Dynamics of Loading the β Sliding Clamp of DNA Polymerase III onto DNA*

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A “minimal” DNA primer-template system, consisting of an 80-mer template and 30-mer primer, supports processive DNA synthesis by DNA polymerase III core in the presence of the β sliding clamp, γ complex clamp loader, and single-stranded binding protein from Escherichia coli. This primer-template system was used to measure the loading of the β sliding clamp by the γ complex in an ATP-dependent reaction. Bound protein-DNA complexes were detected by monitoring fluorescence depolarization of DNA. Steady state and time-resolved anisotropies were measured, and stopped-flow pre-steady state fluorescence measurements allowed visualization of the loading reactions in real time. The rate of loading β onto DNA was 12 s⁻¹, demonstrating that clamp assembly is rapid on the time scale required for lagging strand Okazaki fragment synthesis. The association rate appears to be limited by an intramolecular step occurring prior to the clamp-loading reaction, possibly the opening of the toroidal β dimer.

The replicative DNA polymerase in Escherichia coli is the pol III1 holoenzyme composed of 10 subunits (reviewed in Refs. 1 and 2). The pol III core contains three subunits (3); the α subunit contains the DNA polymerase activity (4), the 3’ to 5’ proofreading exonuclease resides in ε (5, 6), and the third subunit, δ, lacks a well defined function and is nonessential for cell growth (3, 7). The core polymerase is extremely inefficient and SSB enhances processive DNA synthesis by suppressing DNA strand slippage (8). The activity of the accessory proteins γ complex and β tethers the core to the p/t DNA, resulting in a processivity of greater than 5000 nt/template binding event (4, 9).

The γ complex contains five subunits: γ, δ, δ’, χ, and ψ (10, 11). The key function of the γ complex is to load the β subunit onto DNA. Biochemical data suggest that β encircles DNA and interacts directly with the pol III core, tethering the core to the DNA during chain elongation (9). X-ray diffraction analysis reinforces the biochemical data, showing that the β subunit is a doughnut-shaped dimer with an inner diameter sufficient to form a ring around duplex DNA, enabling it to act as a sliding clamp for the polymerase (12). Therefore, proper assembly of the β sliding clamp by the γ complex is an absolute prerequisite for accurate and efficient chromosome duplication. It has recently been shown that the γ subunit acts as a bridge between two core polymerases (13, 14); the γ subunit also brings one γ complex clamp loader into the holoenzyme structure (15, 16). This asymmetric dimer of two polymerases within one holoenzyme is therefore capable of simultaneous replication of both strands of a duplex chromosome as hypothesized (17). E. coli SSB enhances processive DNA synthesis by suppressing DNA secondary structure that would otherwise act to stall the pol III holoenzyme complex (1).

The β clamp slides freely off the ends of linear DNA molecules (9); therefore, previous studies of the γ complex clamp-loading action have been limited to use of circular DNAs, such as large bacteriophage ssDNA circular genomes, as primed templates. Application of biophysical techniques to the study of how the γ complex loads β clamps onto DNA has been hindered by the use of these large DNA templates. In this report we have developed a short linear template with ssDNA overhangs of sufficient length to slow the dissociation of β from the DNA and have used this template to study the interaction of β and γ complex with the DNA template via the technique of fluorescence depolarization.

DNA in solution undergoes rotational diffusion that varies with molecular weight. Rotational motion of DNA is slowed down when proteins are bound. The p/t DNA was labeled with a fluorescent dye molecule, X-rhodamine. The rotational motion of the dye, detected by time-resolved and steady state fluorescence depolarization, was used to measure rotation of the DNA in the presence and absence of combinations of β and γ complex. Pre-steady state stopped-flow measurements were made allowing us to visualize the loading of the β sliding clamp onto DNA in real time.

**EXPERIMENTAL PROCEDURES**

Enzymes—E. coli DNA pol III proteins were purified as described: a, ε, and γ (18); β (12); δ and δ’ (19); χ and ε (20); and θ (7). Subassemblies of γ complex and core were constituted as described (7, 11). Enzyme reaction buffer contained 20 mM Tris-HCl, pH 7.5, 40 μg/ml BSA, 5 mM dithiothreitol, and 8 mM MgCl₂. For fluorescence experiments, 50 mM NaCl was included in the reaction buffer to reduce nonspecific protein-DNA interactions.

Oligonucleotides—Oligonucleotides were synthesized using standard β-cyanoethyl phosphoramidite chemistry. The 80-mer template used for primer extension reaction is 5’-TGA GGG TCG CCG TTC TGA GGG TGG TCG CCG TTC TAA GGG TGG TCG TAC TAA ACC TCC TGA GTA GCG TGA TAC ACC ATT TAT CTC GG 3’.
The primer-template used for fluorescence measurements is as follows.

\[ 5\prime \text{ CT CCT TCT TGA GTT TGA TAG CCG GAA CGA CCA TTA TAG GTC } \]
\[ \text{AAC AAT ATT ACC GCC A } \]
\[ \text{TGG TTA TAA TGG CGG TCG GTA ACG TTG TCC TTT TCG GT } 5\prime \]

**SEQUENCE 1**

A 5′-amino group on a 6-carbon atom linker (Glen Research) was added to oligonucleotides that were to be labeled with rhodamine. X-rhodamine (Molecular Probes, catalog number X-491) was attached to the amino linker as described previously (21). A 30-nt primer and a complementary 80-nt template were labeled with X-rhodamine. The labeled primer was annealed to an unlabeled template, and the double-stranded p/t was purified from the single-stranded oligonucleotides by nondenaturing polyacrylamide gel electrophoresis. The labeled template was annealed to an unlabeled primer in enzyme reaction buffer using a ratio of 1 template to 1.5 primers. This p/t system was not gel-purified.

The template used in reactions with core polymerase was synthesized with a single phosphorothioate linkage to the last nucleotide on the 3′ end of the template. Oligonucleotides of different length were purified by polyacrylamide gel electrophoresis. This was necessary for experiments in which an unmodified isomer, which was resistant to exonuclease cleavage and the second is not. Both T4 DNA polymerase and the ε subunit of pol III show the same stereoelectricity in excision reactions (22). The mixture of phosphorothioate isomers was degraded on a preparative scale by incubation with T4 DNA polymerase to leave only the nonhydrolyzable isomer.

**Primer Extension Processivity Assays**—Primers were 5′-end-labeled with \(^{32}\text{P}\) using T4 polynucleotide kinase (U.S. Biochemical Corp.) in enzyme reaction buffer at 37°C. Primer-templates were annealed in enzyme reaction buffer using a ratio of 1 primer to 1.2 templates by heating to 80°C in enzyme reaction buffer and cooling to room temperature. Four different primers were annealed to the 80-mer template, and six separate primer extension reactions were performed for each p/t combination containing (A) core only, (B) core, \(\beta\), and \(\gamma\) complex, (C) core and SSB, (D) core, SSB, \(\beta\), and \(\gamma\) complex, (E) core, SSB, and \(\beta\), or (F) core, SSB, and \(\gamma\) complex (Fig. 1). Primer-templates were preincubated with SSB for 2 min at 37°C prior to a second preincubation with \(\beta\) and \(\gamma\) complex for 5 min at 37°C. In reactions where SSB, \(\beta\), or \(\gamma\) complex were omitted, preincubations were performed without reaction buffer only.

Reactions were initiated by the addition of core polymerase and SSB, and reactions were performed as follows. The first syringe was loaded with protein in reaction buffer, the second was loaded with reaction buffer only, and the third was loaded with p/t DNA in reaction buffer. Association reactions were initiated by mixing 100 μM of the protein solution with 100 μM of DNA solution. Control preshot reactions were done by mixing 100 μM of DNA with 100 μM of reaction buffer only to measure the anisotropy of DNA alone. Data points were typically taken every 2 ms. Multiple runs (10–25 runs) were averaged to increase signal to noise ratio.

**Association Kinetics at Different Concentrations of γ Complex—**One syringe contained 100 nM p/t DNA (X-rhodamine label on 5′-template end), 800 nM \(\beta\) dimer, and 0.5 mM ATP in reaction buffer with NaCl and less than 1% glycerol. The second syringe contained 200, 400, or 800 nM \(\gamma\) complex in reaction buffer with NaCl and less than 1% glycerol. Control preshots contained 50 nM p/t DNA, 400 nM \(\beta\), and 0.5 mM ATP.

**Association Kinetics for Different Orders of the Addition of Subunits to DNA—**Two reactions were performed. In the first, 100 nM p/t DNA (X-rhodamine label on 5′-primer end), 800 nM \(\beta\) dimer, and 0.5 mM ATP in reaction buffer with NaCl and less than 1% glycerol. Control preshots contained 50 nM p/t DNA, 400 nM \(\beta\), and 0.25 mM ATP. In the second reaction, one syringe contained 100 nM p/t DNA (X-rhodamine label on 5′-primer end) and 0.5 mM ATP in reaction buffer with NaCl and less than 1% glycerol. The second contained 500 nM \(\beta\) dimer, 600 nM \(\gamma\) complex, and 0.5 mM ATP in reaction buffer with NaCl and less than 1% glycerol. Control preshots contained 50 nM p/t DNA and 0.25 mM ATP.

**Dissociation Kinetics—**M13 trap DNA consisted of single-stranded wild type M13 DNA with two 30-nt oligonucleotides annealed to different sites. In this experiment, p/t DNA was preincubated with \(\beta\) and \(\gamma\) complex, and reactions were initiated by the addition of M13 trap DNA. One syringe was loaded with 100 nM p/t DNA (X-rhodamine label on the 5′-primer end), 800 nM \(\beta\) dimer, 400 nM \(\gamma\) complex, and 2 mM ATP in reaction buffer with NaCl and less than 1% glycerol. The second contained 500 nM M13 trap DNA in reaction buffer with NaCl. Control preshots contained 50 nM p/t DNA, 400 nM \(\beta\) dimer, 200 nM \(\gamma\) complex, and 1 mM ATP.

**RESULTS**

A series of protein-protein interactions govern the assembly of the pol III holoenzyme complex, and protein-protein and protein-DNA interactions, coordinated with the use of ATP, are required to load pol III holoenzyme onto p/t DNA. We are investigating protein-DNA interactions accompanying the loading of the \(\beta\) clamp by the \(\gamma\) complex using a sensitive optical technique, fluorescence depolarization, to detect and analyze binding of the proteins to DNA (21, 25). Fluorescence depolarization measures the rotational diffusion of fluorescence-labeled DNA alone or when present in a DNA-protein complex. The rotational motion depends predominantly on the molecular weight of the DNA-protein complex. Therefore, it is possible to distinguish free DNA from DNA bound by combinations of \(\beta\) and \(\gamma\) complex by measuring steady state fluorescence anisotropy. A pre-steady state anisotropy measurement allows protein-DNA complex formation to be visualized in real time.

**Minimal Primer-Template Requirements for Processive Synthesis by E. coli pol III Core Polymerase in the Presence of \(\beta\) and \(\gamma\).**

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2 L. B. Bloom and M. F. Goodman, unpublished results.
\( \gamma \) Complex—To observe maximum changes in rotational anisotropy signals during transitions between free DNA and bound DNA-protein complexes it is important to utilize the smallest possible DNA capable of supporting processive pol III DNA synthesis. On large DNA templates such as \( \phi X \) and M13 DNA, the processivity of DNA synthesis by pol III core DNA polymerase is increased substantially by the addition of \( \beta \) and \( \gamma \) complex. The \( \gamma \) complex loads \( \beta \) on DNA, and \( \beta \) acts as a sliding clamp to tether the core polymerase to the DNA template, reviewed in Ref. 26.

We found that a DNA template as small as 80 nt in length supports processive synthesis by core polymerase in the presence of \( \beta \) and \( \gamma \) complex. Processive synthesis on this 80-mer template requires \( \beta \), \( \gamma \) complex, and single-stranded binding protein, SSB (Fig. 1, lanes 23–28). A 5’-\( ^{32} \)P-labeled 30-nt primer was annealed to an 80-nt template so that 25-nt single-stranded regions were present on both the 5’- and 3’-ends of the template. The primers were extended by core polymerase and different combinations of accessory proteins (\( \beta \), \( \gamma \) complex, and SSB). After 2 min at 37 °C with SSB only, the p/t DNA was preincubated at 37 °C for 5 min with different combinations of accessory proteins in separate reactions. Reactions were initiated by the addition of the core polymerase and quenched after 10 s. Reaction products were analyzed by polyacrylamide gel electrophoresis.

Gel bands represent sites where the polymerase either dissociated or paused after incorporating one or more nt. Since DNA synthesis by the core polymerase alone has been demonstrated to be distributive (8), dark gel bands most likely indicate a high probability of dissociation at a given site. Synthesis by core polymerase alone was essentially distributive. Dark gel bands were present for DNA products that were extended by 1, 2, 3, 4, and 5 nt (Fig. 1, lane 23). Bands caused by the formation of longer products were less intense. The core alone was unable to synthesize DNA to the end of the template.

The addition of \( \beta \) and \( \gamma \) complex to the core polymerase, in the absence of SSB, did not significantly enhance the processivity of the core polymerase, although a small fraction of primers were extended to the end of the template (Fig. 1, lane 24). The addition of SSB alone to the core inhibited synthesis, as shown by faint gel bands (Fig. 1, lane 25). However, processive synthesis was observed by the core polymerase in the presence of \( \beta \), \( \gamma \) complex, and SSB (Fig. 1, lane 26). A dark gel band was present for primers extended to the end of the template, while faint bands due to dissociation of the core prior to reaching the end of the template were also present. The addition of either \( \beta \) and SSB in the absence of \( \gamma \) complex (Fig. 1, lane 27) or \( \gamma \) complex and SSB in the absence of \( \beta \) (Fig. 1, lane 28) did not result in processive synthesis by core polymerase. Reaction products resembled those by synthesis of core in the presence of SSB alone.

The efficiency of synthesis on the 80-nt template depended both on the length of the primer and its position on the template. On an 80-mer template, a 30-nt primer was extended more efficiently by core polymerase in the presence of \( \beta \), \( \gamma \) complex, and SSB than a 20-nt primer (data not shown). A 15-nt primer was not extended at all in a 20-s reaction (data not shown).

To achieve processive synthesis, the 30-nt primer had to be positioned on the 80-mer template so that a single-stranded region of DNA greater than 15 nt long was present at the 3’-template end (Fig. 1, lanes 5, 12, 19, and 26). 5’-\( ^{32} \)P-labeled primers, 30 nt in length, were annealed to an 80-nt template so that the length of the single-stranded region of DNA on the 3’-template end (3’-template overhang) varied. Primer extension reactions on these p/t DNAs were performed with core polymerase along with different combinations of accessory proteins as above. In the presence of \( \beta \), \( \gamma \) complex, and SSB, processive synthesis by the core polymerase was not observed when the 3’-template overhang was 10 or 15 nt long (Fig. 1, lanes 5 and 12). When the 3’-template overhang was increased to 20 nt, a faint gel band representing the extension of primers...
Fig. 2. Increase in steady state anisotropy for X-rhodamine-labeled DNA when γ complex loads β on DNA. A, the change in steady state anisotropy for a X-rhodamine-labeled primer-template when β and then γ complex were added to the DNA. An 80-nucleotide template was labeled at the 5'-end with X-rhodamine and annealed to a 30-nucleotide primer. The steady state anisotropy of the primer-template in reaction buffer containing ATP and MgCl₂ was measured (DNA). The anisotropy of this DNA solution was measured again adding a small aliquot of β (± β) and then γ complex (± γ complex). Concentrations after the addition of γ complex were 50 nM primer-template, 400 nM β (dimer), 200 nM γ complex, and 0.5 mM ATP. The anisotropy of this solution was measured again after the solution stood at room temperature for 15 min (15 min). An aliquot of ATP was then added, and the anisotropy was measured again (A + ATP). B, the change in steady state anisotropy for an X-rhodamine-labeled primer-template when γ complex and then β were added to the DNA. The conditions for this titration were the same as for A except that the order of the addition of β and γ complex was reversed. In this experiment, γ complex was added first, and then β was added to establish that both β and γ complex are required to give the large increase in steady state anisotropy (r = 0.26) for the DNA. A sketch of the reaction scheme is drawn at the top of the figure.

to the end of the template was observed for a reaction containing core polymerase, β, γ complex, and SSB (Fig. 1, lane 19). More efficient primer extension to the end of the template by core in the presence of β, γ complex, and SSB was observed when the 3'-template overhang was increased to 25 nt (Fig. 1, lane 26). This probably reflects a requirement for SSB to prevent β from "sliding off" the end of the template.

Although an excess of polymerase over DNA was used in primer extension reactions (core:p/t = 9:1), only a fraction of primers were extended during our short 10-s reaction time. In reactions with pol III core alone, inefficient primer extension may result from a weak association of core with DNA. In the presence of β, γ complex, and SSB, inefficient primer extension may be caused by a slow association of the core polymerase with β to form a processive βcore complex (or a slow displacement of SSB from the template). Note that it may also be difficult to load β onto all of the p/t molecules because γ complex may actively remove β resulting in a steady state population of DNA containing β and free DNA.

In primer extension assays, the fraction of primers extended increased with concentration of core polymerase in 10-s reactions (data not shown) and also increased with reaction time (data not shown), indicating that essentially all of the primers were extendible. Experiments are under way to measure the kinetics of association of the core polymerase with β to determine if a rate-determining association step limits the rate of primer extension. In the holoenzyme complex, the core and γ complex form part of a larger complex so that association of the core with β is likely to be more efficient, since it is in close proximity to β after β is loaded by γ complex.

Loading of β on Primer-Template DNA by γ Complex—Interactions of β and γ complex with p/t DNA under steady state conditions were observed by monitoring changes in fluorescence anisotropy of p/t DNA labeled with an extrinsic fluorescent probe, X-rhodamine. An 80-nt template was labeled at the 5'-terminus with X-rhodamine. A 30-nt primer was annealed to the center of the template so that 25-nt regions of single-stranded DNA were present on both the 5'- and 3'-ends of the template. This was the optimal p/t configuration supporting processive DNA synthesis by the pol III core in the presence of β, γ complex, and SSB (Fig. 1). A DNA synthesis reaction using a rhodamine-labeled template, carried out as shown in Fig. 1, demonstrated that the rhodamine label has no measurable effect on the activity of the pol III proteins (data not shown).

The steady state anisotropy of the rhodamine-labeled p/t, in the absence of pol III accessory proteins, was 0.166 (Fig. 2A). The addition of β (molecular mass 81 kDa for a β₂ dimer) to the rhodamine-labeled p/t DNA (52 nM DNA, 420 nM β₂ dimer) did not affect the anisotropy of the DNA (i.e. in the absence of γ complex, there was no measurable interaction between β and p/t DNA). The addition of γ complex to the solution of β and DNA (50 nM DNA, 400 nM β₂ dimer, and 200 nM γ complex) resulted in a large increase in the steady state anisotropy of the labeled DNA, from 0.166 to 0.257 (Fig. 2A). The rotational diffusion of the DNA decreased upon addition of β and γ complex.

To determine if the increase in steady state anisotropy in the presence of β and γ complex was caused by γ complex loading...
\[ \text{pol III Accessory Proteins} \beta \text{ and } \gamma \text{ Complex} \]

**pol III Accessory Proteins β and γ Complex**

β on the p/t or by γ complex alone binding to p/t, the order of the addition of γ complex and β to the p/t DNA was reversed (Fig. 2B). The solution of labeled p/t DNA in the absence of β and γ complex gave a steady state anisotropy of 0.165 as observed previously. The addition of γ complex (201 kDa) to DNA (51 nM DNA and 200 nM γ complex) resulted in a small increase in anisotropy to 0.176. However, the subsequent addition of β to the solution (50 nM DNA, 400 nM β dimer, 200 nM γ complex) resulted in a much larger increase in anisotropy to 0.248. Thus, the much larger increase in anisotropy was caused by γ complex loading β on the p/t DNA and not by direct interaction of γ complex alone with DNA.

In both titration experiments above, the anisotropy of the p/t DNA decreased when solutions of DNA, β, and γ complex were added. The solution of labeled p/t DNA in the absence of β and γ complex remained for 15 min at room temperature (Fig. 2). The anisotropy remained at its former level in both experiments when a fresh aliquot of ATP was added (Fig. 2). These results demonstrate that the interactions of β and γ complex with DNA required hydrolysis of ATP and that the ATP initially present was consumed during the 15-min incubation. The γ complex has been shown to be a DNA-dependent ATPase, and ATP is required for γ complex to load β on DNA (27).\(^3\) \(^3\) Hydrolysis of α-\(^32\)P-labeled ATP was measured in solutions containing an unlabeled p/t, β, and γ complex (data not shown). ATP was converted to ADP under these conditions. Loading of β by the γ complex did not take place when ATP was replaced in the reaction with either UTP or the nonhydrolyzable ATP analog AMPPNP (data not shown).

**Binding of γ Complex to DNA**—Association of γ complex and loading of β on DNA by γ complex are not simple equilibrium protein-DNA binding interactions. Association of γ complex with DNA is dependent on ATP hydrolysis, and loading of β onto DNA depends on both the catalytic activity of γ complex and the hydrolysis of ATP. Association constants for these proteins to DNA were not measured under true equilibrium binding conditions; instead, association was measured as a function of protein concentration to give apparent binding affinities under steady state conditions (Fig. 3). The binding of γ complex to different DNA substrates, single-stranded 30-nt oligonucleotide, single-stranded 80-nt oligonucleotide, and 30-mer/80-mer p/t were measured in the presence of 1 mM ATP (Fig. 3A). We previously used 0.5 mM ATP for steady state anisotropy measurements with 200 nM γ complex (Fig. 2). The higher concentration of ATP was used to ensure that it was not completely consumed during the 2–3 min taken to measure anisotropies using up to 800 nM γ complex (Fig. 3). γ complex was found to interact with the single-stranded 80-nt DNA substrate, although this association is weak (apparent \(K_D = 450 \text{ nM}\)). Interaction of γ complex with 30-mer/80-mer p/t DNA or with 30-mer ssDNA was barely detectable (Fig. 3A, lower two curves). However, when β was added to form β-γ complex, the interaction with ssDNA and p/t DNA was strengthened considerably (Fig. 3B). We want to emphasize that for the p/t DNA, the increase in anisotropy with increasing concentration of γ complex is most likely caused by loading of β onto DNA. The replication assays (Fig. 1) show that a processive replication complex can be formed on this p/t, demonstrating that γ complex is fully capable of loading β on this p/t in a biologically relevant manner.

The association of γ complex with either single-stranded DNA or p/t DNA required ATP hydrolysis. In the presence of the nonhydrolyzable ATP analog, AMPPNP, γ complex alone and a β-γ complex did not associate with either single-stranded 80-mer or with 30-mer/80-mer p/t DNA (data not shown). Since γ complex was observed to bind ssDNA and p/t DNA, in the absence of β in an ATP-dependent reaction, a preliminary experiment was carried out to see if binding of individual subunits of γ complex to ssDNA could also take place in the presence of ATP. No observable binding to either a single-stranded 30-mer or 80-mer was observed for γ, δ, δ', or χ-ψ at concentrations of at least 600 nM protein and 50 nM DNA (data not shown).

**Time-resolved Anisotropy Measurements**—Time-resolved intensity and anisotropy measurements (ns time scale) were made on p/t DNA containing a rhodamine-labeled primer-5' terminus. These measurements were made in the presence and absence of β and γ complex. When β and γ complex are present, the system is dynamic, i.e. β and γ complex continuously associate and dissociate from the DNA in an ATP-dependent reaction on a ms time scale. The time-resolved measurements re-

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\(^3\) J. Turner and M. O'Donnell, unpublished results.
fluctuate during the time course of the measurement. The presence of $\beta$ and $\gamma$ complex does not affect the lifetime of the X-rhodamine probe; the decay curves overlap. Therefore, the signal in Fig. 2 is truly reflective of the rotational diffusion of the protein-DNA complex.

Pre-steady State Association of $\beta$ and $\gamma$ Complex with p/t DNA Observed in Real Time—The kinetics of loading the $\beta$ sliding clamp onto p/t DNA by the $\gamma$ complex were observed in real time by measuring the steady state anisotropy of rhodamine-labeled p/t in stopped-flow reactions (Fig. 5). Note that the protein-DNA association kinetics occurring on a pre-steady state (ms) time scale are detected by changes in the steady state rotational anisotropy. Four curves are shown in Fig. 5. The lower horizontal curve shows a constant value for the steady state anisotropy of p/t DNA in the presence of $\beta$, which does not interact with p/t under these conditions (Fig. 2). The upper three data curves illustrate loading of a constant amount of $\beta$ (400 nM dimer) using three levels of $\gamma$ complex (100, 200, or 400 nM). The concentrations of $\beta$, $\gamma$ complex, p/t, and ATP for the reaction containing 200 nM $\gamma$ complex were the same as in the steady state experiment in Fig. 2.

The reaction was carried out using the following scheme (Fig. 5, sketch of reaction). $\beta$ (800 nM $\beta_2$) was present in one syringe along with p/t DNA (100 nM), ATP (1 mM), and buffer containing Mg$^{2+}$. The $\gamma$ complex was present in a second syringe in the same buffer with Mg$^{2+}$ but without ATP. The X-rhodamine label was present at the 80-mer template-5' terminus. The contents of the two syringes were delivered to the reaction chamber, and anisotropy data points were collected at 2-ms intervals (see "Experimental Procedures"). Multiple runs were done to ensure the reliability of the results.

The data collected were then analyzed using a stopped-flow spectrophotometer. The decay in fluorescence intensity, i.e. the fluorescence lifetime of the rhodamine-labeled p/t with an X-rhodamine label on the 5'-primer terminus in the presence and absence of 200 nM $\gamma$ complex and 630 nM $\beta$ dimer. Both reactions contained 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl$_2$, 2 mM ATP, and less than 1% glycerol as described under "Experimental Procedures." Data were collected over 30 min. The concentration of ATP was increased from 0.5 to 2 mM so that ATP would not be completely consumed during the time course of the measurement. The presence of $\beta$ and $\gamma$ complex does not affect the lifetime of the X-rhodamine probe; the decay curves overlap. Therefore, the signal in Fig. 2 is truly reflective of the rotational diffusion of the protein-DNA complex.

Steady state anisotropy values (Fig. 2) are a function of both the lifetime and rotational correlation time of a fluorophore (23, 24). Since the fluorescence lifetime of the rhodamine probe was the same in the presence and absence of $\beta$ and $\gamma$ complex, the changes in steady state anisotropy that we measured using rhodamine-labeled DNA are the result of changes in rotational motion of the probe only. Therefore, the signal in Fig. 2 is truly reflective of the rotational diffusion of the protein-DNA complex.

Pre-steady State Association of $\beta$ and $\gamma$ Complex with p/t DNA Observed in Real Time—The kinetics of loading the $\beta$ sliding clamp onto p/t DNA by the $\gamma$ complex were observed in real time by measuring the steady state anisotropy of rhodamine-labeled p/t in stopped-flow reactions (Fig. 5). Note that the protein-DNA association kinetics occurring on a pre-steady state (ms) time scale are detected by changes in the steady state rotational anisotropy. Four curves are shown in Fig. 5. The lower horizontal curve shows a constant value for the steady state anisotropy of p/t DNA in the presence of $\beta$, which does not interact with p/t under these conditions (Fig. 2). The upper three data curves illustrate loading of a constant amount of $\beta$ (400 nM dimer) using three levels of $\gamma$ complex (100, 200, or 400 nM). The concentrations of $\beta$, $\gamma$ complex, p/t, and ATP for the reaction containing 200 nM $\gamma$ complex were the same as in the steady state experiment in Fig. 2.

The reaction was carried out using the following scheme (Fig. 5, sketch of reaction). $\beta$ (800 nM $\beta_2$) was present in one syringe along with p/t DNA (100 nM), ATP (1 mM), and buffer containing Mg$^{2+}$. The $\gamma$ complex was present in a second syringe in the same buffer with Mg$^{2+}$ but without ATP. The X-rhodamine label was present at the 80-mer template-5' terminus. The contents of the two syringes were delivered to the reaction chamber, and anisotropy data points were collected at 2-ms intervals (see "Experimental Procedures"). Multiple runs were done to ensure the reliability of the results.

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summed to increase the signal:noise ratio, and the data shown were run-averaged over 5 points, while the raw data were fit by a double exponential (Fig. 5, solid curves).

The data showed an increase in anisotropy for the labeled p/t with an observed rate of $12 \pm 2$ s$^{-1}$, resulting from association of $\beta$ and $\gamma$ complex with p/t DNA. In this experiment, $\beta$ was assigned to or in excess of $\gamma$ complex, and both were in excess of p/t DNA. The amplitudes of the reaction times courses increased with an increase in $\gamma$ complex and reflect an increase in the total amount of protein bound to DNA. Although more protein bound to p/t DNA as the concentration of $\gamma$ complex was increased, the observed reaction rate remained constant. Each protein-bound DNA curve exhibited a small, but significant, decrease between 250 and 350 ms prior to reaching steady state ($t > 500$ ms).

There are several possible explanations for this small decrease in anisotropy. First, the dip could reflect the initial sliding off of $\beta$ from the relatively short 30-mer/80-mer p/t DNA in the absence of SSB, relaxing to a steady state characterized by repeated sliding off and reloading of $\beta_2$ onto p/t DNA. Note that SSB which was present in the polymerization reactions to keep the $\beta$ dimer from sliding off the p/t DNA was not present during the anisotropy measurements because the presence of SSB bound to the DNA would limit the increase in anisotropy that would be observed when $\beta$ and $\gamma$ complex bind. A second explanation is that the decreased anisotropy reflects dissociation of some fraction of $\gamma$ complex from the protein-DNA complex. $\gamma$ complex has been shown to dissociate from $\beta$ after loading it on DNA (9, 16). A third possibility is that $\gamma$ complex initially binds the p/t at multiple sites. A nonproductively bound $\gamma$ complex may have to dissociate or may have to be displaced before productive loading of $\beta$ onto the p/t can occur to achieve steady state loading and dissociation. A fourth, much more interesting, possibility might be a dramatic conformational change in the $\beta\gamma$ complex that affects the rotational mobility of the protein-DNA complex.

Stopped-flow anisotropy measurements were made using different orders of the addition of $\beta$ and $\gamma$ complex to 30-mer/80-mer p/t DNA (Fig. 6). Depending on which interactions or steps are rate-limiting, the order of the addition of these components to one another should affect the observed reaction rate. In these reactions, the 5' primer terminus, rather than the template, was labeled with X-rhodamine. Since the rate of association of $\beta$ and $\gamma$ complex with DNA was not affected by the concentration of $\gamma$ complex, but the magnitude of the anisotropy increase was a function of the $\gamma$ complex concentration (Fig. 5), a concentration of 300 nM $\gamma$ complex was used in these experiments to give a large anisotropy signal change during the time course of these reactions. The concentrations of $\beta$, DNA, and ATP were the same as in Fig. 5.

In Fig. 6A, $\beta$ (800 nM dimer) was preincubated with p/t DNA (100 nM) and ATP in one syringe, and $\gamma$ complex (600 nM) and ATP were present together in the second syringe. In Fig. 6B, $\beta$ (800 nM), $\gamma$ complex (600 nM), and ATP were present together in one syringe, and p/t DNA (100 nM) and ATP were in the second syringe. There was a much more rapid increase in anisotropy.
when the solution of β and γ complex were added to p/t DNA (Fig. 6B) than when γ complex was added to β and DNA (Fig. 6A). An estimate of the loading rate of β following preincubation with γ complex is 70 s⁻¹, which is at least 6 times faster than the rate of loading β in the absence of preincubation with γ complex (Figs. 5 and 6A). Inclusion of ATP along with the γ complex prior to encountering β and DNA resulted in the same observed rate of increase in anisotropy, 12 s⁻¹, as in reactions where ATP was added to γ complex at the same time as β and DNA (Fig. 5). Thus, the rate-determining step appears to be different for reactions where β, γ complex, and ATP were preincubated prior to the addition of the p/t in comparison with reactions where either γ complex alone or γ complex and ATP were added to a solution of β and p/t DNA.

The decrease in anisotropy observed between 250 and 350 ms appears to be more pronounced in the reaction in Fig. 6B than in Fig. 6A. Both data sets were fit to the sum of an exponential increase and an exponential decrease in anisotropy. Since the rate of the increase in Fig. 6B is more rapid, more of the decrease in anisotropy is observed.

In a similar experiment, a solution of β and γ complex in the absence of ATP was added to a solution of p/t and ATP (data not shown). The observed rate of increase in anisotropy for this reaction was 11 s⁻¹, indicating that this combination is not sufficient to bypass the rate-limiting step. These results are consistent with previous data showing that a stable multiprotein complex consisting of β, γ complex, and ATP remains in an ATP-dependent reaction in the absence of DNA (28).

Dissociation of β or β-γ Complex from p/t DNA in Real Time—In steady state reactions (Fig. 2), ATP was rapidly hydrolyzed, most likely by repeated loading of β, which rapidly dissociated from the short synthetic p/t DNA. Under our reaction conditions, two possible dissociation pathways may exist: a two-step dissociation process where γ complex dissociates from β and DNA after loading β and then β diffuses off the p/t, or a one-step process where a β-γ complex dissociates from the DNA. The rate of dissociation of β or β-γ complex from the 30-mer/80-mer p/t DNA (depending on which pathway occurs) was measured by preincubating β, γ complex, and ATP, and DNA in one syringe, while in the other syringe was placed an excess of single-stranded M13 DNA with two 30-nt primers. When the contents of the two syringes were mixed together in the stopped-flow reaction chamber, any β and γ complex that had either dissociated from the p/t DNA or had never been bound to the p/t became trapped by the M13 DNA so that it could not reload onto the 30-mer/80-mer p/t.

The rotational anisotropy remained essentially unchanged on a 1-s time scale in the absence of trap DNA (Fig. 7, top trace). A reduction in the steady state rotational anisotropy reflects an increase in the rotational motion of the p/t DNA as β (or β-γ complex) dissociated from the p/t, presumably by sliding off over the end (Fig. 7, bottom trace). Dissociation was rapid, with a first order off-rate constant of 6.4 s⁻¹. The data can be represented by a model in which β rapidly slides off the p/t DNA after being loaded. A cycle of repeated loading of β that requires ATP hydrolysis by γ complex, followed by rapid loss of β, would lead to consumption of ATP with time. A rapid dissociation of β would explain the requirement for SSB in primer extension assays to trap β on a linear p/t for enough time for the core to associate with β (Fig. 1).

DISCUSSION

Here we have shown that a simple DNA oligonucleotide primer-template that supports processive DNA synthesis can be used as a model system for analyzing interactions between pol III accessory proteins and core pol III with DNA. In the present study, we have demonstrated that the β sliding clamp-loading reaction carried out by the five-protein γ complex can be detected, with high sensitivity, by measuring changes in the steady state rotational anisotropy of fluorescence-tagged DNA. A binding analysis, using stopped-flow techniques, has allowed us to visualize the clamp-loading reaction in real time.

Loading the β clamp onto DNA by the γ complex is a prerequisite to achieving processive leading and lagging strand DNA synthesis in E. coli. To study the reaction steps that involve loading of the β sliding clamp onto p/t DNA, we have designed a simple model system that supports processive synthesis by the pol III core polymerase in the presence of β, γ complex, and SSB. We find that processive synthesis is supported by a 30-nt primer annealed to the central region of an 80-nt template, so that 25-nt single-stranded regions of DNA exist on both the 5'- and 3'-ends of the template. This 30-mer/80-mer p/t DNA system is convenient for studying the binding of accessory proteins (this report) and will also make possible study of the effects of processivity on the fidelity of DNA synthesis.

Extensions of ssDNA of at least 20 nt were necessary on both ends of the p/t DNA, in order to support processive synthesis by pol III core polymerase in the presence of β and γ complex (Fig. 1). Processive synthesis was observed only when SSB was included in reactions with core, β, and γ complex. These single-stranded regions are most likely needed to bind SSB efficiently. SSB inhibited synthesis in reactions by core alone except when the single-stranded region of DNA on the 5'-template end was only 10 nt long, suggesting that SSB cannot bind to this short
region of ssDNA. Binding of SSB to single-stranded regions of the template on both sides of the primer may help stabilize β on short p/t DNA long enough for a complex between β and core to assemble.

The 30-mer/80-mer p/t DNA was labeled either at the 5’-primer terminus or the 5’-template terminus with a fluorescent probe, X-hodamine, and the fluorescence anisotropy of this probe was then used to detect interactions with the pol III accessory proteins, β and γ complex. Association of β and γ complex with the labeled p/t DNA decreased the rate of rotational motion of the DNA and thus increased the steady state anisotropy of the probe. When β and γ complex were added to labeled p/t DNA, the anisotropy increased, demonstrating loading of β by γ complex. In contrast to the requirement for SSB to obtain processive synthesis on the short p/t DNA (Fig. 1), SSB was not required for β loading, further supporting the idea that SSB can provide a block keeping β from sliding off the ends of the primer. Consistent with the idea that SSB serves as a block to stabilize β on linear DNA, a rapid rate of dissociation of β was observed in the absence of SSB (Fig. 7). In these experiments, a block is not necessary for loading per se, but rather to keep β on the template so that core can bind to it and harness its ability to serve as a processivity factor.

We investigated binding of γ complex to individual ssDNA and p/t DNA components. In the absence of β clamp, γ complex bound extremely weakly to 30-mer ssDNA and 30-mer/80-mer p/t DNA (Fig. 3A). A somewhat higher affinity binding was observed with 80-mer ssDNA (Fig. 3A). Binding to each of the forms of DNA was significantly enhanced in the presence of β clamp (Fig. 3B). In preliminary experiments with the individual subunits comprising γ complex, we were unable to detect binding of γ, δ, δ’, or χ-φ subunits to the single-stranded 30-mer or 80-mer (data not shown).

Stopped-flow fluorescence anisotropy was used to measure the association of β and γ complex with p/t DNA, on a ms time scale, in real time. Three different molecules, β, γ complex, and DNA, come together in these loading reactions, and γ complex hydrolyzes ATP to load β on DNA. Three types of loading reactions were performed in which the order of the addition of these components to one another was varied. These experiments demonstrated that preincubating γ complex, ATP, and β caused β to load onto DNA at a rate in excess of 70 s⁻¹ (Fig. 6B), which appeared to bypass the rate-limiting step in the overall loading reaction, which occurred at a rate of 12 s⁻¹ (Figs. 5 and 6A).

The rate-limiting step is not likely to be the association of γ complex with β, because in reactions where the concentration of γ complex was varied (Fig. 5), the observed association rate was independent of the concentration of γ complex. Instead, the slow step is more likely to be intramolecular in nature, because it does not depend on the concentration of γ complex. Perhaps a conformational change within β or γ complex may be limiting the rate of loading β. Two prominent possibilities for this step are a conformational change in γ complex or a conformational change in β, such as "opening" of the ring prior to placement on DNA.

The pol III holoenzyme contains two DNA polymerase cores (for simultaneous replication of both strands of a duplex chromosome) and only one γ complex, all connected together by a dimer of the τ subunit (15). The γ complex acts catalytically during lagging strand replication to load β clamps onto RNA primers as they are produced by the helicase/primase (29). The lagging polymerase, upon finishing a fragment, rapidly disengages from its β clamp, leaving it behind on DNA, and cycles back to the newly assembled β clamp on the upstream primer to extend the next lagging strand fragment (Fig. 8). Since the replication fork moves at a rate of approximately 1 kilobase/s and lagging strand fragments average 1–2 kilobase in length, a new fragment is produced every 1 or 2 s. Thus, the γ complex must be capable of clamping β onto primers within 1 s. The speed of clamp assembly observed in this report (12 s⁻¹, t₁/₂ = 58 ms) is well within the speed required for γ complex action on the lagging strand. Perhaps even more relevant is that the rate of clamp assembly when β and γ complex were preincubated together was about 70 s⁻¹ (t₁/₂ ~ 10 ms), which is much faster than required for recycling β clamp onto the lagging strand for rapid synthesis of Okazaki fragments. It is reasonable to suppose that β clamp and γ complex are, in fact, "preincubated" in vivo, prior to the occurrence of the clamp-loading reaction. In vivo, γ complex loads β onto a template primed by primase with RNA. Although the rate of loading β onto a template primed with DNA was rapid enough to be consistent with loading rates required during replication, the efficiency of loading β on a template primed with RNA might be even greater.

It has been shown previously that the γ complex undergoes a structural alteration in the presence of ATP (28). It is believed that this conformational change is necessary for γ complex to bind β, because ATP is required to isolate a β-γ complex interaction in vitro. However, preincubation of γ complex with ATP did not result in an increase in the rate of β loading, suggesting that the ATP-dependent conformational change in γ complex may not be rate-limiting during the process of β loading. Therefore, the rate-limiting step is most likely an intramolecular event that occurs upon the interaction of β with γ complex (which has itself already changed its conformation upon interaction with ATP). The most attractive possibility then is that
the rate-limiting step is opening of the toroidal dimer. To determine if this explanation is correct, we will investigate mutant proteins in this assay that have different oligomeric stability, the prediction being that a tighter dimer interface would decrease the rate of loading, while a weaker interface might result in a faster rate.

In summary, we have shown that a simple DNA oligonucleotide primer-template that supports processive DNA synthesis can be used as a model system for analyzing interactions between pol III accessory proteins and core pol III with DNA. We have demonstrated that the β sliding clamp-loading reaction carried out by the five-protein γ complex can be detected, with high sensitivity, by measuring changes in steady state rotational anisotropy of fluorescence-tagged DNA. A binding analysis, using stopped-flow techniques, has allowed us to visualize the loading reactions in real time. The future use of a series of site-directed β mutants should help in defining the rate-limiting step during clamp loading, and it should also be possible to determine the properties of β that cause it to interact with two dissimilar E. coli DNA polymerases, pol III core and polymerase II (30).

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Nucleic Acids, Protein Synthesis, and Molecular Genetics: Dynamics of Loading the β Sliding Clamp of DNA Polymerase III onto DNA

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