A Model for *Escherichia coli* DNA Polymerase III Holoenzyme Assembly at Primer/Template Ends

DNA TRIGGERS A CHANGE IN BINDING SPECIFICITY OF THE γ COMPLEX CLAMP LOADER*  

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*The abbreviations used are: pol III HE, *E. coli* DNA polymerase III holoenzyme containing 10 subunits including pol III core (α, ε, and θ subunits), β-dimer sliding clamp, γ complex (γ, δ, ε, δ, and ψ subunits), r-dimer; SSB, single-stranded binding protein; PAGE, polyacrylamide gel electrophoresis; ss, single-stranded; p/t, primer/template; nt, nucleotide(s); RhX, X-rhodamine; BSA, bovine serum albumin; DTT, dithiothreitol; ATP-s, adenosine 5′-O-3′-thiotriphosphate; MDCC, N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide; PBP-MDCC, phosphate-binding protein labeled with N-[2-(maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide.

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The γ complex of the *Escherichia coli* DNA polymerase III holoenzyme assembles the β sliding clamp onto DNA in an ATP hydrolysis-driven reaction. Interactions between γ complex and primer/template DNA are investigated using fluorescence depolarization to measure binding of γ complex to different DNA substrates under steady-state and presteady-state conditions. Surprisingly, γ complex has a much higher affinity for single-stranded DNA ($K_d$) in the nM range than for a primed template ($K_d$ in the μM range) under steady-state conditions. However, when examined on a millisecond time scale, we find that γ complex initially binds very rapidly and with high affinity to primer/template DNA but is converted subsequently to a much lower affinity DNA binding state. Presteady-state data reveals an effective dissociation constant of 1.5 nM for the initial binding of γ complex to DNA and a dissociation constant of 5.7 μM for the low affinity DNA binding state. Experiments using nonhydrolyzable ATPγS show that ATP binding converts γ complex from a low affinity “inactive” to high affinity “active” DNA binding state while ATP hydrolysis has the reverse effect, thus allowing cycling between active and inactive DNA binding forms at steady-state. We propose that a DNA-triggered switch between active and inactive states of γ complex provides a two-tiered mechanism enabling γ complex to recognize primed template sites and load β, while preventing γ complex from competing with DNA polymerase III core for binding a newly loaded β-DNA complex.

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*Escherichia coli* pol III HE 1 is responsible for replicating the

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E. coli genome (reviewed in Ref. 1–3). Pol III HE contains pol III core along with accessory proteins. Pol III core is made up of three subunits, α, ε, and θ. The α subunit possesses 5′ → 3′ polymerase activity (4) and the ε subunit contains 3′ → 5′ proofreading exonuclease activity (5, 6), while the function of the θ subunit is not clear (7, 8). Synthesis by pol III core is extremely inefficient owing to its low intrinsic processivity ~10 to 20 nt (9). The addition of accessory proteins dramatically increases pol III core processivity to several thousand nt, thereby providing a high replication efficiency necessary for genome duplication (4, 10).

Accessory proteins enhance pol III core processivity by tethering it to a primed DNA template. Tethering of pol III core to DNA is accomplished by β protein, functioning as a sliding clamp, and by γ complex, required for loading β onto p/t DNA. X-ray data show that β sliding clamp is a ring-shaped homodimer with an inner diameter of about 35 Å, large enough to encircle duplex DNA (11). By encircling DNA, β dimer effectively clamps pol III core to the template through direct protein-protein interactions with the α subunit. Biochemical evidence suggests that β exists as a dimer when free in solution (12). The β dimer must be assembled onto DNA by the activity of the γ complex clamp loader which is composed of 5 different polypeptides having a stoichiometry of γ$_2$δδχψ (13–15). In addition to its clamp loading activity, γ complex also possesses DNA-dependent ATPase activity (16). ATP hydrolysis provides the energy required to complete the assembly reaction which involves loading β onto DNA followed by dissociation of γ complex (17, 18).

The overall efficiency of DNA replication depends in part on the efficiency of loading the β sliding clamp onto DNA and forming a processive replication complex. On the leading strand, where DNA is synthesized in one continuous strand, loading may be performed just once on an undamaged DNA template. However, on the lagging strand, where DNA is synthesized in relatively short Okazaki fragments of 1000–2000 nt, loading takes place at each primer site where synthesis of an Okazaki fragment begins. Thus, on the lagging strand, β dimers must be loaded and unloaded many times.

Kinetic studies have shown that the half-life for loading is on the order of 60 ms (19), rapid enough to support *in vivo* Okazaki fragment synthesis occurring about every 1 to 2 s. Not only is the rate of loading β important to the overall efficiency of synthesis of Okazaki fragments, but the orientation of the β ring on DNA and the site on DNA where the β clamp is placed is also critical. The β sliding clamp has two distinct faces, one that interacts directly with pol III core (20). In order for a
loading reaction to result in formation of a replication competent core-β complex, the β ring must be oriented on DNA so that the face that binds the core is oriented in the proper direction along the DNA axis. If loading were random, half the loading reactions would be unproductive because half the β rings loaded onto DNA would face in the wrong direction. In addition to proper orientation of β rings on DNA, the overall efficiency of DNA synthesis should be enhanced by placing β clamp on DNA at or near a primer 3′-end in anticipation of binding pol III core.

Once β sliding clamp is loaded onto DNA, pol III core requires unimpeded access for binding β. Protein footprinting experiments show that γ complex interacts with the same face of the β sliding clamp required for binding pol III core (20). Thus, if both γ complex and pol III core compete for binding with β, then mutual interference of both binding events is likely to diminish the efficiency of assembling a processive replication complex on DNA. Naktinis et al. (20) have shown that the relative affinities of γ complex and pol III core for β are different when β is free in solution and when it is bound to DNA. In the absence of DNA, γ complex has a higher affinity for β than does pol III core. However, the affinity of pol III core for β clamp is much greater when β has been assembled onto DNA. Indeed, once pol III core binds β, then γ complex is prevented from binding.

Structural features at a replication fork, reflecting interactions between pol III HE and other proteins, clearly play an important role in assembling a replication complex. DNA properties, such as the ss-double-stranded DNA junction formed by a primed template, may play a role in directing the assembly of the pol III HE on DNA. Assembly of sliding clamps specifically at primer DNA junctions would allow for the greatest overall efficiency of DNA synthesis particularly on the lagging strand where clamps must be assembled for synthesis of each Okazaki fragment. In this paper, we report on the presteady-state and steady-state interactions occurring between γ complex and primer and template DNA molecules individually, enabling us to propose a model to account for pol III HE assembly at primer DNA ends.

**EXPERIMENTAL PROCEDURES**

**Enzymes—**All DNA polymerase III proteins were purified and γ complex was reconstituted as described (11, 21), and stored in 20 mM Tris- HCl, pH 7.5, 2 mM DTT, 0.5 mM EDTA, and 10% glycerol. Assay buffers for all experiments contained 0.5 mM ATP, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl2, 5 mM DTT, and 40 μg/mL BSA.

**Oligonucleotides—**Two different DNA substrates were used in these experiments, a ss 50-mer covalently labeled on the 5′-end with X-rhodamine and a 30/105-mer primer/template covalently labeled on the 5′-end. The sequences of the 50-mer and primer template were as follows:

5′-GAG CTA TAA ATG ATT GCC TCT TGT TTA GCC ATG ATG CCA AAC CTA ATT

3′-TCT TCT TCA TGA GGT TAG CGA GGA CGA CTA TAG GTT TGG TTA TAA

ACC GCC A-3′

TGG CGG GTA AGC TGG TCC TTT TGG CGA GTA CTT TTA TGG ATG TAA

AAC TGC GAC-5′

30/105-mer p/TONA

All oligonucleotides were synthesized using standard β-cyanoethyl phosphoramidite chemistry and purified by denaturing polyacrylamide gel electrophoresis (PAGE) and stored in deionized H2O. A 5′-C6 amine linker (Glen Research) was added to the 5′-end of the 50-mer during synthesis. Following synthesis and purification, this 5′-amine group was covalently labeled by addition of X-rhodamine isothiocyanate (Molecular Probes, catalog number X-491) as described (19, 22).

The 105-nt template, labeled on the 5′-end with X-rhodamine, was made by ligating a 55-mer that was chemically phosphorylated during synthesis to the X-rhodamine-labeled 50-mer. For the ligation reaction, a 30-nt oligonucleotide complementary to both the phosphorylated 55-mer and the X-rhodamine-labeled 50-mer was used as a scaffold. When the scaffold was annealed to both the 50- and 55-mer, nicked substrate was formed where 15 nt on the 3′-end of the 50-mer and 15 nt on the 5′-end of the 55-mer were annealed to the 30-mer scaffold. Typical ligation reactions contained 20–30 μM (80–120 nmoL) DNA, 1 mM ATP, and 50 units (1 unit/μL) of T4 DNA ligase (Life Technologies) in 67 mM Tris-HCl, pH 7.8, 10 mM MgCl2, 10 mM DTT, and 25 μg/mL BSA and were incubated overnight at 14–16 °C. Single-stranded 105-mer was purified from unreacted 50- and 55-mer as well as the 30-mer scaffold by denaturing PAGE.

For control experiments, a second 105-nt template covalently labeled with an RhX probe at a site 26 nt from the 5′-end was made as above by ligating a 65-mer to a 40-mer that was covalently labeled with RhX. A commercially available dU phosphoramidite modified with an amino linker at the 5′ position (Amino-Modifier C2 dT, Glen Research) was incorporated into the 40-mer during synthesis at the 26th nt from the 5′-end. This amino linker was labeled with X-rhodamine isothiocyanate as above.

Primer/template DNA consisted of a 30-nt primer annealed to a Xrhodamine-labeled 105-nt template to create a substrate with 30 nt of duplex DNA near the 5′-end. p/t DNA was annealed to the primer and 50 nt of ssDNA on the 5′-end of the primer and 25 nt of DNA on the 5′-end of the primer (refer to sequence above). The p/t DNA was annealed by incubating 1.2 mol eq of 30 nt primer with 1 mol eq of 105 nt X-rhodamine-labeled template in a water bath that was initially at 80 °C and allowed to slowly cool to room temperature. Excess single-stranded DNA was removed by denaturing PAGE. Gel-purified p/t DNA was used in experiments where indicated since the presence of the excess ss 30-mer did not affect assays. DNA concentrations were determined using calculated extinction coefficients for ssDNA. Duplex DNAs were heat denatured in a UV spectrophotometer to determine the absorbance of ssDNAs.

**Steady-state Fluorescence Anisotropy Measurements—**Steady-state anisotropy measurements were taken using a QuantaMaster QM-1 fluorometer (Photon Technology International) equipped with a 75 W xenon arc lamp, an excitation monochromator, dual emission monochromators, photon-counting detectors with Hamamatsu R928 PMTs, and Glen-Thompson polarizers. Samples were excited with vertically polarized light at 580 nm (5 nm band pass) and both vertically and horizontally polarized emission at 610 nm (5 nm band pass) were simultaneously measured (T-format). Typical sample volumes of 80–100 μl were used in a microcuvette (internal dimensions 3 × 3 × 5 mm, Starna Cells).

Titrations experiments were performed by addition of a constant volume of γ complex solution to a cuvette containing a solution of RhX-labeled DNA and assay buffer. Concentrations of γ complex were varied while each assay contained 50 nM RhX-labeled DNA, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl2, 0.5 mM ATP, 5 mM DTT, and 40 μg/mL BSA. Titrations experiments that included β clamp were performed as above except 1.4 μl of 62 μM β clamp was added to 80 μl of γocomplex-DNA solution to obtain a 1 μM β final concentration without significantly changing the concentrations of the other species in solution. Titrations experiments using the nonhydrolyzable ATP analog, ATPγS were done under identical conditions replacing 0.5 mM ATP with 0.5 mM ATPγS.

For each sample, vertically and horizontally polarized emission intensities were measured. Background intensities were measured for solutions containing assay buffer only. Intensities were corrected by subtracting background intensities. The polarization bias (G-factor) of the fluorometer was determined by measuring vertical and horizontal emission intensities when samples were excited with horizontally polarized light (23). Anisotropy values (Iv) were determined at each assay concentration by measuring vertical and horizontal polarization corrected intensity values and the equation, r = (Iv − GIVH)/(Iv + 2gIIVH), were I, and I are the intensities of vertically and horizontally polarized emission, respectively, measured when samples are excited with vertically polarized light and g is the G-factor. Multiple anisotropy values were determined at each concentration of γ complex and sample standard deviations were calculated from the raw anisotropy data.

**Steady-state ATPase Assays—**Labeled phosphate-binding protein was prepared by overexpression of a mutated phoS gene from E. coli and by purifying and labeling the protein with a fluorescent probe as described in Ref. 24. The plasmid carrying the mutated phoS gene was a gracious gift from Martin Webb. The accretion of phosphate, following ATP hydrolysis, was measured on a QuantaMaster QM-1 fluorometer
(Photon Technology) by monitoring the increase in intensity of a fluorescent probe bound to the phosphate-binding protein. Steady-state ATPase assays for both ss 50-mer and p/t DNA were measured in 200-μl reaction volumes. A 100-μl sample of enzyme buffer, 50 nM DNA, 25 nM γ complex, 500 nM ATP, 10 μM PBP-MDCC, 0.05 units/ml purine nucleoside phosphorylase, and 50 μM 7-methylguanosine was placed in a cuvette. The sample was excited at 430 nm and emission data were collected at 474 nm. After 1 min of data accumulation, a 10-μl aliquot of 10 mM ATP was added to the cuvette and an additional 14 min of data was collected. Purine nucleoside phosphorylase and 7-methylguanosine were included in the reaction to remove phosphate contamination prior to ATP hydrolysis. The amount of ATP hydrolyzed throughout the reaction was calculated from the direct measurement of phosphate released and bound by the phosphate-binding protein over time.

Presteady-state Fluorescence Anisotropy Measurements—A Biologic SFM-4 stopped-flow (Molecular Kinetics, Pullman, WA) equipped with four independently driven reagent syringes and a 31-μl cuvette (model number FC-15) with a 1.5-mm path length were used. Dichroic sheet polarizers (380–770 nm, Oriel Corp., Stratford, CT) were mounted directly onto the cuvette. A Quantamaster QM-1 fluorometer, described above, was used as an excitation source by focusing the output from the excitation monochromator (580 nm, 1.25–1.5 nm band pass) onto a fused silica fiber optic consisting of a bundle with dimensions of 0.25 × 2.56 mm containing 9 250-μm fibers (Fiberguide Industries, Stirling, NJ). A sample cuvette was mounted perpendicularly to the stopped-flow cuvette. Vertically and horizontally polarized emission were measured simultaneously in a "T"-format through UVVIS liquid light guides with a 5-nm core diameter (Oriel Corp., Stratford, CT) mounted directly against the stopped-flow cuvette. Fluorescence emission was detected with a photon-counting detection system consisting of two channels each with a filter holder containing a 610-nm cut-on filter (CVI Laser Corp., Albuquerque, NM), mounted onto an ambient PMT housing (Products for Research, Inc., Danvers, MA) with an R4457P PMT (Hamamatsu Corp., Bridgewater, NJ). Signals from both PMTs were detected via a 5X filter holder containing a 610-nm cut-on filter (CVI Laser Corp., Albuquerque, NM), mounted onto an ambient PMT housing (Products for Research, Inc., Danvers, MA) with an R4457P PMT (Hamamatsu Corp., Bridgewater, NJ). Signals from both PMTs were detected via a 5X filter holder containing a 610-nm cut-on filter (CVI Laser Corp., Albuquerque, NM), mounted onto an ambient PMT housing (Products for Research, Inc., Danvers, MA) with an R4457P PMT (Hamamatsu Corp., Bridgewater, NJ). Signals from both PMTs were detected via a 5X filter holder containing a 610-nm cut-on filter (CVI Laser Corp., Albuquerque, NM), mounted onto an ambient PMT housing (Products for Research, Inc., Danvers, MA) with an R4457P PMT (Hamamatsu Corp., Bridgewater, NJ). Signals from both PMTs were detected via a 5X filter holder containing a 610-nm cut-on filter (CVI Laser Corp., Albuquerque, NM), mounted onto an ambient PMT housing (Products for Research, Inc., Danvers, MA) with an R4457P PMT (Hamamatsu Corp., Bridgewater, NJ). Signals from both PMTs were detected via a 5X filter holder containing a 610-nm cut-on filter (CVI Laser Corp., Albuquerque, NM), mounted onto a...
FIG. 1. Steady-state binding of γ complex and both γ complex and β to DNA. A, the relative affinity of γ complex for ss 50-mer (triangles) and p/t DNA (circles) substrates was measured in solutions containing 50 nM DNA and increasing concentrations of γ complex. B, binding of γ complex and β to ss 50-mer (triangles) and p/t DNA (circles) substrates were measured in solutions containing 50 nM DNA, 1 μM β, and increasing concentrations of γ complex. C, binding of γ complex to p/t DNAs where the site of attachment of RhX differed (open and filled circles) or the site where the primer was annealed differed (triangles and squares). In each plot, changes in anisotropy as a function of γ complex concentrations were plotted. For A and B, initial anisotropy values for free DNA in the absence of γ complex were 0.224 for p/t DNA and 0.210 for ss 50-mer. For C, absolute anisotropy values at 0 nM γ complex were 0.222 and 0.227 for open and filled circles, respectively, and 0.245 and 0.246 for triangles and squares, respectively. The anisotropy value for free DNA was subtracted from the anisotropy value for DNA in the presence of γ complex to give the Δ anisotropy values plotted. Error bars show the standard error in two independent measurements. For p/t DNA in A and B, one measurement was made using gel purified DNA and the second was made with DNA containing an excess (0.2 mol equivalents) of 30 nt primer (“Experimental Procedures”). Both the ss 50-mer and p/t DNA were almost saturated with protein in the presence of 1 μM γ complex and 1 μM β, and similar overall increases in anisotropy were observed. All solutions contained 0.5 mM ATP, 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl2, 4 μg/ml BSA, and 5 mM DTT.

...taining both β and γ complex. These assays contain 1 μM β dimer + 0.5 mM ATP with increasing concentrations of γ complex (Fig. 1B). Much lower concentrations of γ complex in the presence of β are required to saturate each DNA substrate with bound protein compared with binding assays with γ complex alone. This difference is most striking for p/t DNA. In assays with γ complex alone, very little p/t DNA remains bound at steady-state, even at 1 μM γ complex, while in assays with γ complex + β, the p/t DNA is essentially saturated with protein at concentrations exceeding 1 μM γ complex. In addition to the
overall increase in protein binding to DNA in reactions with β + γ complex, there is a much smaller difference in the apparent affinities of these proteins for the ss 50-mer and p/t DNA. Apparent $K_d$ values for protein binding to DNA are 45 nM for the ss 50-mer and 210 nM for p/t DNA. These results suggest that β may increase the affinity of γ complex for DNA so that a complex between β and γ complex (βγ complex) has a higher affinity for DNA than γ complex alone.

One possible explanation for the apparent higher affinity of γ complex for ss 50-mer than p/t DNA is that γ complex may bind the larger p/t DNA at a site that is farther removed from the RhX probe so that it affects the anisotropy of the probe to a lesser degree. To rule out this possibility, we measured binding of γ complex to four p/t DNA substrates that differed in the site of attachment of the RhX probe and location of the primer. Two of the 30/105-mer p/t substrates were identical to the p/t used in Fig. 1, A and B, except for the position of the RhX probe. In one case, it was placed on the 5′ primer end, and in the other, it was covalently attached to an amino linker on the 5′ position of a template dU located 26 nt from the 5′ template end (Fig. 1C, sketch). Binding of γ complex to each of these p/t was measured under steady-state conditions. Observed anisotropy changes are the same regardless of the location of the probe; very small increases are seen for each of the p/t DNAs (Fig. 1C, open and filled circles). This suggests that the small increases in anisotropy seen when p/t DNA is titrated with γ complex are real, reflecting a small population of DNA bound by γ complex, and not a function of the sensitivity of the probe to binding.

Two additional 30/105-mer p/t's differed in the site where the primer was annealed, either at the 3′ template end or at the 5′ template end (Fig. 1C, sketch). Both of these p/t's were labeled with a RhX probe 26 nt from the 5′ template end. The location of the primer on the template does affect binding of γ complex to p/t DNA. When the primer is located at the 3′-end of the template, very small increases in anisotropy are observed on addition of γ complex as when the primer is annealed near the center of the template. However, when the primer is located on the 5′-end of the template, a greater increase in anisotropy is observed. It is interesting to note that the apparent affinity of γ complex is weakest for the p/t DNA substrates which have primer 3′-ends that can be extended by a DNA polymerase (Fig. 1C, circles and triangles), whereas γ complex has a higher affinity for the p/t DNA substrate (Fig. 1C, squares) that cannot be extended by a DNA polymerase.

The curious finding that γ complex apparently fails to bind p/t DNA in the absence of β at steady-state led us to measure ATP hydrolysis. Steady-state ATPase assays are carried out for both ss 50-mer and p/t DNA in reactions containing 50 nM DNA, 25 nM γ complex, 500 μM ATP, and 10 μM PBP-MDCC. Rates of phosphate release following ATP hydrolysis are $5.3 \times 10^{-8}$ μM/s for ss 50-mer DNA and $2.3 \times 10^{-8}$ μM/s for p/t DNA. In the absence of DNA, ATP hydrolysis by γ complex is negligible (data not shown). The rate of DNA-dependent ATP hydrolysis by γ complex in the presence of p/t DNA is roughly half that of ssDNA, suggesting that γ complex interacts almost as well with p/t DNA despite the small fraction of p/t DNA bound in steady-state binding assays. Steady-state rates of ATP hydrolysis were also measured in assays containing 50 nM β, and are $5.9 \times 10^{-8}$ and $4.1 \times 10^{-8}$ μM/s for reactions with ss 50-mer DNA and p/t DNA, respectively.

In the presence of β, ATPase rates do not change significantly for ss 50-mer and increase by a factor of 1.8 for p/t DNA. However, addition of β + γ complex causes a significant increase in the apparent binding of these proteins to p/t DNA, while ATP hydrolysis rates increase by a factor of about 2. These results are consistent with previously reported data for DNA-dependent ATPase activity for γ complex using DNA substrates in the presence and absence of β (16).

The seeming lack of interaction of γ complex with p/t DNA, based on the absence of a change in steady-state anisotropy, contrasts with a significant increase in ATP hydrolysis, suggesting that a γ-p/t DNA interaction must indeed be taking place. These apparently conflicting results point up the difficulty in relying on steady-state measurements alone to interpret specific protein-DNA interactions. However, a detailed and consistent picture of the clamp loading reaction can be obtained by performing presteady-state rotational anisotropy measurements.

Presteady-state Kinetics of γ Complex Binding to P/T DNA—In order to define the interaction of γ complex with p/t DNA more completely and to explain these apparently contradictory results between steady-state binding and ATPase assays, we investigated the kinetics of binding of γ complex to p/t DNA on a presteady-state time scale. In these experiments, reactions were initiated by mixing γ complex and DNA rapidly in a stopped-flow apparatus. Polarized emission of the RhX probe on DNA was measured in real time at 1-ms intervals during the time course of the binding reaction. These measurements were then used to calculate the steady-state anisotropy of the probe on a presteady-state enzyme kinetic time scale. Binding reactions were initiated by mixing equal volumes of a solution of 100 nM p/t DNA and 0.5 mM ATP from one stopped-flow syringe with a solution of 600 nM γ complex and 0.5 mM ATP from a second stopped-flow syringe (Fig. 2). Dramatic changes in anisotropy are observed during the time course of the binding reaction. The anisotropy initially increases rapidly from about 0.22 to 0.27 at a rate of about 100 s⁻¹ and then decreases at a rate of about 4 s⁻¹, almost back to the initial starting value of 0.22. It is this final anisotropy value that was observed in steady-state experiments. It is important to note that in these experiments the concentration of γ complex is in excess (6-fold) over DNA.

Presteady-state data show that a significant population of p/t DNA bound by γ complex is formed rapidly but then this population decays to a level where very little DNA is bound by γ complex. Thus, γ complex does indeed bind to p/t DNA, but this interaction is dynamic in nature. Such reaction kinetics are not consistent with a simple two-state binding reaction. Instead, the kinetic data suggest that γ complex initially binds p/t DNA avidly, but upon binding DNA, γ complex is altered so that its affinity for DNA decreases markedly.

Presteady-state Association Kinetics of γ Complex with P/T DNA as a Function of γ Complex Concentration—In order to define the interaction between γ complex and p/t DNA, presteady-state binding kinetics were measured at three different concentrations of γ complex, 100, 200, and 400 nM, and at a constant concentration of p/t DNA (50 nM). At each concentration of γ complex, a rapid rise in anisotropy is followed by a much slower decrease (Fig. 3). The amplitude of the rise phase increases with increasing γ complex concentration. These data were fit to the sum of an exponential rise and fall (not shown). Rates of the rise phases were 65 s⁻¹, 66 s⁻¹, and 130 s⁻¹ for 100, 200, and 400 nM γ complex, respectively. Although initial increases are very rapid and a small part of the rise was missed in the dead time of our instrument, these observed rates are on the right order of magnitude. The largest error is in the observed rate for the reaction at 100 nM γ complex where a small anisotropy change occurred. Going from 200 to 400 nM γ complex rates double with concentration. Apparent rates of the down phases are 8.6 s⁻¹, 9.0 s⁻¹, and 4.8 s⁻¹ for 100, 200, and 400 nM γ complex, respectively. Analysis of these data directly
in terms of a second-order on-rate, with cycling and inhibition, was then performed.

These data at all three concentrations of γ complex were simulated by a minimal kinetics model using the rate constants shown in Fig. 3 (solid curves through data) and the kinetics simulation program, KinSim (26). For these simulations, the anisotropy for free DNA was set at the experimentally determined value of 0.224 and the anisotropy for DNA bound by γ complex was set at 0.34. The value of 0.34 was chosen for bound DNA because this is the experimentally observed value when DNA is saturated with either SSB or β and γ complex, and is reasonable for a complex of this size. In this model, γ complex is assumed to be present initially in an “active” DNA binding state. Upon binding p/t DNA rapidly, γ complex is then converted to an “inactive” binding state and dissociates from the DNA. The $K_a$ values predicted by the model for γ complex binding to DNA in its active versus inactive state are about 2 nM and 6 μM, respectively. The affinity of γ complex for DNA changes by about 3 orders of magnitude. Following conversion from active to inactive state, γ complex then slowly returns to an active state. The conversion from the inactive-to-active DNA binding state is likely to be the rate-determining step in the cycle. Although γ complex is able to cycle through this pathway, at steady-state, the rate-limiting step is conversion from the inactive to active DNA binding state. Thus, the largest population of γ complex is present in the inactive state, and only a minuscule fraction of γ complex is bound by p/t DNA at steady-state.

This simple kinetic model is consistent with both the pre-steady-state and steady-state anisotropy measurements. The key feature of this model is the cycling of γ complex between two states, one which is active and the other inactive for DNA binding. However, the actual mechanism must have an additional level of complexity because ATP is required for DNA binding, and ATP hydrolysis occurs when γ complex is incubated with DNA. We speculate that ATP binding is associated with forming the active DNA binding state while ATP hydrolysis may be involved in forming the inactive state. Each of these steps in the minimal model represents a composite of steps that may include processes such as ATP binding, ATP hydrolysis, and enzyme conformational changes. If ATP hydrolysis occurs during each cycle, then this model also provides an explanation for why p/t DNA effectively stimulates ATP hydrolysis while a very small fraction is bound by γ complex at steady-state.

**Competitive Binding of γ Complex to P/T and ssDNA Substrates**—Reaction time courses for association of γ complex with p/t DNA (Fig. 3) suggest that γ complex cycles into an inactive DNA binding state in the presence of p/t DNA. We performed a series of competition experiments to explore this possibility further.

In the competition experiments, γ complex (400 nM) was added to a mixture of RhX-labeled ss 50-mer DNA (50 nM) and unlabeled competitor DNA. Both p/t DNA and ss 50-mer DNA were used as competitors in separate experiments. The anisot-
Binding of the RhX probe on the ss 50-mer DNA was then measured in the presence of increasing concentrations of unlabeled competitor DNA (Fig. 4). When ss 50-mer DNA is present as a competitor, the results are consistent with simple competitive binding of unlabeled DNA to free γ complex. At equimolar concentrations of labeled and unlabeled 50-mer (50 nM each), the fraction of labeled ss 50-mer DNA bound by γ complex is reduced by about half.

Although steady-state experiments (Fig. 1A) indicate that only a very small fraction of p/t DNA is bound by γ complex, p/t DNA acts as a much better competitor than the ss 50-mer! Addition of 10 nM p/t DNA competitor to a solution containing 400 nM γ complex and 50 nM RhX-labeled ss 50-mer reduces the concentration of bound ss 50-mer (50 nM) by about half. This decrease is much larger than can be explained by simple competition between p/t DNA and ss 50-mer for binding to free γ complex, and is consistent with a mechanism in which interaction of γ complex with p/t DNA cycles γ complex into an inactive DNA binding state.

**Binding of γ Complex to P/T DNA in Reactions with ATPγS**—Our data suggest that γ complex is in an active DNA binding state in the presence of ATP but is subsequently converted to an inactive state following binding to p/t DNA. To determine if ATP hydrolysis is necessary for DNA binding or conversion between active and inactive states, steady-state and presteady-state measurements can be carried out with either ATP or its nonhydrolyzable analog, ATPγS. Hingorani and O’Donnell (27) have shown that hydrolysis of ATPγS by γ complex does not occur on the time scale of our measurements.

Steady-state binding assays were carried out as in Fig. 1A except that p/t DNA was titrated with γ complex in the presence of either 0.5 mM ATP or 0.5 mM ATPγS. The presence of ATPγS has a dramatic effect on p/t DNA binding. The steady-state population of p/t DNA bound by γ complex is much greater in reactions with ATPγS (Fig. 5A, squares) than in reactions with ATP (Fig. 5A, circles), as indicated by the larger anisotropy values. These results are consistent with previous data showing that both β and γ complex bind to p/t DNA in the presence of ATPγS (17), and further demonstrate that ATP binding is sufficient to convert γ complex to an active DNA binding state. Taken alone, these data would seem to suggest that γ complex has a greater affinity for p/t DNA in the absence of ATP hydrolysis. These data are also consistent with a model where cycling of γ complex into an inactive DNA binding state is associated with ATP hydrolysis.

In presteady-state reactions, γ complex was preincubated with either ATPγS or ATP in one stopped-flow syringe prior to addition of p/t DNA and ATPγS or ATP from a second syringe. Final concentrations of 500 nM γ complex, 50 nM p/t DNA, and 0.5 mM ATPγS or 0.5 mM ATP were present in the reactions. As expected from the steady-state results, substitution of ATPγS for ATP dramatically alters the kinetics of γ complex binding to p/t DNA (Fig. 5B). In reactions with ATPγS, the anisotropy increases from about 0.22 to about 0.28 and then remains constant rather than decreasing.

Binding kinetics in reactions with ATPγS resemble typical second order binding and approaches equilibrium at a rate of 5.6 s⁻¹. Since γ complex binds but fails to hydrolyze ATPγS on this time scale (data not shown), it is likely that the decrease in anisotropy in the presence of ATP is associated with ATP hydrolysis. Thus, conversion of γ complex from an active to inactive DNA binding state is likely to require hydrolysis of ATP, whereas formation of an active binding state requires ATP binding but not hydrolysis. A second difference in kinetics is observed as a decrease in the apparent rate of binding γ complex to p/t DNA when ATPγS is present. The difference in association rates may be caused by differences in interactions of ATP and ATPγS with γ complex. It is possible that γ complex binds ATPγS in place of ATP, but that ATPγS is not as effective in producing a form of γ complex active for DNA binding.

**Kinetics of Loading β onto P/T DNA**—If the interaction with p/t DNA converts γ complex from an active to inactive binding state, and if return to the active state is rate-limiting, then the kinetics of loading β onto p/t DNA should depend on whether γ complex is present initially in an active or inactive state. In order to test this supposition, the kinetics of loading β onto p/t DNA were measured in experiments in which reactions were initiated either by adding a solution of γ complex + ATP to a solution of β + p/t DNA (Fig. 6A) or by adding a solution of γ complex + p/t DNA + ATP to a solution of β + ATP (Fig. 6B). In the first protocol, γ complex is postulated to be in its active DNA binding state when added to β + p/t DNA + ATP. In the second experiment, preincubation of γ complex + p/t DNA + ATP should convert the majority of active γ complex into an inactive DNA binding state.

As is readily apparent, the observed reaction kinetics are substantially different depending on whether the reactions are initiated with a predominantly active (Fig. 6A) or inactive (Fig. 6B) population of γ complex. In the first protocol, where active γ complex is added to β + p/t DNA (Fig. 6A), a biphasic increase in anisotropy is observed followed by a small decrease to reach a final value of about 0.3. Rates for the biphasic increase in anisotropy are about 200 s⁻¹ and 9 s⁻¹ and the rate of the decrease in anisotropy is about 4 s⁻¹. In the second protocol, where γ complex cycles into an inactive state prior to initiating the loading reaction, an increase in anisotropy to about 0.3 is observed following an initial lag phase of about 70 ms. During
the lag phase, the anisotropy remains constant so that an overall sigmoidal increase in anisotropy is observed.

**DISCUSSION**

The γ complex, powered by ATP hydrolysis, functions as a "clamp loader" assembling β the "sliding clamp" onto DNA. Ideally, DNA synthesis is most efficient if γ complex loads β onto DNA specifically at primers where synthesis by the core polymerase begins. Recognition of these sites is most important on the lagging strand since a new β clamp must be loaded for synthesis of each Okazaki fragment during replication. In this work, the question of specific interactions between γ complex and primed template DNA is addressed. The approach to answering this question utilizes a fluorescence anisotropy based assay to measure the binding of γ complex with p/t DNA under both steady-state and presteady-state conditions.

The affinity of γ complex for p/t DNA compared with ss 50-mer DNA was first measured in steady-state anisotropy binding experiments. The apparent affinity of γ complex appears to be much greater for ssDNA compared with p/t DNA in the steady-state assay, even though the p/t DNA contains a single-stranded 50 nt end having identical sequence to the ss 50-mer DNA (Fig. 1A). This observation implies that γ complex binds the ss 50-nt strand of DNA with greater affinity when free in solution rather than when present in the context of a primed DNA template. Although, it may appear that γ complex binds ssDNA with greater affinity than p/t DNA, it is nevertheless also observed that both DNA substrates are effective in stimulating the DNA-dependent ATPase activity of γ complex. The rate of ATP hydrolysis by γ complex in the presence of p/t DNA is about half that of ssDNA suggesting that interaction of γ complex with p/t DNA is nearly as great as that of ss 50-mer DNA.

Presteady-state kinetics of γ complex binding to p/t DNA were measured to resolve these seemingly contradictory results obtained using steady-state binding and ATPase assays (Fig. 2). Only by examination of the presteady-state interaction of γ complex with p/t DNA is it possible to determine that any protein-DNA interaction is occurring at all. These data show that γ complex indeed binds p/t DNA rapidly and avidly but then rapidly dissociates. Thus, association of γ complex with p/t DNA is not a simple second order binding reaction, but is instead a binding reaction in which interaction with DNA results in a change in the affinity of γ complex for DNA. A simple second order binding reaction would not have a transient "overshoot" of the equilibrium population of bound DNA and would only approach the equilibrium population by a monotonic increase in the fraction of DNA bound. The decrease in γ complex binding affinity for DNA is so severe (3600-fold) that upon reaching steady-state, only a small fraction of p/t DNA is bound by γ complex.

When the presteady-state analysis is performed as a function of γ complex concentration (Fig. 3), a rapid increase in anisotropy followed by a slower decrease is observed at each γ complex concentration, with a rise in amplitude occurring with increasing γ complex concentration. Simulation of the data demonstrate consistency with a model in which γ complex exists initially in an active state for rapidly binding p/t DNA. Once bound, γ complex is then quickly converted to an inactive DNA binding state and dissociates. Since recycling of γ complex back to an active DNA binding state is relatively slow, it is likely to be the rate-limiting step in the cycle and explains why a very small population of DNA is bound by γ complex at steady-state.

Additional evidence suggesting formation of an inactive DNA binding state can be obtained from competition binding assays in which γ complex is added to a solution of RhX-labeled ss 50-mer DNA and unlabeled DNA competitor. As expected, addition of ss 50-mer as a competitor decreases the fraction of bound RhX-labeled 50-mer DNA caused by competitive binding to γ complex. In contrast, addition of unlabeled p/t DNA as a competitor decreases the population of bound 50-mer DNA much more than expected based on the small fraction of p/t DNA bound by γ complex in steady-state binding assays. These results are consistent with a mechanism in which binding of 

**FIG. 5. Binding of γ complex to p/t DNA in reactions containing either ATP or ATP₅S.** A, steady-state binding of γ complex to p/t DNA was measured in solutions containing either 0.5 mM ATP (circles) or 0.5 mM ATP₅S (squares), 50 nM p/t DNA and increasing concentrations of γ complex. The change (increase) in anisotropy for the RhX probe covalently attached to DNA is plotted as a function of γ complex concentration. All solutions contain 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl₂, 5 mM DTT, and 4 μg/ml BSA. Error bars show the standard error in two independent measurements. B, presteady-state kinetics of γ complex binding to p/t DNA in reactions containing either 0.5 mM ATP or 0.5 mM ATP₅S. Reactions contained final concentrations of 50 nM p/t DNA and 500 nM γ complex in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 8 mM MgCl₂, 5 mM DTT, and 40 μg/ml BSA.
would also be consistent with these results. However, since the presteady-state kinetic data demonstrate the formation of γ complex in a state both active and inactive for DNA binding, the first mechanism seems by far to be the most likely.

Steady-state binding experiments using ATPγS, a nonhydrolyzable ATP analog, can be used to address the question of whether conversion of γ complex from an active to inactive DNA binding state is caused by ATP hydrolysis. These experiments establish two important facts. First, since ATP is essential for any interaction between γ complex and DNA, binding of ATP, without hydrolysis, is sufficient to form a state in which γ complex can bind DNA. Second, ATP hydrolysis is required to convert γ complex from an ATP-bound and active state one which is inactive in binding DNA. γ Complex binds p/t DNA with high affinity at steady-state when ATP is replaced with ATPγS. However, we find that γ complex is not converted into its inactive DNA binding state in the absence of hydrolysis (Fig. 5).

Strong evidence for the ATP hydrolysis-dependent conversion of γ complex from an active to inactive p/t DNA binding state is obtained in presteady-state binding reactions using ATPγS. Here we observe that the anisotropy of labeled p/t DNA increases on addition of γ complex but does not fall as was seen with ATP. Our previous data have similarly shown that loading reactions containing both γ complex and β form a stable “locked-on” complex when ATPγS is replaced by ATP (17, 27). An effect on the rate of binding is also observed when ATPγS is replaced by ATP. Binding reactions performed in the presence of ATP are on the order of 20 times faster than reactions using ATPγS. The slower association rate may be caused by ATPγS being much less effective in generating an active ATP-bound state of γ complex, so that there is a smaller population of γ complex initially present which can bind p/t DNA. Once bound, however, a relatively stable complex is formed between ATPγS-bound γ complex and p/t DNA. Since the increase in anisotropy is much slower in reactions with ATPγS, it cannot be determined from these data alone whether conversion of γ complex to the inactive state is less efficient or is completely abolished in the absence of ATP hydrolysis. The slower increase may effectively mask the biphasic nature and fall in anisotropy occurring at a rate of about 5 s⁻¹ in reactions with ATP.

In experiments in which γ complex is added directly to β + p/t DNA, a rapid biphasic increase in anisotropy is observed followed by a small decrease (Fig. 6A). The fast component of the biphasic increase in anisotropy is caused most likely by γ complex binding directly to p/t DNA, while the slower component is most likely caused by loading of β-dimer onto p/t DNA. Earlier results suggest the rate of loading β in this type of binding assay may be limited by the rate of opening the β-dimer ring (19). When γ complex was added to β and DNA, the observed rate of the loading reaction was independent of the concentration of γ complex and slower (12 s⁻¹ compared with 70 s⁻¹) than when β and γ complex were preincubated prior to the addition of ATP (19). The decrease in anisotropy is most likely caused by dissociation of γ complex from the p/t DNA. In contrast, in presteady-state kinetic assays where γ complex is preincubated with p/t DNA prior to addition of β, there is a lag of about 70 ms before an increase anisotropy (Fig. 6B). In this experiment, preincubation of γ complex with p/t DNA cycles γ complex into its inactive DNA binding state. The lag prior to the increase in anisotropy is consistent with a relatively slow recycling of γ complex to a state which is active for DNA binding and for loading β onto DNA. The anisotropy after reaching steady-state in both reactions is about the same, ~0.3.

When considered in their entirety, our results demonstrate...
that p/t DNA modulates the affinity of γ complex both for DNA and for a β-DNA complex. We propose a model in which an ATP-bound form of γ complex rapidly binds p/t DNA, is quickly converted to a state inactive for DNA binding, and then slowly recycles to a state which can once again bind DNA (illustrated in Fig. 7). While our model explains the global features of this binding reaction, many more discrete steps are likely to exist. Examples include sequential binding of 2 ATP molecules, the change in enzyme conformation upon binding ATP, and ATP hydrolysis or ADP release which may be associated with slow recycling of γ complex to an active binding state. Although binding and hydrolysis of ATP are not included in our model since these rates were not measured directly, the model does explain key features of the reaction of γ complex with p/t DNA.

What is clearly evident in these experiments is the dynamic nature displayed by γ complex when interacting with a primed template. The dynamic interactions achieve two major goals: first, they allow for the recognition of a proper site on DNA for template. The dynamic interactions achieve two major goals:

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