DNA sliding clamps attach to polymerases and slide along DNA to allow rapid, processive replication of DNA. These clamps contain many positively charged residues that could curtail the sliding due to attractive interactions with the negatively charged DNA. By single-molecule spectroscopy we have observed a fluorescently labeled sliding clamp (polymerase III charged DNA) loaded onto freely diffusing, single-stranded M13 circular DNA annealed with fluorescently labeled DNA oligomers of up to 90 bases. We find that the diffusion constant for the β clamp diffusing along DNA is on the order of 10^{-14} m^2/s, at least 3 orders of magnitude less than that for diffusion through water alone. We also find evidence that the β clamp remains at the 3’ end in the presence of *Escherichia coli* single-stranded-binding protein. These results may imply that the clamp not only acts to hold the polymerase on the DNA but also prevents excessive drifting along the DNA.

DNA sliding clamp proteins increase enzymatic processivity of DNA polymerases during DNA synthesis and are required by all cellular replicases for duplication of long chromosomes. This paper focuses on DNA polymerase III of *E. coli*, in which the β subunit (also known as the β clamp) tethers the polymerase to the DNA, thereby enabling the polymerase to add thousands of bases without detaching from the DNA (1, 2). The remarkable ring-shaped structure of the eubacterial, Archaeal, and eukaryotic DNA sliding clamps (3–5) provides a beautifully simple mechanism for sliding on DNA without falling off; the proteins form a closed ring around DNA and slide along like a washer, or possibly a nut, on a very long bolt.

The major clamps associated with DNA replication are the homodimeric β clamp loaded onto freely diffusing, single-stranded M13 circular DNA annealed with fluorescently labeled DNA oligomers of up to 90 bases. We find that the diffusion constant for the β clamp diffusing along DNA is on the order of 10^{-14} m^2/s, at least 3 orders of magnitude less than that for diffusion through water alone. We also find evidence that the β clamp remains at the 3’ end in the presence of *Escherichia coli* single-stranded-binding protein. These results may imply that the clamp not only acts to hold the polymerase on the DNA but also prevents excessive drifting along the DNA.

To investigate these issues, we have measured the position and diffusion of the β clamp on DNA by single-molecule fluorescence resonance energy transfer (FRET) combined with alternating laser excitation (ALEX) (17). These technologies allow us to observe the dynamic, unsynchronized movement of the sliding clamp on DNA (Fig. 1). Previous single molecule measurements of other proteins sliding on DNA have been performed by tracking the position of the proteins on long DNA metabolism (6–9). Seen to interact with a wide variety of proteins and protein complexes, they have been described as molecular tool belts and moving platforms (10, 11).

The β clamp is loaded at the primer-template junction of DNA by a clamp loader, the γ complex, via a process requiring ATP hydrolysis (12). The γ complex also unloads the β clamp from DNA, again requiring ATP hydrolysis. As long as ATP remains available, both the loading and unloading processes continue.

When not being actively unloaded, a loaded DNA sliding clamp remains stably attached to the DNA and moves with the polymerase (13). Even in the absence of a polymerase, DNA sliding clamps move along DNA by random thermal motion. In experiments where the β clamp was loaded onto a nicked, circular 7200 base pair DNA plasmid (active unloading was prevented), the β clamp remained on the circular plasmid with a half-life of an hour, two to three times the period of *E. coli* cell division (14). When the plasmid was linearized by a site-specific endonuclease, the β clamp disassociated within a few minutes, apparently by diffusing or “sliding” off one of the free ends of the DNA (15).

Beyond these experiments, very little is known about the motion of the sliding clamps on DNA, and analysis of the protein structure creates a mixed impression. On the one hand, DNA sliding clamps have a large, overall negative charge (the β clamp dimer has a formal charge of −44) that could create electrostatic repulsion between the clamp and the DNA and, therefore, rapid, “frictionless” diffusive motion. On the other hand, there is a prevalence of positively charged (basic) residues on the inner surface capable of forming attractive ionic bonds (electrostatic interactions) with DNA (3, 4, 16). Such favorable interactions would suggest energetic barriers to the clamp sliding along DNA that would slow the rate of diffusion along DNA.

The remarkable ring-shaped structure of the eubacterial, Archaeal, and eukaryotic DNA sliding clamps (3–5) provides a beautifully simple mechanism for sliding on DNA without falling off; the proteins form a closed ring around DNA and slide along like a washer, or possibly a nut, on a very long bolt.
molecules that have been tethered to a surface (18–20). The FRET measurements performed here are complementary to such experiments in two important aspects. First, we do not tether the DNA to any surface, thereby avoiding any possible interference from surface interactions. Second, we monitor short range (1–10 nm) movements of the protein on DNA.

EXPERIMENTAL PROCEDURES

Preparation of DNA Substrates—M13mp18 phage was prepared by two consecutive bandings in cesium chloride as described (21). Each M13-ss/dsDNA was created by annealing purified M13-ssDNA (single-stranded M13mp18) with one of eight 5′-labeled DNA oligomers as described in Laurence et al. (22), and two 3′-labeled oligomers. The M13-ss/dsDNA products were purified by gel filtration (Bio–Gel A-15m).

Cloning, Bacterial Expression, and Fluorescent Labeling of the β Clamp—Cloning and bacterial expression of the β clamp are as described in Laurence et al. (22). Protein expression and purification was done in two separate ways. In the O’Donnell laboratory (3) the protein was cloned, expressed, and purified as reported earlier. In the Camarero laboratory (22), the protein was also produced by an intein fusion system (IMPACT).

The gene fragment encoding the β clamp was amplified by PCR using a plasmid from the O’Donnell laboratory (3) containing the β clamp genes from E. coli K12 genomic DNA as template. The 5′-primer (5′-GGT GGT CAT ATG AAA TTT ACC GTA GAA CGT GAG CAT TTA TTA AAA-3′) introduced a Ndel site and the 3′-primer (5′-GGT GGT TGC TCT TCC GCA GCC CAG TCT CAT TGG CAT GAC AAC ATAT-3′) introduced a SapI restriction site and an extra Gly residue at the C terminus of the DNA β clamp. The PCR-amplified DNA was purified, digested simultaneously with Ndel and SapI, and then ligated into a Ndel, SapI-treated pTXB1 plasmid (New England Biolabs) to generate pEY10. The purified β clamp was characterized as the desired product by electrospray-MS (expected mass (average isotopic composition) = 40,642 Da; measured mass, 40,659 ± 15 Da). The isolated yield for purified β clamp was around 20 mg/liter.

Fluorescence Labeling Reaction—15 μl of Alexa 488-maleimide in N,N-dimethylformamide (1 mg/100 μl) was added to 500 μl of 50 μM β clamp in phosphate-buffered saline (50 mM phosphate, 150 mM NaCl, 2 mM Tris-(2-carboxyethyl)phosphine, pH 7). The smaller fluorophore was chosen to label the β clamp to minimize potential effects; the larger acceptor (Alexa 647) was used to label the DNA. The reaction was allowed to proceed for 2 h in the dark at room temperature. Excess glutathione was added to terminate the reaction, and the reaction mixture was applied to a Sephadex G-25 gel filtration column and then eluted with buffer to separate the β clamp from the small molecular weight reactant. The reaction resulted in an ~15–20% fluorescently labeled β clamp, and under these conditions no multiple labeling was observed (data not shown).

Trypsin Digestion and MS Analysis—The pH of a solution of fluorescently labeled β clamp (5 μM, 450 μl) was adjusted to ~8.0 by adding 50 μl of 1 M Tris-HCl buffer at pH 8. Sequencing grade modified trypsin (Promega) was added to the protein solution to a final trypsin/protein ratio of 1:20. The trypsin digestion was kept overnight at 37 °C and then analyzed by RP-C18 HPLC. The proteolytic peptides having absorption at 490 nm were analyzed by electrospray-MS and MS/MS.

MS/MS analysis was performed at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry. Peptide fractions collected from HPLC were diluted with an equal volume of 0.2% formic acid in methanol. Aliquots (5 μl) were analyzed by static nanospray infusion using an Advion NanoMate (0.3 pounds/square inch, 1.45 kV; Advion Biosciences, Ithaca, NY). MS and MS/MS data were collected on a Q-Tof API US hybrid quadrupole-time of flight mass spectrometer (Micromass–Waters Corp., Milford, MA). Peptide sequence and modifications were confirmed by comparison of the observed and theoretical peptide fragment ions. In particular, the mass and position of the attached dye was confirmed to the specific amino acid by ions from both the b- and y-ion series.

Loading of β Clamp on DNA—Loading of the β clamp was performed as in Laurence et al. (22). A 50-μl reaction mixture was formed in a 0.1 mM EDTA, 8 mM MgCl₂, 20 mM Tris-HCl buffer at pH 7.5 containing 4% glycerol and 40 μg/ml bovine serum albumin. NaCl was added to a final concentration of 65 or 15 mM depending on the experiment. 60 fmol (1.2 nM) of
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M13-ss/dsDNA was added with 330 pmol (6.6 μM) of SSB (or human RPA). 100 fmol (2 nM) of γ clamp loading complex and 20 fmol (0.4 nM) of β clamp (labeled monomer) were then added. Finally, ATP was added to a final concentration of 1 mM. For each experiment, 10 μl of this reaction mixture was placed into a well formed on a glass coverslip using a silicone gasket (Grace BioLabs), covered with another coverslip, and heated from room temperature to 35 ± 2 °C over a period of 2 min using a microscope-based heater (Warner Instruments, Hamden, CT). Single-molecule spectroscopy via ALEX was then performed for the specified times (75–180 min) at 35 ± 2 °C. ALEX has been successfully used to study DNA-protein interactions of RNA polymerase (23, 24), and single molecule measurements have been used to study clamp loading pathways in the assembly of the T4 bacteriophage DNA polymerase holoenzyme (25).

Single-molecule FRET Spectroscopy of Diffusing Species—Solution-based single molecule measurements were performed as in Kapanidis et al. (17). The ALEX experiments were performed using two laser lines: 488 nm (Innova 90C, Coherent Inc., Santa Clara, CA) and 633 nm (1163P, Uniphase, Milpitas, CA). The lasers were alternated with a period of 25 μs using an acoustooptical modulator (AOTF 48062-2.5-.55, NEOS technologies, West Melbourne, FL).

The excitation was reflected using a dichroic mirror (488–633 DBDR, Omega Optical, Brattleboro, VT). A 1.4 NA objective (60×1.4 NA oil immersion Plan Apochromat, Nikon, Tokyo, Japan) on a Nikon TE300 inverted microscope was used for the excitation; a 100-μm pinhole was used on the emission path. A second dichroic mirror (580 DRLP, Omega Optical) split the emission. The donor (Alexa 488) and acceptor (Alexa 647) channels were filtered using a bandpass filter (535DF45, Omega Optical) and a longpass filter (665AGLP, Omega Optical), respectively. The photodetectors, timing electronics, and software were as described previously (17). Fluorescence bursts were identified by searching for consecutive 10-ms time bins containing numbers of FRET photon counts exceeding a threshold (3.5–5.0 kHz) which depended on the concentrations of labeled M13 ssDNA and β.

FRET Efficiency \( E \) was calculated using the formula:

\[
E = \frac{n_{\text{FRET}}}{\left(n_{\text{FRET}} + \gamma n_{\text{D}}\right)},
\]

where \( n_{\text{FRET}} \) is the number of FRET photon counts, \( n_{\text{D}} \) is the number of Donor photon counts, and \( \gamma \) is a correction factor for differences in quantum and detection efficiencies for the donor and acceptor emission. The median count rate of each channel was used to subtract background counts from \( n_{\text{D}} \) and \( n_{\text{FRET}} \) before calculating \( E \). To calibrate \( \gamma \) we obtained single molecule FRET measurements for the 18-base pair dsDNA region using nanosecond-scale ALEX (nsALEX) (26). We used pulsed donor and acceptor excitation lasers and time-correlated single-photon counting electronics to acquire the data (LDH-P-C-470, LDH-P-635, and PicoHarp 300, Picoquant, Berlin, Germany). Selecting for FRET bursts and subtracting background, we determined that for the 18-base pair dsDNA region, \( E = 0.8 \pm 0.1 \). For \( E \) calculated using photon counts to match this value within the error we used a value of \( \gamma = 0.33 \).

**Purified Fluorescence Correlation Spectroscopy (FCS)**—In our experiments the vast majority of the fluorescence bursts were not from β clamp-DNA complexes. We used purified fluorescence correlation spectroscopy to obtain FRET autocorrelation signals only from bursts from β clamp-DNA complexes (22). Bursts in the FRET channel were sorted into those that have a large coincident burst in the donor channel (indicating a β clamp aggregate) and those that do not (indicating a β clamp-DNA complex). Additionally, the FRET autocorrelation was calculated only over small regions surrounding FRET bursts (100 ms before and after each burst). With the burst selection and the 100-ms region expansion, only relevant signals contribute, and standard FCS models may be applied to the result. The data were fit using the model,

\[
m_{\text{diff}}(\tau) = 1/N(1 + \tau/\tau_{\text{D}}) + c,
\]

where \( N \) is the effective number of molecules in the observation volume, \( \tau_{\text{D}} \) is the diffusion correlation time, and \( c \) is an additive factor that can deviate from 1 in purified fluorescence correlation spectroscopy. Any deviations from this model would indicate other dynamics such as distance-related FRET dynamics.

**Fluorescence Cross-correlation Spectroscopy**—We used sensitive, reduced background cross-correlations allowed by ALEX or the equivalent pulsed-interleaved excitation (27). Cross-correlations were formed from acceptor photons that were detected while the acceptor excitation laser was on and from donor photons detected while the donor excitation laser was on. In this way leakage of donor signal into the acceptor channel and direct excitation of the acceptor by the donor excitation laser were excluded from the analysis, eliminating these sources of spurious cross-correlation signals.

**One-dimensional Diffusion Simulations**—We performed a series of convolved random walk (Monte Carlo) simulations of our FRET events. In one dimension, an idealized donor diffuses to and from the acceptor with diffusion constants \( D \) ranging from \( 10^{-11} \) m²/s to \( D = 10^{-15} \) m²/s. At the same time, in three dimensions the donor and acceptor diffuse together in and out of the confocal volume over a period of 1000 s. The simulations were performed as described in Lawrence et al. (28) and were designed to mimic single molecule measurements of proteins sliding on short DNA regions which themselves diffuse into and out of a confocal detection volume (see Fig. 4). In the simulations the three-dimensional diffusion time in the confocal volume was set to 3 ms, based on observed values obtained from FCS of M13-ds/ssDNA diffusion (data not shown).

In the simulations the sliding clamp was assumed to undergo free one-dimensional diffusion on DNA without sticking at either end of the dsDNA region (reflecting boundary conditions). The size of the one-dimensional region corresponded to the various lengths of the dsDNA used in the experiments (18, 30, 45, and 90 base pairs where each base pair contributes 0.34 nm to the length) after subtracting out the width of the β clamp (~4 nm, see Fig. 2). The three-dimensional diffusion of the “β clamp-DNA complex” was simulated in time steps of \( \Delta t_{\mu,\text{DNA}} = 1 \) μs. During this interval photon excitation events were generated via a simulated Poisson process where the rate was determined by the three-dimensional position of the complex in the confocal detection volume. Between each photon excitation event there was a time between excitation of \( \Delta t_{\mu,\text{DNA}} \). The distance that the β clamp diffused on dsDNA during this interval
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FIGURE 2. A, the β clamp is labeled with Alexa 488 (D) at an available cysteine. Analysis of trypsin digestions indicate that the Cys-180 is preferentially labeled (cf. supplemental Figs. S2–S4). According to a simple model where B-DNA is placed axially inside the β clamp, and the distance between the donor (D) and the acceptor (A) is the same as the distances between the cysteine residues and axis (indicated parenthetically for each cysteine), the closest approach between the donor (D) and the acceptor (A) is ~3 nm. B, the above model can be simplified such that the β clamp is represented by a D-labeled ring with a 4-nm width (along the DNA). C, range of FRET efficiency values expected in our experiments. Four different lengths of dsDNA are used in our experiments (18, 30, 45, and 90 base pairs), each with a different range of possible E values, indicated by the black curve. Because of the significant width of the β clamp, about 12 base pairs or 4 nm, the range of motion (and E values) available for the different dsDNA regions is limited to that left of the respective vertical gray line.

was determined by a simulated gaussian random variable $x$ that satisfied $\langle x^2 \rangle = 2D\Delta s_{\beta-DNA}$, where the values of $D$ range from $10^{-11}$ to $10^{-15}$ m$^2$/s.

Simulations of slide-off experiments (15) were also performed by assuming a random initial position of the clamp on a linearized dsDNA plasmid. Random diffusive motion was simulated as above until the clamp slide off either end of the linearized DNA. Using 10,000 repetitions, we obtained the same slide-off time $\tau$ as given by the “survival probability” analysis (29), where $\tau \sim L^2/(D\pi^2)$, where $D$ is the diffusion constant sought, and $L$ is the length of the interval (~7200 base pairs).

RESULTS

FRET Labels for β Clamp and DNA—After fluorescently labeling the β clamp with a donor fluorophore D (Alexa 488), we determined the extent and position of the labeling and thereafter verified the activity of the labeled protein. To avoid multiple labels per protein subunit, the thiol-maleimide labeling reaction was run for 2 h (see “Experimental Procedures”), after which mass spectrometry (electrospray-MS) revealed that 15–20% of the protein contained a single label (supplemental Fig. S1). The rest of the protein remained unlabeled, and aggregates were observed very infrequently (<1 per 15 min observation on average for 0.4 nm labeled β clamp). Trypsin digestion of the labeled β clamp and subsequent MS/MS analysis of the corresponding fragments established that, out of four possible cysteine residues, Cys-180 was the labeled residue (see supplemental Figs. S2–S4). This result differs from an earlier study where Cys-333 was predominately labeled under similar conditions (30), possibly because the Alexa 488 used here is more hydrophilic than the fluorescein used in the earlier study.

Oligomers of single-stranded DNA 18, 30, 45, and 90 bases in length were commercially manufactured with an acceptor (A) fluorophore label (Alexa 647) at the 5′ end (see “Experimental Procedures”). For each of the four lengths, two labeled oligomers were designed to be complementary to one of two different sites on the M13 single-stranded, circular DNA (M13-ssDNA). We used two sites to exclude the possibility of sequence specificity in our measurements; none were identified in this way. Two additional oligomers of length 18 and 45 bases were labeled at the 3′ end. Each of the 10 labeled oligomers is uniquely identified by the oligomer length and the designation of site 1 or site 2 on the M13-ssDNA and whether the label was at the 3′ or 5′ end. Unless specifically stated, the specified oligomer is assumed to be labeled at the 5′ end. The labeled oligomers were each annealed to M13-ssDNA, creating 10 batches of A-labeled M13-ss/dsDNA with short, 18–90 bp double-stranded (ds) regions of DNA. Single-stranded binding protein (SSB) from E. coli or human RPA was then added to coat the single-stranded region and facilitate clamp loading. Once loaded onto the double-stranded region of the M13-ss/dsDNA, the motion of the β clamp should be restricted to the length of the dsDNA region due to the presence of SSB or RPA. We describe the product of this preparation as a “loaded clamp” or a β clamp-dsDNA complex, and for simplicity, each M13-ss/dsDNA is denoted simply by the corresponding dsDNA region length in base pairs.
Details of the labeling positions for the β clamp and for the DNA oligomers are shown in Fig. 2. In Fig. 2A, a molecular model of DNA and the β clamp is used to estimate expected distances between D on the sliding clamps and A on the 5′ end of the DNA oligomers. The distance of half-maximum FRET signal, $R_0$, is 5.1 nm for Alexa 488 (D) and Alexa 647 (A), based on spectroscopic analysis (Molecular Probes, Invitrogen). We estimate a distance of ∼3 nm between the D and A at the closest approach of the sliding clamp to the 5′ end of dsDNA region. In addition to the 3-nm offset at closest approach, the model of the sliding clamp on DNA yields a footprint on the DNA of about 4 nm (Fig. 2A). This leads to the simplified schematic of the sliding clamp on DNA shown in Fig. 2B. The recently observed tilt of the sliding clamp on DNA of 22° adds additional uncertainty to the values used in our simplified model; either monomer may be labeled, and the donor may be as much as 1 nm closer or further from the acceptor due to the tilt (16).

The FRET efficiency $E$ (cf. "Experimental Procedures") is given by the relation $E = 1/(1 + (R/R_0)^6)$, and thus, the geometric considerations above allow us to estimate the FRET efficiencies we would expect. We point out that expected autocorrelations and trends expected are insensitive to typical variations in photophysical characteristics and errors in distances because we are observing relatively large changes in distances, leading to large changes in $E$. Most important to notice in Fig. 2C is the rather fast transition from $E = 1$ to $E = 0$ over a short distance, relative to the lengths of the dsDNA regions.

**Single Molecule Observation of a β Clamp-DNA Complex—**After initiating the reaction that loads the β clamp on to DNA ("Experimental Procedures"), we observed via single molecule FRET that the β clamp was loaded on DNA. We first observed that a control reaction mixture containing the β clamp, the ATP-driven clamp-loader γ, and the annealed M13-ss/dsDNA coated with SSB but without ATP produced no single molecule fluorescence bursts indicative of FRET (spikes in Fig. 3A). After 1 mM ATP was added to the reaction mixture, many FRET bursts were observed (Fig. 3A). In other control experiments, the omission of other components, including the γ complex and SSB, resulted in a dramatically reduced number of FRET bursts (data not shown). These data demonstrate that the β clamp is being loaded onto the dsDNA region of the annealed M13 ss/dsDNA and can be detected at the single molecule level. Finally, data from an independent polymerization assay suggest that the labeling of β clamp not only allows loading of the β clamp on DNA but also does not significantly interfere with its being able to facilitate replication by binding to the polymerase (supplemental Fig. S6).

Single molecule measurements generally require concentrations substantially less than 1 nM. Unfortunately, the biochemical reaction is inefficient at these concentrations. We balanced these demands by using a concentration of β clamp (0.4 nM), which still allows single molecule analysis (albeit approaching the boundary), and using a higher concentration (1.2 nM) of M13 ssDNA with annealed DNA oligomer.

We observed that the clamp loading reaction is much more efficient at low NaCl concentrations; thus (in addition to 8 mM MgCl$_2$), we used relatively low NaCl concentrations (15 and 65 mM) in order to favor loading of the β clamp. Except for the...
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Results from Simulations Guide Our Analysis of Fluorescence Correlations and E Histograms—Before examining the results of further experiments, it is helpful to know what to expect based on results from simulations. Fig. 4 shows data from four sets of simulations performed with different diffusion constants $D$ for the β clamp on DNA, ranging from $D = 10^{-12}$ to $10^{-15}$ m$^2$/s. At each value of $D$, a Monte Carlo simulation was performed for each dsDNA length: 18, 30, 45, and 90 bp. FRET autocorrelation functions for different diffusion constants were extracted from the simulations using the technique of purified fluorescence correlation spectroscopy (Fig. 4, upper panels). For all but the slowest diffusion (i.e. except for $D \leq 10^{-15}$ m$^2$/s), the curves for each dsDNA length are distinguishable. Beyond that, for $D \geq 10^{-12}$ m$^2$/s, two time scales for the fluctuations (evident from an inflection in the autocorrelation function) are clearly visible; less so for $D = 10^{-14}$ m$^2$/s. The autocorrelations are indistinguishable for $D \leq 10^{-15}$ m$^2$/s because the FRET fluctuations caused by β clamp diffusion on the DNA occur on time scales longer than the M13-ss/dsDNA diffusion time. Because the M13-ss/dsDNA diffusion time is the time that the β clamp–DNA complex remains in the observable confocal volume, all correlations look the same, dependent of dsDNA length. The simulations also show that fluctuations in FRET affect the histograms of energy transfer efficiency $E$ (Fig. 4, lower panels). Note that by searching for photon bursts with only the FRET channel, loaded β clamps with low $E$ are found much less efficiently (in the simulation, there were the same number of loaded clamps in each case).

β Clamp Stays at 3′ End in the Presence of SSB—Once we had observed FRET between the β clamp and DNA (as in Fig. 3), we repeated the experiment for all four oligomer lengths using, first, SSB, and then using RPA and in each case at two different ionic strengths, one at 65 mM NaCl, which favored unloading of the β clamp, and another at 15 mM NaCl, that favored loading of the β clamp (and 8 mM MgCl$_2$ in both cases).

Beginning with a series of experiments where the acceptor was at the 5′ end of the DNA oligo, we were surprised to find that the β clamp appeared to stick to the ss/ds junction where it is loaded, i.e. at the 3′ end of the short DNA oligomer that has been annealed to M13-ssDNA. It is known that by coating the single-stranded region of the M13-ss/dsDNA, SSB enhances loading of the β clamp (31). In the presence of SSB, however, our experimental results (Fig. 5, solid and dotted black lines) did not match the simulation results in Fig. 4. Values for $E$ were significantly lower than expected for the 30-bp dsDNA relative...
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FIGURE 5. Histograms showing the number of observed complexes with FRET efficiency $E$ as a function of the dsDNA region site and length. Acceptor label is at 5' end of DNA. A, these histograms provide evidence for the clamp sticking to the 3' end of DNA with SSB but not human RPA (illustration). In B–E, higher $E$ indicates shorter distances. The site of the dsDNA region is denoted by line type. Solid, site 1; dotted, site 2 (note that the experiments for site 1 are twice as long as those for site 2). The use of E. coli SSB or human RPA is denoted by shading. SSB: black; RPA: gray. Ionic strength was also varied, 15 mM NaCl (favoring loading of clamp) on the left, and 65 mM NaCl (favoring unloading of clamp) on the right. B, 18-bp dsDNA. C, 30-bp dsDNA. D, 45-bp dsDNA. E, 90-bp dsDNA. For the two ionic strengths used, the peak positions in the $E$ histograms are the same, but the number of bursts is significantly larger at 15 mM NaCl. The annealing of the DNA to the M13 ssDNA was less efficient (by a factor of 3) for the 30-bp DNA in site 2 than in the other cases.

to the 18-bp dsDNA (Fig. 5, B and C); rather than the small change in $E$ shown in Fig. 4 (from 0.9 to 0.7 for $B–D$ or from 0.9 to 0.8 for A), a comparatively large shift was seen in Fig. 5 (from 0.8 to 0.4).

One explanation would be that our molecular model predicted $E$ values too high and that the $E$ value shifts were simply due to averaging due to diffusive motion of the clamp. However, no fluctuations were observed using the FRET autocorrelation (see below), excluding the possibility of a fast diffusion constant (Fig. 4, C and D). Moreover, the slowest diffusion case with $D = 10^{-15} \text{m}^2/\text{s}$ (Fig. 4A) can be excluded because the 30-bp dsDNA does not show a peak at high $E$. The remaining case (Fig. 4B) is also unlikely as there are significantly fewer FRET photon bursts observed for the 45- and 90-bp dsDNA, with a wide range in $E$ observed. Nonetheless, there was significant loading of the clamp in all cases as shown by cross-correlation analysis and single-molecule FCS (see Figs. 8 and supplemental Fig. S7).

As paradoxical as this initially appears, there is a simple explanation consistent with the above data; namely, the clamp has to remain stationary at the 3' end of the DNA. This would explain the lower $E$ observed for the 30-bp dsDNA compared with the 18-bp dsDNA in terms of the larger distance between the A-labeled 5' end of the 30-bp dsDNA and the D-labeled clamp (see Fig. 2). For the even longer distances encountered with the 45- and 90-bp dsDNA, there would only be residual FRET due to a small number of clamps that occasionally become free from the 3' end.

To verify the observation above and test the role of SSB in the 3'-sticking of the clamp, we replaced SSB with the human ortholog RPA, which will also coat the single-stranded region of the DNA. We found that this substitution changed both the number of FRET bursts for all cases and the distribution of $E$ for the 30- and 45-bp dsDNA (Figs. 5, medium gray lines). Indeed, with RPA the $E$ distribution measured for the 30-bp dsDNA nearly matches the $E$ distribution measured for the 18-bp dsDNA. Also, with RPA a much larger number of FRET bursts were detected for the longer stretches of DNA, especially for the 45-bp dsDNA (Fig. 5). The simultaneous increases in the number of FRET bursts and in the measured $E$ indicate that using RPA freed the clamp from the 3' end.

RPA also affected the number of FRET bursts observed as a function of time. When using SSB, the number of FRET bursts observed as a function of time decreased over the 1.5–3-h experiment (data not shown). When using RPA, the number of FRET bursts in most cases increased over time. This may mean that freeing the clamp from the 3' end decreases the efficiency of the clamp unloading pathway of the clamp loader complex. This issue requires additional investigation that can be performed using bulk-phase methods and will not be discussed further here.

We further tested the hypothesis of clamp sticking to the 3' end by performing experiments with the acceptor at the 3' end (Fig. 6). With the 3'-labeled oligomers, the loading was significantly less efficient, likely because the fluorophore at the 3' end interferes with loading. That loading takes place primarily at the 3' end is a reasonable assumption based on the structure of the clamp loader complex alone and with the clamp and DNA (33, 34). Thus, we were only able to obtain a statistically significant number of FRET bursts at 15 mM NaCl. The FRET efficiency for the 18-bp dsDNA region is significantly lower than that found with the 5' label. This is because the donor and acceptor are now on opposite sides of the sliding clamp (4-nm width, plus offset from DNA axis and linkers). The FRET efficiency histograms for the 3'-labeled 45-bp dsDNA region and the 3'-labeled 18-bp dsDNA region are nearly identical, with a
peak at $E = 0.4$. This is in stark contrast to the results at the 5' end, supporting our conclusion that the sliding clamp stays at the 3' end.

**First Estimate of $\beta$ Clamp Diffusion Constant**—The use of RPA allowed us to observe $\beta$ clamp on DNA that is freely diffusing, and by comparing the histograms of the observed FRET efficiency $E$ with those from simulations (Fig. 4), we can derive a lower limit for the diffusion constant. The 45-bp dsDNA in Fig. 5D for RPA has a peak at lower $E$ than for the 30-bp dsDNA in Fig. 5C for RPA. This implies a diffusion constant of $D > 10^{-15}$ m$^2$/s because, as we see from the simulations, if we had $D = 10^{-15}$ m$^2$/s (see Fig. 4A), there would be a distribution peak at high $E$ (near 1) independent of dsDNA length.

**Autocorrelation Analysis Indicates Slow $\beta$ Clamp Diffusion on DNA**—As well as analyzing the distributions in the FRET efficiency $E$, the fluctuations in $E$, indicative of fluctuations in distance between the D-labeled $\beta$ clamp and A-labeled 5' end of the dsDNA region, can also be analyzed to further determine the diffusion constant. To determine the time scale of these fluctuations, we extracted autocorrelations of the FRET signal using the technique of purified fluorescence correlation spectroscopy (22).

The results for the FRET autocorrelation curves are shown in Fig. 7. We observe no significant difference between the correlation curves for different dsDNA lengths, just as we saw for the simulations where $D = 10^{-15}$ and $10^{-14}$ m$^2$/s. Unfortunately, the variability of the data due to noise and photobleaching (supplemental Fig. S8 and supplemental information) blurs any distinction between these two values. A FCS model with a single diffusing component fits well to all experiments, implying that the movement of $\beta$ on DNA occurs on time scales similar to or longer than the diffusion of the M13 ssDNA through the observation volume. To exclude the possibility that the fluctuations were on time scales shorter than the 25-$\mu$s minimum resolution shown in Fig. 7, we also corrected the autocorrelations for the 25-$\mu$s laser switching used for ALEX (data not shown). There was no evidence of dsDNA length-dependent fluctuations down to $10^{-7}$ s. We also found no evidence of fluctuations using donor-FRET cross-correlations (data not shown). Additional measurements with higher concentrations of $\beta$ clamp also found no evidence of fluctuations on time scales shorter than the M13 ssDNA diffusion (see the supplemental material and supplemental Fig. S9).

Hence, we conclude that the fluctuations occurred on time scales longer than our observation time, and therefore, we have $D \approx 10^{-14}$ m$^2$/s. Putting this together with the last result, we conclude that the diffusion constant $D$ of the $\beta$ clamp is about $10^{-14}$ m$^2$/s.

**Cross-correlation Analysis Reveals Binding When There Is No FRET**—The FRET signal demonstrates loading of the $\beta$ clamp to DNA for
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The β Clamp May Be a 3’ Anchor—We found that in the presence of SSB, the β clamp tends to remain stationary at the single-strand/double-strand junction (the 3’ end of the labeled DNA oligo where the clamp is loaded and replication begins). This result was independent of the acceptor label being at either the 3’ or the 5’ end. With the acceptor label at the 5’ end, substituting RPA for SSB caused increases both in the number of FRET bursts and in the measured $E$, indicating that the β clamp was no longer stationary at the 3’ end. Moreover, by using RPA to free the β clamp from the 3’ end, we were able to deduce a diffusion constant for the β clamp moving on DNA.

The 3’ sticking may indicate an important role for the β clamp as an anchor at the site where replication begins. For example, if there is a time delay of just 10 ms between when the clamp loader ejects the sliding clamp and when the DNA polymerase binds, a diffusion constant of $10^{-14} \text{m}^2/\text{s}$ means that the sliding clamp would diffuse away an average distance of about 40 base pairs (~14 nm), approximately three lengths of the β clamp on DNA. To remain at the 3’ terminus, the sliding clamp may either bind to the dsDNA-ssDNA junction or SSB at the junction. There are two possible explanations for the observed release from the 3’ end when RPA has been substituted for SSB.

The simplest explanation is that the β clamp binds to SSB but not to the ortholog RPA. This is likely since bacterial and human orthologous proteins are not expected to be interchangeable in protein complexes (i.e. β clamp-SSB versus β clamp-RPA). The second possibility is that there is a β clamp-DNA interaction that holds the β clamp in place in the presence of SSB, but the interaction is somehow disrupted by RPA. A recent structure of a β sliding clamp-DNA co-crystal structure

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1 corresponds to 1–2 nM. The x-axis indicates the oligomer length of each batch of four measurements, shaded as follows. Light gray, E. coli SSB and 65 mM NaCl. Medium gray, SSB and 15 mM NaCl. Dark gray, RPA and 65 mM NaCl. Black, RPA and 15 mM NaCl.
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indicates the possibility of direct interaction between β and the template DNA at the dsDNA-ssDNA junction (16).

Although the details of the mechanism await further studies, the result that the β clamp remains at the 3′ end is significant for a number of processes. Interactions that hold the sliding clamp in place may be especially important in lagging strand synthesis, since replication at each Okazaki fragment must be started anew, with an additional clamp loading and polymerase binding event. Also, polymerase I, which can interact with β, must remove RNA primers and complete the Okazaki fragments. Finally, ligase binds β and may target β to join Okazaki fragments together. Retention of the sliding clamp at this junction may allow efficient completion of this task. Binding to SSB at the dsDNA-ssDNA junction would provide a simple way for the sliding clamp to remain at the 3′ end, waiting for the polymerase to bind.

Reducing Drift by Diffusing More Slowly?—When not stuck at the 3′ end, the one-dimensional diffusion constant of the β clamp along dsDNA is rather slow, on the order of \(10^{-14}\ m^2/s\), at least 3 orders of magnitude slower than free diffusion, as shown below. We argue here that the rate of this sliding along DNA should affect the function of the sliding clamp, and its value can provide insight into how DNA replication and repair is coordinated.

The mean square distance \(\langle \Delta x^2 \rangle\) that the clamp diffuses in a time \(\Delta t\) is given by the Einstein relation \(\langle \Delta x^2 \rangle = 2D\Delta t\), where \(D\) is the diffusion constant. Here it is useful to consider \(\Delta t = 1\ ms\); the average time it takes polymerase III (including the β clamp) to extend the DNA by one base (36); the diffusion constant determines how far the sliding clamp can diffuse during that time.

At one extreme one can imagine no friction (i.e. no attractive/adhesive forces) between the clamp and DNA, whereby the diffusion rate of the sliding clamp on DNA would be limited only by the viscosity of water. Based on the size of the β clamp, the diffusion constant can be estimated by Stokes-Einstein relation:

\[
D = \frac{k_BT}{6\pi\eta a}
\]

where \(k_B\) is the Boltzmann constant, \(T\) is the temperature, and \(a\) is a friction term that can be found by solving the Navier-Stokes equations. For a sphere, \(a\) is given by \(a = 6\pi\eta a\), where \(\eta\) is the viscosity of water, and \(a\) is the radius of the sphere. Using \(a = 4.5\ nm\), the approximate cylindrical radius of the β clamp, at temperatures 25–40 °C (i.e. 298–313 K) this gives an estimate of \(D\) in the range of \(5–8 \times 10^{-11}\ m^2/s\). To increase confidence in this estimate, one can solve the Navier-Stokes equations numerically for the molecular structure of the β clamp (3) using the program hydropor (37). At 27 °C this calculation gives \(D = 5.7 \times 10^{-11}\ m^2/s\), which agrees very well with our previous estimate.

Above we considered what might be seen as a washer sliding on a bolt, but what if the clamp moves more like a nut on a bolt? Even if there were no friction between the clamp and the DNA, the sliding clamp would be slowed down if it were constrained to follow the groove of the DNA and, therefore, required to rotate once around the DNA for every 10 base pairs in linear movement (38). We can account for this hypothetical constraint by calculating the rotational diffusion constant of the protein as it rotates on its axis. The hydropor program gives a rotational diffusion constant \(D_{rot} = 2.3 \times 10^6\ to\ 3 \times 10^6\ radians^2/s\), depending on the angle of the rotation. Using the average value and the rotational analogue to the Einstein relation, \(\langle \theta^2 \rangle = 2D_{rot}\Delta t\), we find that it takes 7 \(\mu s\) to make a full rotation around the DNA helix. By contrast, using the diffusion constant \(D\) estimated above, the time it would take to linearly diffuse the distance of one helical turn by simple linear diffusion is just 0.1 \(\mu s\). Thus, if the clamp was constrained to follow the groove of DNA and, therefore, rotate as it moved along the DNA, the viscosity of water would slow the clamp down by a factor of about 70 relative to simple linear diffusion. This leads to an effective linear diffusion constant of \(D_{eff} \sim 10^{-12}\ m^2/s\). A similar slow down (a factor of 110) was estimated several decades ago for the lac repressor, which binds to DNA with an altogether different topology (38).

We measure a diffusion constant 2 orders of magnitude smaller than \(D_{eff} \sim 10^{-12}\ m^2/s\). Hence, we conclude that diffusion of the sliding clamp is slowed down by significantly more than merely its rotation around DNA. Although it seems reasonable that the clamp follows the groove while attached to the polymerase, our measurements neither confirm nor deny this tendency in the absence of the polymerase. In fact, it may be argued that since the sliding clamp has been observed to slide over 13-nucleotide ssDNA loops and other secondary structures of DNA (15), it need not always follow the groove of the DNA. If the clamp is a nut, it is loose-fitting.

It is known that in the absence of the β clamp, the main polymerase, the α-subunit, loses its grip on the template and rapidly falls off the DNA. It is, therefore, plausible that a letting go of the DNA occurs often during synthesis, especially during polymerase swapping events (10, 11), upon which it would fall to the β clamp to maintain the correct position on the DNA rather than sliding away from the replication site. We propose here that the β clamp itself, by sticking significantly as it passes along the DNA, may help prevent such rapid drift.

Is the sticking to the DNA in contradiction to the rapid synthesis of DNA observed? After all, if the diffusion constant is very low, there may be a significant drag on the polymerase. We define the realm of significant drag as that where β diffuses only a distance of one base pair or less during the extension by polymerase III of one base pair. Using the nominal distance of a base pair,
the slide-off experiments could have been used to show that the sliding clamp does not create significant drag on the polymerase (as defined above) but left a range of 4 orders of magnitude possible for the diffusion constant, i.e. $10^{-15}$ m$^2$/s $\leq D \leq 5 \times 10^{-11}$ m$^2$/s, which was not resolved until the present experiments.

In summary, on the one hand, the value of $10^{-14}$ m$^2$/s implies significant friction (attractive or sticky interactions) between the protein and DNA, as it is at least 3 orders of magnitude slower than would be predicted for a protein of this size diffusing through water alone. On the other hand, this diffusion constant is large enough to imply almost no friction for the DNA polymerase to work against; by itself the β clamp moves from 1 base pair to the next ($\sim0.4$ nm) in 1/100th the time the polymerase takes to insert a single base ($\sim1$ ms) (the polymerase presumably also diffuses randomly on a sub-millisecond time scale, but it constitutes a Brownian motor where the last inserted DNA base is the ratchet-tooth that prevents backward motion).

Even though the β clamp easily moves from one base to the next in just 10 μs, longer distances require much more time due to the nature of diffusion. Because the β clamp spans a distance of about 12 base pairs on dsDNA ($\sim4$ nm, see Fig. 2A), the time to move just one clamp-width away is about a millisecond. It is possible then that the discovery of this relatively slow diffusion may expand the understood role of the clamp beyond that of just tethering the polymerase to the DNA to that of a reducing drift in the polymerization process. In other words the slow motion of the β clamp on DNA could mean that the polymerase does not have to maintain contact with the DNA at all times and may be required for efficient switching between polymerases and other DNA replication and repair factors.

The Selective Pressure to Stick and to Slide—What role might natural selection have in the observed dynamics? By multiple sequence alignment we have identified many residues of the clamp that are strongly conserved throughout the bacterial kingdom. Some of these residues are positively charged residues on the inner ring of the β clamp, and in molecular dynamics simulations those residues have been seen to contact the DNA via ionic bonds to phosphate groups, and one of these, Arg-24, has also been observed in a recent crystal structure contacting the DNA (16). What is more, the same pattern of conserved, positively charged residues on the inside of the ring occurs for the eukaryotic ortholog proliferating cell nuclear antigen. Therefore, there seems to be an evolutionary advantage in having certain residues that likely play a role in the attraction of the β clamp to DNA.

As we have discussed, less attraction between the β clamp and DNA means less friction or drag for the polymerase to work against but also less resistance to drift during polymerization. A third, not yet discussed issue is the role of attractive interactions in the loading and stability of the β clamp on DNA. It is reasonable that a number of attractive interactions may be required for successful loading onto DNA. In fact, a mutation of Arg-24 to Ala in the β clamp has a reduced effect on clamp loading. In the end there seems to be a number of competing factors that may put selective pressure in both directions with regard to the diffusion constant of a DNA sliding clamp. Further studies could improve on the specifics of these factors.

The Power of FCS Methods Applied to Single Molecule Dynamics—We have used solution-based single-molecule fluorescence spectroscopy and analysis to determine the dynamical action of a DNA sliding clamp on DNA. The solution-based experiment, where the motion of the clamp on DNA is convoluted with the motion of the entire plasmid in the confocal volume, has been somewhat more complex to analyze, but the benefits have been the straightforwardness of the measurements and the absence of interference from surfaces. The approach of combining single molecule fluorescence with modeling is opening up many new areas of study and will prove increasingly useful as structural biology moves toward dynamical biology, i.e. the study not only of the structure-to-function relationship of biomolecules but also the dynamics-to-function relationship.

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DNA: Replication, Repair, Recombination, and Chromosome Dynamics:
Motion of a DNA Sliding Clamp Observed by Single Molecule Fluorescence Spectroscopy

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