-24 and Gln-149 (Fig. 7*B*) (18). This may be the underlying basis by which β participates in orienting DNA within the clamp loader for 3′ *versus* 5′ clamp loading. Study of a β mutant, in which these residues are replaced with alanine, results in a 2–3-fold lower specificity in loading the mutant β at a 3′ *versus* a 5′ P/T junction. This supports the hypothesis that the clamp acts directly on DNA to specify a 3′ *versus* 5′ P/T junction. Loss of specificity using the β mutant is mostly due to a lower rate of clamp loading at a 3′ P/T junction. Thus mutations in β may reflect loss of necessary β-DNA interactions needed to discriminate 3′ *versus* 5′ primed sites.

The clamp binds to the clamp loader and to ssDNA via a hydrophobic pocket located on the C-terminal face of the clamp (18, 28). Mutation of residues in the hydrophobic pocket of one protomer of the β dimer reduces the specificity of clamp loading at a 3′ *versus* 5′ P/T junction. This may be the result of an altered interaction of the β clamp with the clamp loader, or perhaps the residues of the pocket that are closest to the edge of the central cavity of the β ring interact with DNA when the clamp is in the open configuration during the clamp-loading process.

In summary, use of β mutants suggests that the β clamp participates in determining clamp-loading polarity. The clamp may do so directly, by binding and positioning the DNA duplex, and/or indirectly by enabling γ complex to discriminate between 3′ and 5′ P/T junctions.

Does 5′ P/T Clamp Loading Occur in the Cell?—This report documents that the β clamp becomes loaded at a 5′ P/T junction. Previous studies may not have detected 5′ loading given the fact that clamp loading is reduced at least 20-fold at a 5′ P/T relative to a 3′ P/T (15). This may simply reflect a natural leakiness inherent in the clamp-loading mechanism. However, it also seems possible that 5′ end loading may be physiological, and that the 5′-loaded β clamp could serve a biological role.

Clamps that are loaded at a 5′ P/T would not be expected to function with a DNA polymerase. However, other types of proteins may target to a 5′ P/T junction. For example, DNA ligase functions to seal a 5′ terminus to a 3′ terminus, and ligase is known to bind β (29). Certain proteins involved in recombinative repair are thought to target a 5′ P/T junction, such as RecF, in which case an interaction with β loaded at a 5′ P/T terminus may increase the specificity of reaction (30, 31). In fact, the amounts of γ complex (200 nM; 6 pmol) and β (200 – 400 nM) needed to observe a 5′ loading are within the range of the intracellular concentrations of γ complex (200 nM) and β (600 nM) (32).

This report also shows that the clamp loader forms a stabile complex at a 5′ P/T junction with β in the presence of ATP and magnesium. This interesting finding suggests that the β-γ complex may bind 5′ P/T junctions in living cells. A possible role for a protein complex bound to a 5′ P/T could be to protect the 5′ P/T and inhibit other proteins from acting upon it, such as nucleases, helicases, or even ligase. Further studies will be needed to determine whether clamp loader-clamp complexes perform intracellular functions by binding to 5′ P/T junctions.

Why Does the Clamp Loader Prefer an RNA-primed Site?—Sliding clamps are utilized by many different proteins, including DNA polymerases and factors involved in DNA repair. The substrate for clamp loading during repair is a DNA-DNA hybrid, while clamp loading during replication mainly utilizes RNA-primed sites produced by primase. We show here that clamp loading at a RNA-DNA P/T junction is about 5-fold more rapid than clamp loading at a DNA-DNA P/T junction. This increased rate may reflect the fact that bacterial replication forks move at a rate of ~650 bp/s (33). Assuming an Okazaki fragment size of 1–2 kb, a new RNA primer will be formed every few seconds, and β clamps must be efficiently loaded onto new RNA primers in order for lagging strand synthesis to keep pace with the leading strand.

The fact that γ complex binds only the template strand at a P/T junction may enable it to accommodate both DNA-DNA and RNA-DNA substrates, since the template strand is DNA in both structures. Indeed, modeling of an A-form RNA-DNA hybrid in place of the DNA-DNA hybrid in the γ complex-primed site structure indicates that an RNA-DNA hybrid fits quite well.

Implications of These Studies to Eukaryotes—The eukaryotic clamp loader, replication factor C (RFC), is a AAA+ assembly composed of five distinct subunits arranged in a spiral similar to *E. coli* γ complex (3, 4). The eukaryotic PCNA clamp has a similar structure to *E. coli* β, and is known to function with a wide variety of DNA polymerases and repair factors. Like *E. coli* γ complex, RFC loads the PCNA clamp onto 3′ P/T junctions in an ATP dependent reaction. Unlike bacteria, eukaryotic cells contain alternate clamp loaders in which the RFC1 subunit is replaced by another protein. The Rad17 subunit (Rad24 in yeast) replaces RFC1 in an alternative clamp loader involved in the DNA damage checkpoint response (34–36). Interestingly, Rad17-RFC loads a different type of clamp onto DNA, referred to as the 911 clamp. The 911 clamp is a heterotrimer ring involved in the DNA damage checkpoint signaling pathway, but its exact function in the pathway is not yet clear.

Studies of Rad17 RFC show that it has an opposite specificity of clamp loading from RFC and *E. coli* γ complex; it loads the 911 clamp onto a 5′ P/T junction instead of a 3′ P/T junction (35). The 5′ direction of the 911 clamp suggests that the 911 clamp does not function with a DNA polymerase. In addition, the 911 clamp is not known to slide on DNA. Perhaps the 911 clamp protects a 5′ P/T junction from the action of other proteins, or it may target a factor to a 5′ P/T junction. It is tempting to speculate that the stabile formation of β-γ complex at a 5′ P/T junction in the bacterial system (or a 5′-loaded β clamp) may fulfill a similar role as the eukaryotic 911 clamp at a 5′ P/T junction.

The distinctive 5′ P/T clamp-loading polarity of the 911 clamp is thought to be conferred by the replacement of the Rad17 for RFC1. However, the idea that the Rad17 subunit underlies the specificity of clamp loading at a 5′ P/T junction may need to be revised in light of the current study which shows that the clamp itself participates in the specificity of clamp load-

ing at a 3′ *versus* a 5′ P/T junction. Therefore, on the basis of the current study, one may predict that the 911 clamp plays a role in specifying its own assembly at a 5′ P/T junction.

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