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The δ Subunit of DNA Polymerase III Holoenzyme Serves as a Sliding Clamp Unloader in *Escherichia coli**

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In *Escherichia coli*, the circular β sliding clamp facilitates processive DNA replication by tethering the polymerase to primer-template DNA. When synthesis is complete, polymerase dissociates from β and DNA and cycles to a new start site, a primed template loaded with β. DNA polymerase cycles frequently during lagging strand replication while synthesizing 1–2-kilobase Okazaki fragments. The clamps left behind remain stable on DNA ($t_{1/2}$ ~ 115 min) and must be removed rapidly for reuse at numerous primed sites on the lagging strand. Here we show that δ, a single subunit of DNA polymerase III holoenzyme, opens β and slips it off DNA ($k_{unloading}$ = 0.011 s^{-1}) at a rate similar to that of the multisubunit γ complex clamp loader by itself (0.015 s^{-1}) or within polymerase (pol) III* (0.0065 s^{-1}). Moreover, unlike γ complex and pol III*, δ does not require ATP to catalyze clamp unloading. Quantitation of γ complex subunits (γ, δ, δ′, χ, ψ) in *E. coli* cells reveals an excess of δ, free from γ complex and pol III*. Since pol III* and γ complex occur in much lower quantities and perform several DNA metabolic functions in replication and repair, the δ subunit probably aids β clamp recycling during DNA replication.

Sliding clamps are ring-shaped proteins that completely encircle DNA and slide freely along the double helix. This unique topological linkage between protein and DNA allows the clamp to tether DNA polymerase to the template and move along with the polymerase as it extends a new DNA strand (1). Thus, the circular clamp serves as a processivity factor, allowing DNA polymerase to replicate several thousand nucleotides without dissociation from the template (2, 3).

The DNA polymerase (pol)[1] III holoenzyme replicates the *E. coli* genome processively with the help of its dimeric circular sliding clamp, β (4–6). The holoenzyme comprises two copies of a core DNA polymerase, αεθ (7, 8), in which α is the DNA polymerase (9), ε is the proofreading 3′–5′ exonuclease (10), and the function of θ is yet unknown. The two cores are connected by a scaffold protein, τ (8, 11). τ also binds the sliding clamp loader, γ complex ($\gamma_{2-4}\delta_1\delta'_1\chi_1\psi_1$) (12, 13), as well as the DnaB helicase (14, 15). The two core polymerases function in a coordinated fashion, with one replicating the leading strand DNA and the other replicating the lagging strand DNA (Fig. 1) (14, 16). When the holoenzyme is assembled at the DNA replication fork, the cores assume their roles as leading or lagging strand polymerase and consequently perform in distinctly different styles. The leading strand polymerase remains continuously associated with its sliding clamp and the DNA template during replication. Conversely, the lagging strand polymerase must repeatedly bind and release DNA as it synthesizes discrete Okazaki fragments, due to the antiparallel nature of the double helix (17, 18).

The mechanism underlying this "polymerase hopping" behavior is based on the ability of the β clamp to serve as a target for the polymerase on DNA (17). The γ complex clamp loader within pol III* (holoenzyme without β), or γ complex free in solution, assembles β around newly primed sites on the lagging strand DNA template, in preparation for the polymerase. When the polymerase finishes an Okazaki fragment, it releases both the DNA and sliding clamp and cycles rapidly upstream to the new primer and clamp to continue DNA replication (Fig. 1) (17, 18). This model mechanism predicts that if β remains stably associated with DNA, the clamps abandoned by the polymerase will accumulate along the lagging strand DNA. Indeed, *in vitro* rolling circle assays performed with pol III* detect increasing amounts of β on the lagging strand DNA as replication proceeds, confirming that clamps left behind by the polymerase remain on the DNA (14).

Given that one clamp is used per Okazaki fragment and the *Escherichia coli* genome size is 4.4 megabases, about 2000–4000 clamps are needed for one round of DNA replication. There are, however, only about 300 β clamps in each cell (19). Consequently, each of these clamps must be used more than once during replication of the *E. coli* genome. Previous studies have shown that β remains stably encircled around DNA with a half-life greater than 70 min (20). Therefore, β does not recycle by simply falling off DNA; rather, there exists an active mechanism for retrieving and reusing the clamps during DNA synthesis. *In vitro* studies suggest there are at least three pathways by which β can be actively removed from DNA. The clamp loader, γ complex, can unload β from DNA in an ATP-dependent reaction (Fig. 1) (20, 21). pol III* also unloads β from nicked DNA in an ATP-dependent reaction, presumably via the unloading activity of its clamp loading component, γ/τ complex (17). Finally, we have noted that the δ subunit of γ complex appears capable of removing β from DNA by itself, in the absence of any nucleotide cofactors (22).

The observation that γ complex can unload β from DNA was surprising, since the clamp loader is known primarily for catalyzing β assembly onto DNA. Clearly, these opposing clamp

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[1] The abbreviations used are: pol, polymerase; SSB, single-stranded binding protein; RF-C, replication factor C; PCNA, proliferating cell nuclear antigen.

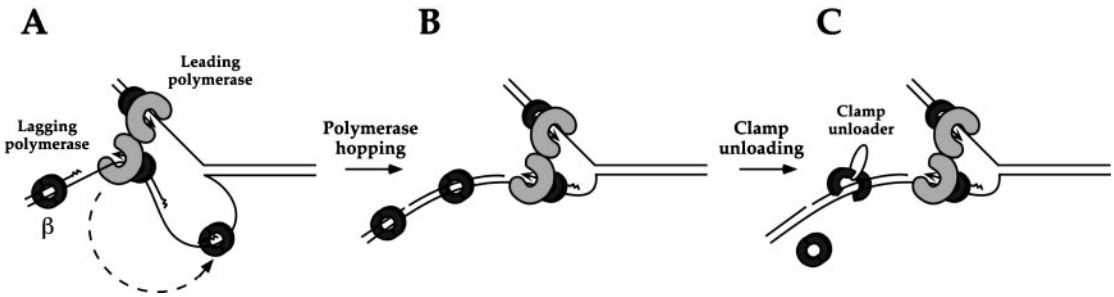

FIG. 1. **Recycling of sliding clamps during DNA replication.** As illustrated in the scheme, (*A*), the core polymerase replicates an Okazaki fragment to completion, after which it "hops" onto an upstream primer loaded with β to start a new fragment and continue lagging strand DNA synthesis. *B*, the β clamp left behind remains stably bound to DNA. *C*, "used" clamps accumulate on the lagging strand until removed by a clamp unloader (γ complex, pol III*, or δ) for reuse at new primed sites.

loading and unloading activities must be regulated such that γ complex performs the right function at the right time. Previous studies have shown that the core DNA polymerase and γ complex compete for interaction with β (21). β binds preferentially to γ complex when free in solution, but once γ complex places β on primed DNA, the polymerase binds β with higher affinity, barring further contact between the clamp and clamp loader (21). Presumably, only when the lagging strand polymerase abandons β after completing an Okazaki fragment does γ complex regain access to β and unload it from DNA.

The process of β loading is complex and requires that multiple subunits of the clamp loader function together. First, the γ subunits bind ATP and trigger conformational changes in γ complex that expose the δ subunit (which is normally occluded by δ′ in the absence of ATP) for interaction with β (22, 23). The ATP- and β-bound γ complex also binds a primed DNA template with high affinity (24, 25). The δ subunit opens the β ring before the γ complex hydrolyzes ATP (22, 24). Finally, two ATP molecules are hydrolyzed, coupled to conformational changes that lead to closure of the clamp around DNA and release of β·DNA from γ complex (22, 26). A minimal subcomplex of $\gamma_{2-4}\delta_1\delta'_1$ without χ and ψ appears sufficient for clamp loading (27), although their presence facilitates holoenzyme activity under high ionic conditions (28). This effect may be due to a stabilizing interaction between the χ subunit of γ complex and single-stranded DNA-binding protein (SSB) on DNA (29). The χ-SSB interaction also helps displace the primase from a new primer, allowing γ complex to access the DNA and assemble β around it in preparation for the polymerase (30).

In comparison with clamp loading, the clamp unloading process appears relatively simple in that it only requires opening of the β ring such that it can slip free from DNA (22). Although the mechanism of γ complex-catalyzed clamp unloading has not been defined in detail, it is an ATP-dependent reaction; therefore, the initial steps are probably similar to those of clamp loading. In this case, ATP binding to γ subunits and consequent conformational changes expose the δ subunit, which binds β on DNA and opens the ring, but then instead of closing the ring back around DNA, γ complex allows it to slip free from DNA. The most critical step in this process is ring opening, and δ can open the ring by itself (independent of other proteins as well as ATP). Therefore, it is likely that δ functions just as well as γ complex for clamp unloading and perhaps with greater efficiency, since it does not require ATP to power its activity; nor does it catalyze the reverse reaction and reassemble β onto DNA.

In this study, we examined the clamp unloading activities of both γ complex and the δ protein, in order to determine whether δ can serve as the physiological clamp unloader in *E. coli*. Quantitative Western analyses of cellular protein content were used to determine if there is any free δ protein in *E. coli*, and quantitative β unloading assays were designed to directly compare γ complex and δ activities. The results show that δ unloads β from DNA as fast as γ complex does, and it is present in 5–7-fold excess over γ complex in *E. coli* cells. Thus, δ probably performs the clamp unloading function in *E. coli*, sparing the limited number of γ complex and pol III* molecules in the cell for other important functions such as processive DNA replication.

EXPERIMENTAL PROCEDURES

Proteins and Other Reagents—Radioactive nucleotides were purchased from PerkinElmer Life Sciences, and unlabeled nucleotides were purchased from Amersham Pharmacia Biotech. Bio-Gel A-15m gel was purchased from Bio-Rad. Restriction enzymes were purchased from New England Biolabs. Oligodeoxynucleotide primers were synthesized by Life Technologies, Inc., and gpII-nicked pBluescript DNA was prepared as described earlier (20). Proteins were purified as described: α, ε, and γ (31), δ and δ′ (32), χ and ψ (33), θ (34), and SSB (35). γ complex and pol III* (a subcomplex of pol III holoenzyme that lacks the β subunit) were reconstituted from individual subunits and purified as described in Refs. 12 and 13, respectively. β^{PK} (β with a C-terminal kinase site: NH_2-RRASVP-COOH; Refs. 21 and 36) and $\beta_{monomer}$ (I272A,L273A) were purified according to previously described protocols for wild type β (4). β^{PK} was labeled with [^{32}P]ATP using cAMP-dependent protein kinase to a specific activity of 100 cpm/fmol as described (36). Hexokinase was purchased from Sigma. The catalytic subunit of cAMP-dependent protein kinase produced in *E. coli* was a gift from Dr. Susan Taylor (University of California, San Diego).

Buffers—Buffer A is 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 2 mM dithiothreitol, 4% (v/v) glycerol, 8 mM $MgCl_2$, and 0.5 mM ATP. Buffer B is buffer A without ATP. Buffer C is 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 2 mM dithiothreitol, and 10% (v/v) glycerol. SDS cell cracking buffer contains 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol. TTBS is 25 mM Tris-HCl, pH 7.4, 136 mM NaCl, 2.5 mM KCl, and 0.05% Tween 20.

Quantitative β-Unloading Reactions—The unloading substrate, β·DNA, was prepared as follows. β^{PK} (2 μM), ^{32}P-labeled at the C-terminal kinase site (21), was incubated with 1 μM γ complex and 1.25 pmol of gpII nicked pBluescript plasmid DNA (20) in buffer A. Following a 10-min incubation at 37 °C, the reaction was applied to a 5-ml A-15m gel filtration column equilibrated in buffer B at 4 °C in the cold room (to maximize stability of the β·DNA complex), and 200-μl fractions were collected. ^{32}P-β·DNA (in the excluded fractions) separates from free ^{32}P-β, γ complex, and ATP (in the included fractions). Two peak fractions containing ^{32}P-β·DNA were pooled for use in the unloading reactions.

Clamp-unloading reactions (45 μl in buffer B) contained approximately 15 nM ^{32}P-β·DNA and were initiated by the addition of γ complex (0.18, 0.3, 0.5, 1, and 1.5 μM) in the presence of 0.5 mM ATP, pol III* (0.18 μM) in the presence of 0.5 mM ATP or by the addition of δ (0.3, 0.5, 1, 1.5, 2 μM) in the absence of ATP. The reactions were incubated at 37 °C for 0–1500 s. Reactions containing γ complex or pol III* were quenched by rapid depletion of ATP upon adding 0.5 units of hexokinase and 500 nmol of glucose. Reactions containing δ were quenched by the addition of 5 μM $\beta_{monomer}$. ^{32}P-β was separated from ^{32}P-β·DNA by electrophoresis on a 1.5% agarose gel for 2 h (100 V) at 4 °C in Tris borate buffer. Gels were fixed with 5% acetic acid for 10 min; placed under DE-81 paper, nitrocellulose membrane, and paper towels; and

flattened under a heavy weight. Bands corresponding to ^{32}P-β on and off DNA were visualized and quantitated on a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The amount of total β bound to DNA at time 0 was measured (with no unloader in the reaction), and the fraction of β remaining on DNA (after adding the unloader) was then plotted *versus* time of reaction. Unloading assays testing the efficacy of $\beta_{monomer}$ as a quencher of δ activity were performed as follows: 5 μM $\beta_{monomer}$ was added to a 45-μl unloading reaction (containing 15 nM β·DNA in buffer B), followed by the addition of 0.3–2 μM δ. After 25 min, the reaction was analyzed for β unloading as described above. No detectable unloading was observed, similar to the negative control with ^{32}P-β·DNA alone, in contrast to the positive control performed with δ without the addition of $\beta_{monomer}$.

In qualitative assays comparing the unloading activity of γ complex and δ, 80 nM ^{32}P-β·DNA was mixed with increasing concentrations of γ complex or δ (final concentrations: 0, 250, 500, 750, and 1000 nM) in buffer A (25 μl) or buffer B (25 μl), respectively. The reactions were incubated at 37 °C for 5 min and loaded directly onto a 1.2% agarose gel, and, following electrophoresis, ^{32}P-β was analyzed as described above.

Antibody Production and Purification—Antibodies used to determine the cellular abundance of γ complex subunits (γ, δ, δ', χ, and ψ) and β were produced by collecting serum from rabbits injected with purified proteins. Sources for the rabbit polyclonal sera were as follows. δ sera was produced by BabCo (Berkley, CA); β and γ sera were produced by Poccono Rabbit Farms; and sera for δ', χ, and ψ were produced by Covance (Radnor, PA). The antibodies were purified further using a modification described in Ref. 37. Each highly purified protein was subjected to SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Schleicher and Schuell), and detected by staining with Ponceau S. A narrow strip of the membrane containing the protein was cut out and blocked with SuperBlock (Pierce) for 15 min, washed with TTBS (2 times, 5 min each), and layered with 200–600 μl of crude serum. The depleted serum was removed after incubation for 2 h at 25 °C (with gentle shaking). The membrane was washed with TTBS and then layered with 200 μl of 0.2 M glycine, 1 mM EGTA (pH 2.5) to recover the antibody. After 20 min, the liquid was collected and neutralized immediately with an equal volume of 100 mM Tris (pH 7.5).

Quantitative Analysis of the Intracellular Concentrations of γ Complex Subunits and β—E. coli cells (Strain C600) were plated from a glycerol stock and grown overnight at 37 °C, and a single colony was used to inoculate 1 liter of liquid Luria broth. The *E. coli* were grown at 37 °C with continuous shaking, and cell growth was monitored by measuring absorbance at 600 nm at various times. At A_{600} = 0.44, 0.88, and 4.87, aliquots were removed, and serial dilutions of 1 in 10^4, 10^5, 10^6, 10^7, and 10^8 were prepared. Next, 100 μl of each dilution was plated onto LB agar plates, and colonies were counted the next day to determine the number of cells per ml of culture at each A_{600}. The cells were also counted directly under a high power microscope (Olympus IX70) using a hemacytometer (Hausser-Scientific). Additionally, 1- and 10-ml aliquots of the cells were removed at A_{600} = 0.44, 0.88, and 4.87 and collected by centrifugation at 13,000 × *g* for 5 min. The cells were resuspended in SDS cell cracking buffer and frozen rapidly at −70 °C to prevent proteolysis. These aliquots of crude cell extract (containing known quantities of cells) were used to quantitate intracellular amounts of γ complex subunits and β by Western analysis.

Protein standards for quantitative Western analysis were generated by subjecting known concentrations of pure γ, δ, δ', χ, ψ, and β proteins to SDS-polyacrylamide gel electrophoresis. Protein concentrations were calculated from absorbance at 280 nm and known molar extinction coefficients of 20,940 (γ monomer), 46,830 (δ), 60,440 (δ'), 29,400 (χ), 24,280 (ψ), and 15,130 (β monomer) as well as by Bradford assays. The proteins were diluted in SDS cell cracking buffer containing 40 μg/ml bovine serum albumin, and varying amounts of each protein were applied to 12% gels to prepare a standard curve. Additionally, samples of the crude extracts, prepared as described above (A_{600} = 0.44, 0.88, and 4.87), were also applied to the same gels and subjected to SDS-polyacrylamide gel electrophoresis (100 V for 45 min). The proteins were transferred to nitrocellulose membranes at 100 V for 2 h (4 °C). The membranes were blocked by a 15-min incubation with SuperBlock, washed with TTBS (2 × 5 min), and incubated overnight with a 1:2000 dilution of purified antibody in TTBS plus 1% gelatin. The antibody was washed off with TTBS (3 × 5 min), followed by incubation with a 1:10,000 dilution of anti-rabbit horseradish peroxidase-conjugated antibody (Sigma) for 30 min. The membranes were washed again with TTBS, developed with an ECL detection kit (Amersham Pharmacia Biotech), and exposed to film for 15–60 s. The films were scanned on a densitometer (Molecular Dynamics), and bands corresponding to protein standards as well as bands of appropriate molecular weight in the crude extract were quantitated. The signals were plotted *versus* protein concentration (ng) to yield standard plots. The linear slope from the standard plot of each protein was used to calculate the amount of that protein present in the crude cell extract. Finally, the number of protein molecules per cell (C) was calculated using the equation,

$$C = M \times A/L \times N \quad \text{(Eq. 1)}$$

where M is the amount of protein in the crude cell extract (grams), A is Avogadro's number (6.023×10^{23} molecules/mol), L is the molecular mass of the protein (g/mol), and N is the number of cells (in the volume of crude cell extract used for Western analysis). The molecular masses of the proteins are as follows: γ (tetramer), 190 kDa; δ, 38.7 kDa; δ', 36.9 kDa; χ, 16.6 kDa; ψ, 15.2 kDa; β (dimer), 81.2 kDa.

Analysis of Excess δ Subunit in E. coli Cells—E. coli strain C600 was grown in 1 liter of Luria broth and harvested at A_{600} = 0.88. Cells were collected by centrifugation and resuspended in 20 ml of 50 mM Tris-HCl, pH 7.5, and 10% sucrose. The following procedures were carried out at 4 °C. Cells were lysed with lysozyme (38) and two passes through the French press at 20,000 psi. The crude lysate was centrifuged for 20 min at 17,000 × *g*, and the supernatant was collected and diluted with buffer C (0 NaCl) to lower the conductivity to that of 50 mM NaCl. The supernatant was loaded onto an 8-ml Mono-Q column (Amersham Pharmacia Biotech) and washed with 80 ml of buffer C (100 mM NaCl). Proteins were eluted from the column using a 100–500 mM NaCl gradient (160 ml total). 2-ml fractions were collected and split into 0.5- and 1.5-ml aliquots. SDS cell cracking buffer (0.5 ml) was added to the smaller aliquots (for Western analysis), and the fractions were frozen rapidly at −70 °C to prevent proteolysis. The larger aliquots were stored frozen at −70 °C for activity assays.

20-μl aliquots of every other Mono-Q column fraction from 8 to 48 (saved for Western analysis as described above) were subjected to electrophoresis on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed for δ by Western blotting as described above. The state of δ in the fractions (free or in γ complex) was determined by DNA replication activity assays as follows. Reactions testing for γ complex contained 35 fmol of primed M13mp18 single-stranded DNA; 0.2 pmol of β; 0.12 pmol of polymerase core ($\alpha\epsilon\theta$); 60 μM dATP, dGTP, and dCTP; 20 μM [α-^{32}P]dTTP (final concentrations); 10.5 pmol of SSB (tetramer); and no γ complex in 20 μl of buffer A. 5-μl aliquots of the fractions were added to the reactions, and the amount of DNA synthesized after 30 min at 37 °C was quantitated as described (31). To test for free δ in the fractions, a similar experiment was performed using the same reaction mixture plus 300 fmol of $\gamma\delta'\chi\psi$ without δ. The incubation time was reduced to 3 min to minimize background DNA synthesis (due to γ complex in the crude lysate), and the reactions were analyzed as described above.

RESULTS

The Clamp Loader (γ Complex), Free and in pol III, Unloads Circular Clamps from DNA*—In earlier studies, we found that γ complex, alone and in pol III*, unloads clamps from DNA (17, 20, 22). This unloading activity requires ATP and releases β from DNA much faster than the rate of spontaneous dissociation of β from DNA. In the first experiment, we quantitate and compare the rates of β release from DNA in the absence and presence of γ complex and pol III*. Fig. 2, *A–C*, show the time course of β dissociation and of γ complex-catalyzed and pol III*-catalyzed β release from DNA, respectively. The clamp-unloading substrate was prepared by loading ^{32}P-β onto a circular nicked DNA with γ complex and ATP, followed by separation of ^{32}P-β·DNA from free ^{32}P-β, γ complex, and ATP by gel filtration on a Biogel A-15m column. This large pore resin includes the free proteins and ATP, but the large ^{32}P-β·DNA complex elutes in the excluded fractions. The gel-filtered ^{32}P-β·DNA complex was incubated at 37 °C, and aliquots of the reaction were removed at various times and subjected to agarose gel electrophoresis. Free ^{32}P-β migrates faster than ^{32}P-β·DNA in the gel, providing an assay for detecting β clamps on and off DNA. The gel in Fig. 2*A* shows that β is mostly present on DNA at time 0, but over time it slowly dissociates from the DNA (a small fraction of β runs as free protein even at time 0, possibly due to some β dissociation in the time interval

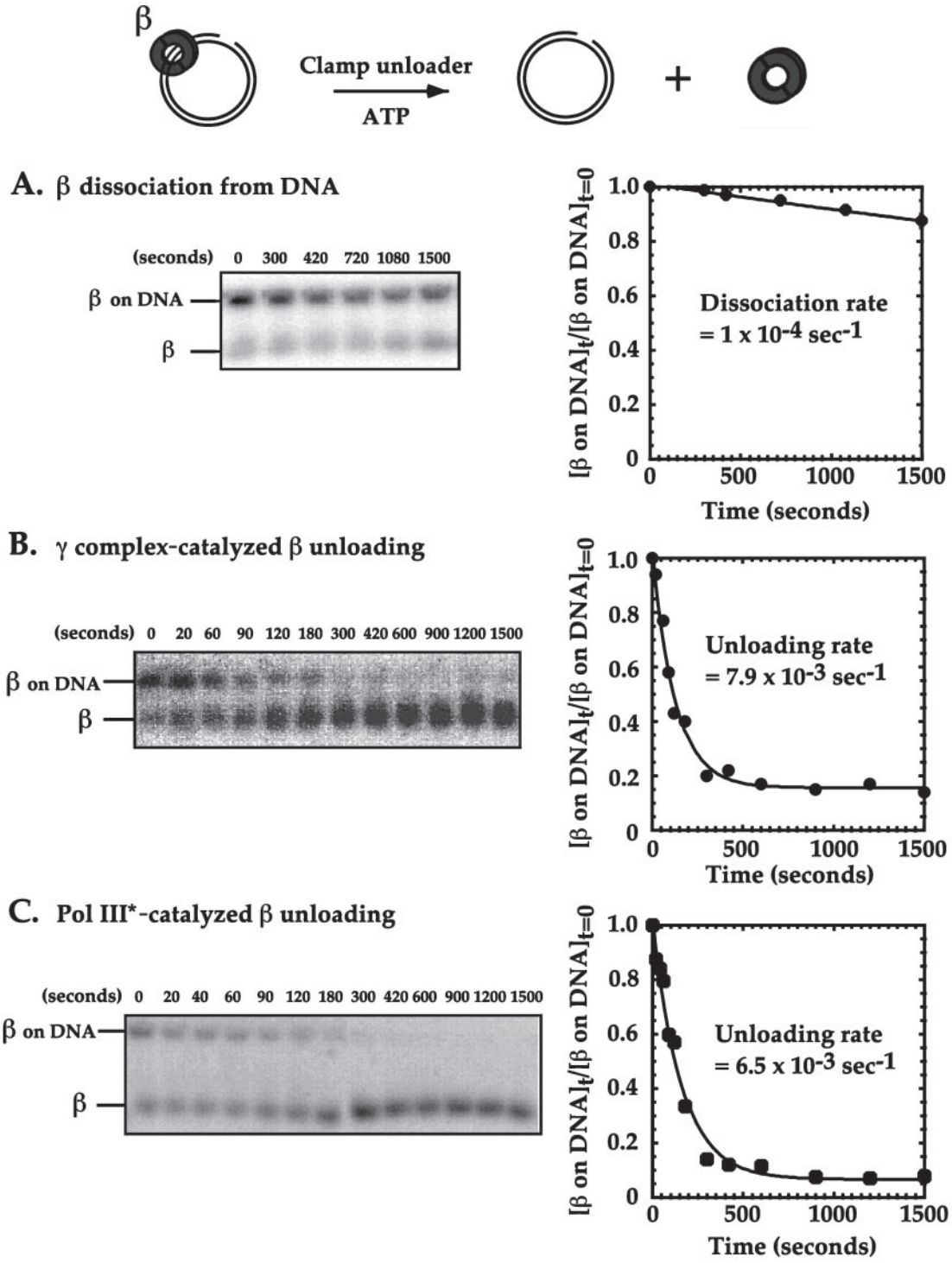

FIG. 2. **Removal of sliding clamps from DNA.** Quantitative clamp-unloading assays performed with ^{32}P-β·DNA (^{32}P-β assembled on nicked circular DNA) used agarose gel electrophoresis to separate β on DNA from free β (released from DNA). The fraction of β remaining on DNA (relative to β on DNA at time zero) was plotted *versus* time of reaction (*A*). In the absence of a clamp unloader, β remains highly stable on DNA with a half-life of ~115 min ($k_{dissoc} = 1 \times 10^{-4}$/s). Similar assays performed with clamp unloaders indicate that γ complex (0.5 μM) (*B*) and pol III* (0.18 μM) (*C*) remove β from DNA rapidly, in an ATP-dependent reaction ($k_{unloading} \sim 7.9 \times 10^{-3}$/s for γ complex and 6.5×10^{-3}/s for pol III*).

between isolation of ^{32}P-β·DNA and performing the β-unloading experiment). A plot of the fraction of ^{32}P-β remaining on DNA (relative to that at time 0) *versus* time of reaction shows that dissociation occurs with a $t_{½} \sim 115$ min ($k_{dissoc} = 1 \times 10^{-4}$/s).

To measure γ complex-catalyzed unloading, ^{32}P-β·DNA was incubated with 0.5 μM γ complex in the presence of ATP, which is required for its activity. The reaction was quenched at various times by removal of ATP with hexokinase and glucose and analyzed on an agarose gel (Fig. 2*B*). The results show that γ complex at 0.5 μM catalyzes rapid removal of β from DNA with a $t_{½} \sim 88$ s (the observed unloading rate, $k_{unloading} = 7.9 \times 10^{-3}$/s). The β clamp unloading probably occurs as a result of ATP-driven opening of the dimeric ring by γ complex. We have shown earlier, and confirm here, that the topological link between β and DNA is severed by breaking open the protein ring and not the DNA (22). The γ complex can function by itself but is also an integral part of the DNA polymerase holoenzyme

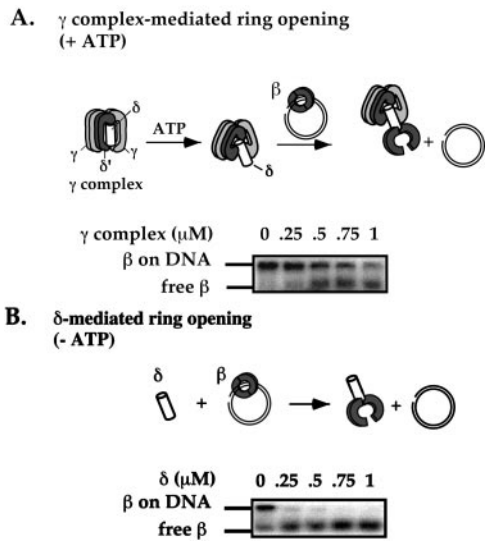

FIG. 3. **δ opens the β ring and unloads β from DNA.** Clamp-unloading assays performed with increasing amounts of γ complex (*A*) in the presence of ATP or δ (*B*) in the absence of ATP show that, like γ complex, the single δ subunit unloads β from a nicked circular DNA, but unlike γ complex, δ activity is independent of ATP. The schematic diagrams illustrate how γ complex subunits function together to open the β ring. ATP binding to γ leads to conformational changes that displace δ′ and expose δ for interaction with β (*A*), while the free δ protein can simply bind the ring and open it by itself (*B*).

assembly (pol III*). The experiment in Fig. 2*C* demonstrates that pol III* can also unload clamps with high efficiency from a nicked DNA substrate ($t_{½}$ = 106 s; $k_{unloading} = 6.5 \times 10^{-3}$/s).

δ, the "Clamp Opener" Subunit of γ Complex, Can Unload β from DNA—ATP binding to γ induces a conformational change in γ complex, exposing the δ subunit that binds and opens the dimeric β ring, as illustrated in the schematic in Fig. 3. In the next experiment, we examined the ring opening activity of γ complex and δ protein by measuring their ability to remove β from DNA. Removal of ^{32}P-β from circular nicked DNA was tested with increasing amounts of γ complex (Fig. 3*A*) or δ (Fig. 3*B*). Agarose gel analysis of the reactions show that δ alone is apparently as active as γ complex in unloading β from DNA, consistent with an earlier report indicating that δ can unload β clamps from DNA (22). Furthermore, unlike γ complex, δ does not require ATP to fuel its unloading activity. The apparent ability of the single δ protein to function as well as the five-subunit γ complex (or the nine-subunit pol III*) raises the possibility that it might be primarily responsible for removing and recycling sliding clamps during DNA replication in *E. coli*.

*Most of the δ Protein in E. coli Cells Exists as Free Protein, Separate from γ Complex and pol III**—The δ subunit is an integral component of γ complex as well as pol III*. Since δ alone is as active as a β unloader, we investigated whether there is δ protein in the cell that exists free from γ complex or pol III* and may serve as a clamp unloader *in vivo*. In the next experiment, the amounts of δ and other γ complex subunits present in *E. coli* cells were quantitated by Western analysis. First, *E. coli* cells were counted, and the content of each γ complex subunit in a known number of cells was measured to determine the number of protein molecules per cell. Fig. 4*A* shows the growth curve of *E. coli* cells at 37 °C, with optical density (at 600 nm) plotted *versus* time of growth. Cells were collected at A_{600} = 0.44 and 0.88 (logarithmic growth phase) and at A_{600} = 4.87 (stationary phase). Two different methods were used to count the number of cells; either cells were plated and then the colonies counted, or cells were counted directly using a high power microscope. The data (Fig. 4*B*) show that both methods yielded similar results of 1.6×10^8 cells/ml at A_{600} = 0.44, 4.2×10^8 cells/ml at A_{600} = 0.88, and 2.1×10^9 cells/ml at A_{600} = 4.87.

Next, aliquots of the cells were lysed, and proteins in the whole cell lysate were fractionated using SDS-polyacrylamide gel electrophoresis (Fig. 4, *C–H*). Then antibodies specific to either γ, δ, δ′, χ, ψ, or β, were used to detect these proteins in the cell lysate. Known quantities of each protein, analyzed at the same time, were used to prepare standard concentration curves. The results of the Western analysis are shown in Fig. 4, *C–H*, for γ, δ, δ′, χ, ψ, and β, respectively, and the data are quantitated in Table I as the number of molecules of each protein present in an actively growing *E. coli* cell. There appear to be about 1000 molecules/cell of the δ and χ proteins (930 δ and 1250 χ; the average of results at A_{600} = 0.44 and 0.88), about 200 molecules of γ (as a tetramer), and about 350 molecules of ψ in an actively growing cell (logarithmic growth phase). The number of β dimers (~350) is consistent with the earlier estimate of about 300 dimers (19) and confirms the need for rapid clamp recycling during DNA replication. Interestingly, there are only about 140 copies of the δ′ protein present per cell. Since the δ′ subunit is an essential structural and functional component of the clamp loader, the amount of γ complex ($\gamma_{2-4}\delta_1\delta'_1\chi_1\psi_1$), free or in pol III*, is limited to about 140 molecules/cell. Moreover, the δ subunit interacts primarily with δ′ (as depicted in Fig. 3), not the other proteins of the clamp loader, and is incorporated into γ complex by direct association with the δ′ subunit (12). Thus, the presence of an about 7-fold excess of δ over δ′ implies that a substantial amount of δ remains as free protein in the cell after γ complex is formed.

The following experiments were performed to test the above hypothesis directly. *E. coli* cells were grown to A_{600} = 0.88, harvested, and lysed, and the crude lysate was fractionated on a Mono-Q ion exchange column using a salt gradient. Previous studies have shown that the δ subunit resolves from γ complex on a Mono-Q column, while γ complex and pol III* co-elute (12). The fractions were analyzed for δ, free and in complex with other proteins (*i.e.* γ complex and pol III*), by Western blotting with a δ-specific antibody and by the requirements for reconstituting efficient DNA replication activity. The Western blot in Fig. 5*A* shows that δ elutes off the column in two peaks: fractions 12–25 (130–200 mM NaCl) and fractions 26–42 (210 - 300 mM NaCl). The first peak contains ~80 pmol δ, and the second peak contains about 5–6-fold less δ (~15 pmol). The first peak corresponds to the elution position of free δ, and the second peak corresponds to the elution positions of γ complex and pol III* (data not shown; Ref. 12).

Next, we analyzed the fractions for δ, γ complex, and pol III* by performing DNA replication assays. The assay for γ complex includes primed single-stranded M13mp18 DNA, SSB, β, ATP, dNTPs, and core DNA polymerase III ($\alpha\epsilon\theta$) but excludes γ complex. Under these conditions, there is no β loading and negligible DNA replication, since the core polymerase is highly inefficient without β (especially in the presence of SSB; Ref. 39). If any fraction contains γ complex, its addition to the reaction will lead to β loading and consequent stimulation of core polymerase activity and increase in DNA synthesis. The results, in Fig. 5*B*, show high levels of DNA synthesis in fractions 26–42, indicating that γ complex is present in these fractions. Thus, the second peak of δ observed in the Western blots (fractions 26–42 in Fig. 5*A*) is consistent with δ present in complex with the clamp loader. Under these conditions, DNA synthesis occurs only at the background level across fractions 12–25, indicating that δ protein in the first peak (Fig. 5*A*) is not associated with a functional clamp loader. Fig. 5*C* shows data from a similar experiment, except in this case a complex of

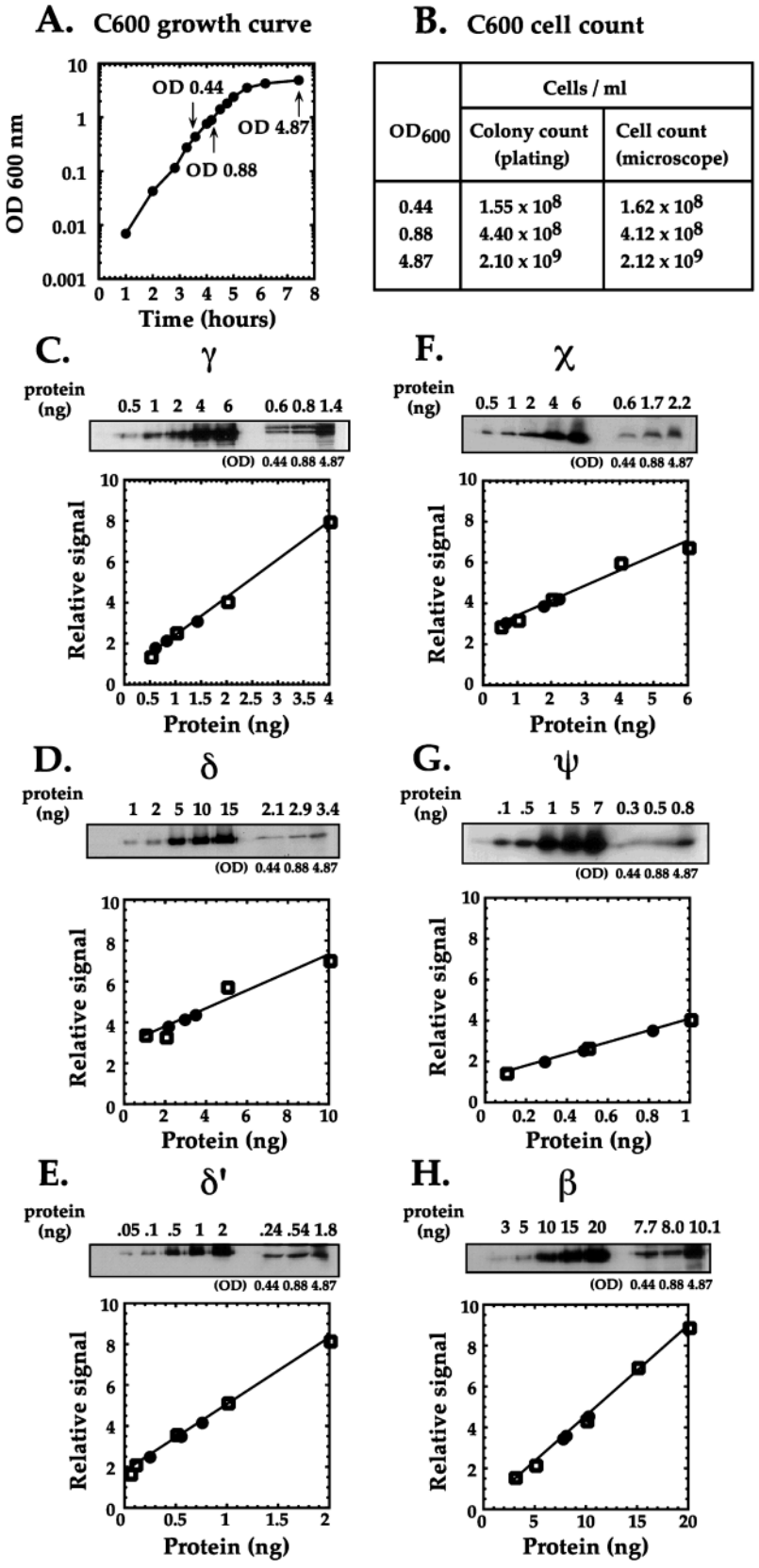

FIG. 4. **Intracellular abundance of γ complex subunits and β.** *A*, *E. coli* (C600) were grown at 37 °C in LB media, and samples were taken at A_{600} = 0.44, 0.88, and 4.87; *B*, cells were counted by plating or direct visualization under a microscope, as described under "Experimental Procedures." *C–H*, Western blots of the whole cell lysate as well as known amounts of pure γ, δ, δ′, χ, ψ, and β proteins, respectively. The relative signals from the Western blots (in the linear range) were plotted *versus* the amount of protein (nanograms) to prepare the standard curves. The *numbers above* each gel indicate the amounts of protein in the standard curves (*open squares*) and the calculated amounts of protein in each cell sample (*closed circles*).

$\gamma\delta'\chi\psi$ (without δ) was added to the reaction mix. The $\gamma\delta'\chi\psi$ complex cannot load β onto DNA without δ; hence, this assay is designed to detect any free δ protein in the fractions that can complement $\gamma\delta'\chi\psi$ to form a functional clamp loader (40). DNA synthesis is stimulated in fractions 12–26, confirming that δ is present in the first peak and that it can work with $\gamma\delta'\chi\psi$ to assemble β onto DNA. These data are consistent with results of the Western analysis in Table I and Fig. 5*A*, with both experiments indicating that δ is present in excess over the γ and δ′ subunits and probably exists as free protein in *E. coli* cells.

δ Serves as an Efficient Unloader of Sliding Clamps from DNA—The above experiments demonstrate that there is an

TABLE I
Molecules of γ complex subunits and β per E. coli cell

The number of molecules of each protein γ_4, δ, δ′, χ, ψ, and β_2 per *E. coli* cell was determined by quantitating the amounts of these proteins in whole cell lysate by Western analysis, as described under "Experimental Procedures" and in the legend to Fig. 4.

Proteins	Molecules per cell		
	A_{600} = 0.44	A_{600} = 0.88	A_{600} = 4.87
γ tetramer	240	170	110
δ	1150	710	400
δ′	130	150	180
χ	1030	1480	980
ψ	380	330	200
β dimer	350	360	460

excess of free δ protein in *E. coli*. We have hypothesized that this δ protein may have a significant role as the cellular clamp unloader (in addition to or instead of the γ complex). In order to test this hypothesis further, the following experiments quantitatively compared the clamp unloading activity of δ and γ complex by measuring the rate of β release from DNA at various δ and γ complex concentrations. Since δ does not require ATP for its activity, the δ-catalyzed β unloading reaction could not be quenched with hexokinase and glucose as described in the legend to Fig. 2 for γ complex and pol III*. Instead, we utilized a monomeric version of the clamp ($\beta_{monomer}$ = I272A,L273A mutant of β) that binds δ with high affinity, thereby forming a δ·$\beta_{monomer}$ dead end complex.[2] A control reaction shows that the addition of $\beta_{monomer}$ to ^{32}P-β·DNA prior to adding δ blocks the unloading action of δ completely (Fig. 6*D*, *lane 2*). Reactions at different δ concentrations (0.3–2 μM) were initiated by mixing δ with ^{32}P-β·DNA and quenching at various times with $\beta_{monomer}$, then analyzed by gel electrophoresis. Fig. 6*A* shows the gel analysis and corresponding plots of the amount of β on DNA *versus* reaction time for two δ concentrations. The β unloading rate increases from 3.7×10^{-3} s^{-1} at 0.3 μM δ to 8×10^{-3} s^{-1} at 1.5 μM δ. A plot of the β-unloading rate *versus* δ concentration fit to a hyperbola yields a maximum rate of 0.011 s^{-1} and an apparent K_d = 0.46 μM for the interaction between δ and β·DNA.

Similar assays were performed with γ complex, except in this case the reactions were quenched by depleting ATP with hexokinase and glucose; Fig. 6*D* (*lane 5*) shows a control reaction in which the addition of hexokinase and glucose to the reaction prior to γ complex effectively blocks β unloading. The unloading reactions were performed with increasing γ complex concentrations (0.18–1.5 μM). Fig. 6*B* shows the gel analysis and quantitation of reactions containing 0.3 and 1.5 μM γ complex, and Fig. 6*C* shows a plot of the β-unloading rate *versus* γ complex concentration. As seen for δ, the rate of β unloading from DNA increases with γ complex concentration to a maximum of 0.015 s^{-1} with an apparent K_d of 0.28 μM for the interaction between γ complex and β·DNA. These results indicate that both δ and γ complex are capable of catalyzing rapid removal of β clamps from DNA, and their activities are comparable.

DISCUSSION

β Clamps Can Be Recycled by at Least Three Proteins—The β clamp is formed by the association of two semicircle-shaped monomers (4, 6). A core of hydrophobic amino acids and ion pairs at the interface between the monomers results in a closed circular clamp that maintains its dimeric structure even at nanomolar concentrations (20).[3] In order to assemble the clamp on DNA, γ complex must pry open the dimer at the interface and then allow it to reclose around the DNA. Once the β dimer closes around DNA, it remains stably linked to the DNA and has to be retrieved by an active mechanism rather than by simple dissociation from DNA. There are only about 350 β clamps in each *E. coli* cell, yet approximately 2000–4000 Okazaki fragments are produced during one round of genomic DNA replication, and each fragment requires one β clamp. Replication of the *E. coli* genome (wild type) is completed in about 40 min (41), making it imperative that the clamps be recycled every 3.5–7 min ($k_{unloading} \sim 0.008 - 0.016$ s^{-1}; 0.693/0.008 = $t_{½} \times 5$ = 7 min). This rate is substantially faster than that of spontaneous dissociation of β from DNA (k_{dissoc} = 0.0001 s^{-1}). In this study, we demonstrate that γ complex (the clamp loader), pol III* (core polymerase plus γ/τ complex), and, most notably, δ (a single subunit of γ complex) can unload β at relatively high speeds of about 0.015/s, 0.007/s, and 0.011/s, respectively.

Intracellular Concentrations of γ Complex Subunits and β and Implications for β Recycling—Quantitative measurements of the intracellular amounts of each γ complex subunit indicate that δ′ is the limiting subunit in the complex. The γ subunit forms a tetramer in solution (13, 42, 43) and is estimated at 2–4 copies per γ complex (12, 43, 44). Each of the other subunits, δ, δ′, χ, and ψ, is present at one copy per γ complex (12, 43). The δ′ subunit plays an important structural role in γ complex by bridging the γ and δ subunits (12). It also plays an important functional role by modulating the interaction between δ and β (the δ′ subunit preferentially binds δ and blocks its interaction with β in γ complex without ATP, while in the presence of ATP a change in γ complex conformation allows δ-β interaction to proceed). Therefore, the presence of only about 140 δ′ molecules in a cell limits the number of γ complex molecules to 140 also.

We also show that there are about 800 free δ molecules (930 molecules total, less 140 molecules in γ complex), equivalent to a concentration of about 1.4 μM δ in the cell (assuming an *E. coli* cell volume of 10^{-15} liters). This concentration is about 3-fold higher than the K_d for interaction between δ and β·DNA (0.46 μM); therefore, the δ-catalyzed β unloading rate in the cell should be near the observed maximal rate of 0.011/s. In contrast, there are only about 120 molecules of free γ complex in the cell, since up to 20 function as clamp loaders in the DNA polymerase holoenzyme (estimated at 10–20 molecules) (45, 46). Free γ complex molecules may contribute to β unloading from DNA, although the unloading rate would be limited by equilibrium interaction between γ complex and β·DNA, since 120 molecules equal only about 0.2 μM γ complex in the cell, which is close to its K_d of 0.28 μM for interaction with β·DNA. The ability of γ complex to recycle β in the cell may be further compromised by the fact that its clamp loading activity is essential at sites of DNA repair or recombination and perhaps even to assist pol III* in loading β at upstream primed sites on the lagging strand during DNA replication.

As discussed earlier, β clamps need to be recycled at close to 0.01/s, or even faster, given that more than two replication forks are probably active during bidirectional DNA replication; origins can initiate prior to completion of one round of DNA replication (41), and there may be 2–4 bidirectional forks in progress at the same time (41, 47). Thus, the presence of excess δ protein in the cell ensures that clamps are recycled efficiently and within the required time frame for replication of the *E. coli* genome.

Implications for Sliding Clamp Dynamics in Other Organisms—The bacteriophage T4 replicase also utilizes a circular sliding clamp, gp45 (48), and a clamp loader, gp44/62, which uses ATP to assemble gp45 around DNA (49, 50). The trimeric gp45 clamp is less stable on DNA than the β dimer (20), and *in*

[2] J. Andjelkovic and M. O'Donnell, unpublished results.
[3] J. Andjelkovic and M. O'Donnell, unpublished data.

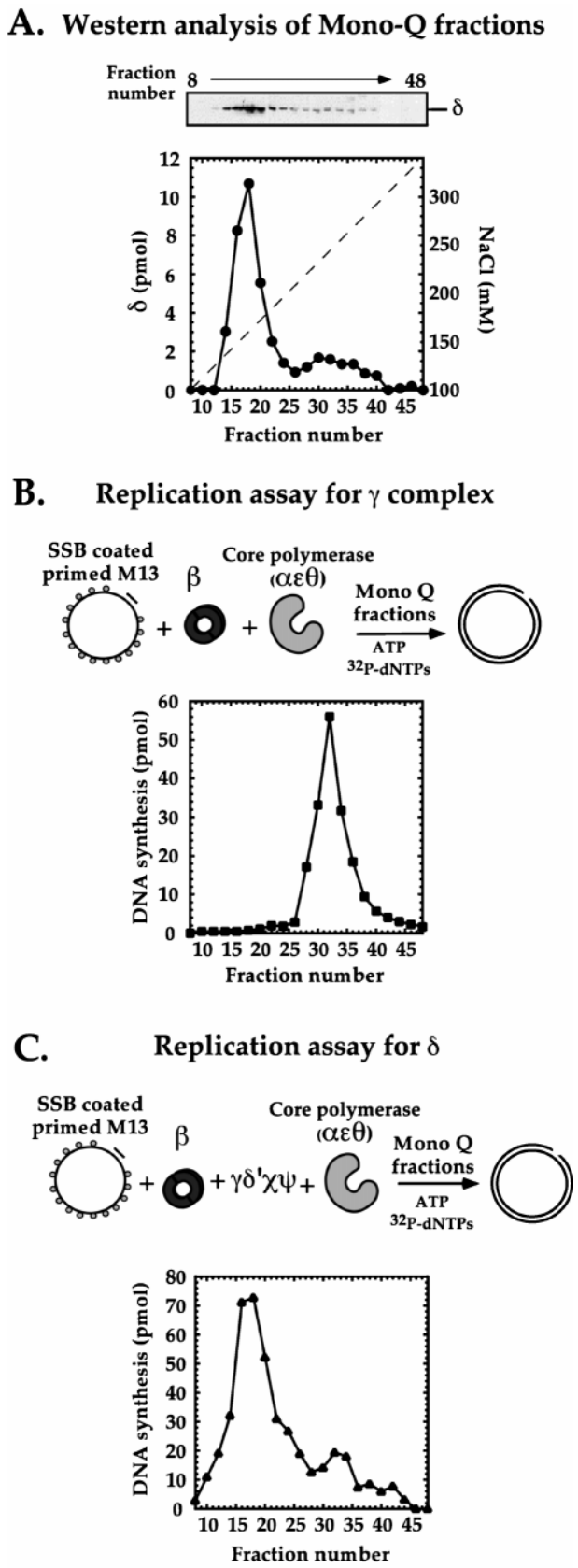

FIG. 5. ***E. coli* cells contain an ~5-fold excess of free δ protein over γ complex.** Fractionation of a crude lysate of *E. coli* (C600) on an anion exchange column (Mono-Q) yields δ protein in two different fractions. *A*, a Western analysis shows ~80 pmol of δ in the first peak (fractions 12–25) and ~15 pmol of δ in the second peak (fractions 26–42). *B* and *C*, DNA replication assays were performed with the fractions in the absence of γ complex or in the presence of $\gamma\delta'\chi\psi$ (no δ) to examine the state of δ in the two peaks (free or in γ complex). *B*, in reactions performed without externally added γ complex, fractions 26–42 stimulate DNA synthesis, indicating that δ in the second peak exists as part of γ complex. *C*, in reactions performed with $\gamma\delta'\chi\psi$ (no δ), fractions 12–26 stimulate DNA synthesis, indicating that the first peak contains free δ that complements $\gamma\delta'\chi\psi$ to form γ complex, which stimulates DNA polymerase activity.

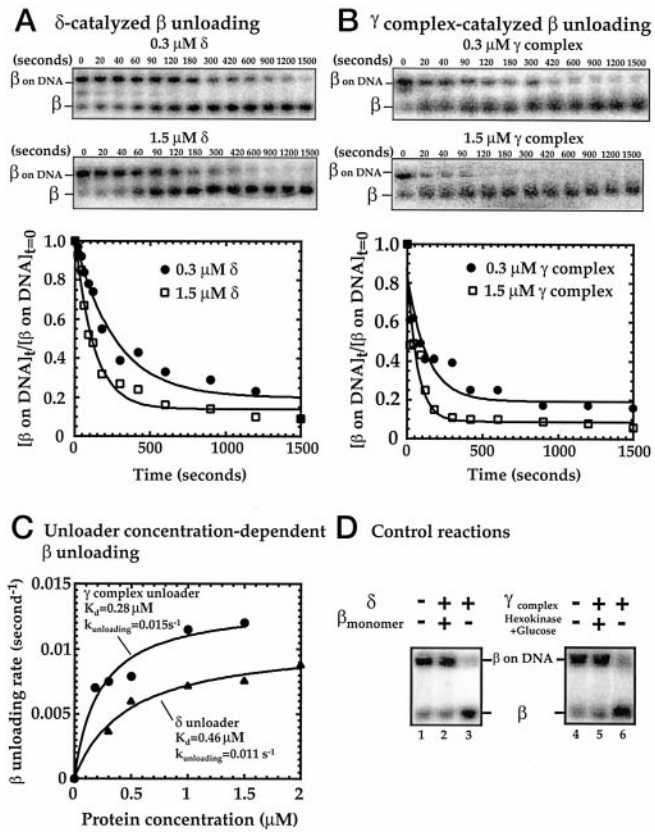

FIG. 6. **δ and γ complex unload β from DNA with comparable speeds.** Quantitative clamp-unloading assays were performed by incubating increasing amounts of δ (*A*) or γ complex (*B*) with ^{32}P-β, quenching the reactions with $\beta_{monomer}$ or hexokinase and glucose, respectively, followed by agarose gel electrophoresis. *A*, the time course of β unloading at 0.3 μM (0.0037 s^{-1}) and 1.5 μM (0.008 s^{-1}) δ concentrations; *B*, β unloading at 0.3 μM (0.0075 s^{-1}) and 1.5 μM (0.013 s^{-1}) γ complex concentrations. *C*, a plot of β unloading rates *versus* δ (*closed triangles*) or γ complex (*closed circles*) concentrations. The data fit to a hyperbola yields a K_d of 0.46 μM for the interaction between δ and β·DNA and an apparent maximal β unloading rate of 0.011 s^{-1}. A similar fit yields a K_d of 0.28 μM for interaction between γ complex and β·DNA and an unloading rate of 0.015 s^{-1}. *D*, control reactions testing the efficiency of $\beta_{monomer}$ or hexokinase and glucose as quenchers of δ or γ complex-catalyzed β unloading, respectively. *Lanes 2* and *5* show negligible clamp unloading when the quencher is added to the reaction prior to the clamp unloader. *Lanes 3* and *6* show nearly complete β unloading when no quenchers are added to the reaction.

vitro assays show that gp45 rapidly falls off DNA once the polymerase completes DNA synthesis (51). These data imply that an active unloading mechanism may not be necessary for clamp recycling in bacteriophage T4. However, gp45 does have a residence time on DNA, as measured by DNA footprinting assays and evidenced by its ability to track along DNA and help activate transcription by RNA polymerase at late gene promoters (52, 53). Therefore, the possibility that gp45 does remain on DNA for a few minutes and that its removal is facilitated by gp44/62 cannot be ruled out. It is not clear yet which of the two proteins, gp45 or gp62 or both, interact with the clamp (54), and it should be interesting to determine if either plays a role analogous to the *E. coli* δ protein in clamp unloading.

Eukaryotic DNA replicases also utilize sliding clamps for

processive DNA replication. The PCNA clamp (in *S. cerevisiae* and humans) is a trimeric ring (55, 56) that is assembled at primed DNA sites by RF-C, the eukaryotic clamp loader. RF-C is a five-subunit complex, and all five proteins share significant amino acid homology with the γ and δ′ subunits (57, 58). Like γ complex, RF-C opens PCNA and places it around DNA in a reaction driven by ATP (59). A recent study has shown that, once assembled, human PCNA clamps remain bound to DNA ($t_{½} \sim 24$ min; Ref. 20) although less stably than β ($t_{½} \sim 100$ min). There are, however, only about $1–2 \times 10^5$ PCNA clamps in HeLa cells for $\sim 2 \times 10^7$ Okazaki fragments (assuming each is about 200 nucleotides in length) (60). Hence, they must be reused about 100 times per cell cycle, and given an S phase of about 6 h, PCNA must be retrieved from DNA every 3–4 min (20). Thus, the eukaryotic sliding clamp also requires an active clamp-unloading mechanism for recycling. Human RF-C (and a subcomplex of three of the five subunits) has been shown to unload PCNA from DNA in a reaction fueled by ATP (20, 61). Moreover, similar to the δ subunit in *E. coli*, the 40-kDa subunit of RF-C is capable of removing PCNA from DNA by itself (61). At this time, it is not known exactly how PCNA is recycled, and there is no quantitative analysis of clamp unloading activity in eukaryotic cells. Observed similarities between the structure and clamp loading function of γ complex and RF-C predict that their mechanisms of clamp unloading may also be similar. It will be interesting to determine whether eukaryotes have an excess of the 40-kDa subunit or an equivalent clamp unloader protein and whether such a protein can function efficiently to recycle the PCNA clamps.

Recently, circular sliding clamps have been implicated in several DNA metabolic processes in addition to serving as processivity factors in DNA replication (62). Human and *S. cerevisiae* PCNA have been identified as targets for a number of proteins including DNA ligase (63), the DNA flap-processing enzyme Fen1 (64), DNA cytosine 5-methyltransferase (65), and DNA mismatch repair proteins (66) among others. It has been hypothesized that sliding clamps left behind on DNA, after the polymerase moves on, serve to mark the newly replicated strand for further processing such as DNA methylation and repair (59). Furthermore, PCNA-binding proteins might also use the clamp for rapid transport to their sites of action on DNA, and/or to stabilize their interaction with DNA (much like the DNA polymerase). If this hypothesis is valid, then changes in the equilibrium between clamps on and off DNA could influence processes other than DNA replication, and regulation of the clamp loading and unloading reactions could have a wide ranging impact on DNA metabolism. For example, suppression of clamp unloading can inhibit DNA replication and facilitate DNA repair (by preserving clamps on DNA for use by repair proteins) at the same time. This study reveals a unique clamp unloader protein that could serve as an ideal target for such regulation. Changes in γ complex or RF-C activity would probably affect both clamp loading and unloading processes equally. However, a change in the free δ protein (or a eukaryotic δ-like protein) would have a predominant effect on clamp unloading and thus strongly affect the equilibrium between clamps on and off DNA.

In summary, we have identified a single protein which could be dedicated to the task of unloading clamps from DNA to ensure efficient recycling of sliding clamps during DNA replication, and may influence subsequent processing of DNA in *E. coli* as well as other organisms.

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