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The internal workings of a DNA polymerase clamp-loading machine

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Replicative DNA polymerases are multiprotein machines that are tethered to DNA during chain extension by sliding clamp proteins. The clamps are designed to encircle DNA completely, and they are manipulated rapidly onto DNA by the ATP-dependent activity of a clamp loader. We outline the detailed mechanism of γ complex, a five-protein clamp loader that is part of the *Escherichia coli* replicase, DNA polymerase III holoenzyme. The γ complex uses ATP to open the β clamp and assemble it onto DNA. Surprisingly, ATP is not needed for γ complex to crack open the β clamp. The function of ATP is to regulate the activity of one subunit, δ, which opens the clamp simply by binding to it. The δ′ subunit acts as a modulator of the interaction between δ and β. On binding ATP, the γ complex is activated such that the δ′ subunit permits δ to bind β and crack open the ring at one interface. The clamp loader–open clamp protein complex is now ready for an encounter with primed DNA to complete assembly of the clamp around DNA. Interaction with DNA stimulates ATP hydrolysis which ejects the γ complex from DNA, leaving the ring to close around the duplex.

Keywords: ATPase/clamp loader/DNA polymerase/processivity/sliding clamp

Introduction

Replicases are efficient DNA-synthesizing enzymes that duplicate long chromosomes with high speed and processivity. These biological machines are comprised of three functional components in both prokaryotic and eukaryotic organisms: (i) a DNA polymerase, (ii) a processivity factor or sliding clamp protein and (iii) a multiprotein clamp loader. Replicases from *Escherichia coli*, *Saccharomyces cerevisiae*, humans and bacteriophage T4 have these three components. The DNA polymerases lack high processivity alone but upon association with their respective sliding clamps, they can replicate several thousand bases continuously (reviewed in Kelman and O'Donnell, 1995). The sliding clamps are ring-shaped homo-oligomers that encircle duplex DNA (Stukenberg *et al*., 1991; Kong *et al*., 1992; Burgers and Yoder, 1993; Krishna *et al*., 1994; Gulbis *et al*., 1996; Yao *et al*., 1996). These protein rings are assembled onto DNA by their respective clamp loaders in an ATP-dependent reaction (Kelman and O'Donnell, 1995). Unlike sequence-specific DNA-binding proteins, the circular clamps form a topological link with DNA and slide along the duplex without localizing to a specific region. Therefore, when the DNA polymerase binds its clamp, it is endowed with high processivity, allowing it to move continuously along the template during chain extension.

The *E.coli* replicase, DNA polymerase III holoenzyme, consists of 10 different polypeptide chains (Kornberg and Baker, 1992; Kelman and O'Donnell, 1995). Within the holoenzyme are two core polymerases (αεθ) and a clamp loader (γ complex), that are held together by τ, a connector protein (Onrust *et al*., 1995a). Both core polymerases become highly processive when tethered to DNA by β sliding clamps (Stukenberg *et al*., 1991). As the holoenzyme moves along the replication fork, the processive polymerase extends DNA continuously on the leading strand. On the lagging strand, the other polymerase releases its sliding clamp upon completion of each Okazaki fragment, and is cycled back to the replication fork by targeting to a new clamp assembled at an upstream RNA primer by the clamp loader (O'Donnell, 1987; Stukenberg *et al*., 1994).

The *E.coli* clamp loader, γ complex, consists of five different subunits: γ, δ, δ′, χ and ψ (Maki and Kornberg, 1988). Within the γ complex are two to four γ subunits and one each of δ, δ′, χ and ψ (Maki and Kornberg, 1988; Onrust *et al*., 1995b). The proteins are assembled such that both δ′ and ψ bind directly to γ, while δ and χ bind the δ′ and ψ subunits, respectively (Onrust *et al*., 1995b). Previous studies have shown that a γδδ′ complex is sufficient to load β onto DNA (Onrust *et al*., 1991). The χ and ψ subunits are not essential for β loading onto DNA; χ binds single-stranded DNA binding protein (SSB) and facilitates displacement of the primase from RNA primers, an event that must occur prior to clamp loading on the lagging strand (Kelman *et al*., 1998; Yuzhakov *et al*., 1999). The δ subunit is the major contact point between γ complex and β, and δ binds tightly to β in the complete absence of the other clamp loader proteins (Naktinis *et al*., 1995). When δ is part of the γ complex, however, it exhibits only a low affinity for β in the absence of ATP. In the presence of ATP, the γ complex undergoes a conformational change that now allows δ to bind β with an affinity comparable with that of the free δ–β interaction (Naktinis *et al*., 1995). γ is the only clamp loader subunit that binds and hydrolyzes ATP (Maki and Kornberg, 1988; Tsuchihashi and Kornberg, 1989; Onrust *et al*., 1991). Recent studies show that ATP binding powers a change in γ subunit conformation (Hingorani and O'Donnell, 1998), which may underlie the ATP-induced change in γ complex conformation that leads to tight interaction with

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β (Naktinis *et al.*, 1995; Hingorani and O'Donnell, 1998). Thus, the γ subunit transduces the energy from ATP to expose the δ subunit for interaction with the β ring.

This report describes the mechanics of the γ complex-catalyzed process of β assembly onto DNA. We show that the energy of ATP binding powers the γ complex machinery to bind β and open the circular clamp at one interface. Once γ complex binds β, its ATPase activity is supressed until it binds DNA. The correct DNA substrate stimulates ATP hydrolysis, which is coupled to the release of γ complex from β on DNA. On DNA, β reassumes its lowest free energy state and forms a closed ring, now with DNA passing through the center.

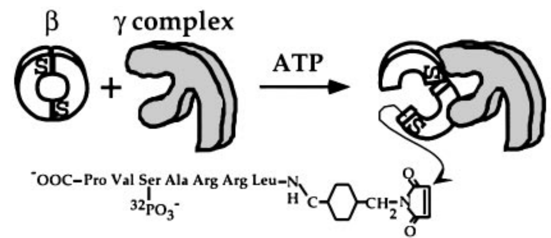
The molecular details that underlie the clamp loader activity have been explored and as a result, specific functions have been assigned to the three integral components of the γ complex: γ is the 'motor', δ is the 'ring opener' and δ′ is the 'modulator'. The γ subunits are the only components of γ complex to interact with ATP, and their function is to drive ATP-induced conformational changes of other subunits in the clamp loader. The δ subunit contains the intrinsic clamp-opening activity, as it can open the β ring in the complete absence of ATP and the other subunits. The δ′ subunit binds δ and probably modulates the ability of β to gain access to δ. The ATP-driven γ motor either moves or alters the δ′ modulator such that the δ clamp opener gains access to the β clamp and opens the ring. This action requires only ATP binding to the γ complex. The resulting clamp loader–open clamp composite then binds primed DNA with high affinity. Upon interaction with primed DNA, two or three molecules of ATP are hydrolyzed, resulting in closure of the β ring around DNA and ejection of γ complex off the DNA and back into solution.

Results

The γ complex uses energy from ATP binding to open the β ring

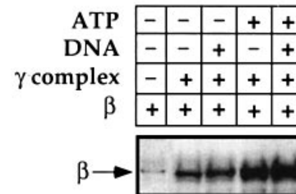
The β sliding clamp is composed of two crescent-shaped protomers arranged in a tight head-to-tail dimer (Kong *et al.*, 1992). This circular protein clamp is transferred onto circular DNA by the ATP-dependent clamp-loading activity of the γ complex. How does γ complex assemble the β ring around DNA? How is ATP used in this reaction? Is ATP binding sufficient, or is hydrolysis necessary? What is the role of DNA in this process? To begin addressing these questions, we developed a novel 'Cys-labeling' assay to detect and characterize the ring-opening step in clamp assembly.

Leu273, a buried hydrophobic residue in the β dimer interface, was substituted with cysteine (β crystal structure; Kong *et al.*, 1992). This buried cysteine residue should not be accessible to a thiol-reactive reagent, such as a maleimide, when the L273C-β ring is closed. When the interface is opened, however, the buried cysteine should become accessible to the surface and reactive to maleimide. To follow this reaction, we synthesized a radioactive reagent by linking the N-terminus of a ^{32}P-labeled peptide with an *N*-hydroxysuccinimide (NHS) ester coupled to maleimide (Figure 1). If the dimer interface opens, the interface cysteine residue will react with the [^{32}P]maleimide reagent, resulting in a radiolabeled β clamp. One other

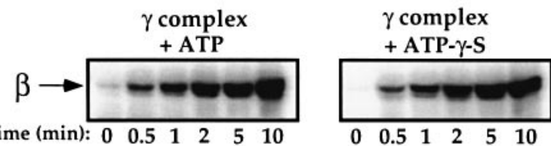


A γ complex catalyzes β ring opening

ATP	–	–	–	+	+
DNA	–	–	+	–	+
γ complex	–	+	+	+	+
β	+	+	+	+	+



B ATP binding leads to ring opening



C γ complex-catalyzed hydrolysis of ATP and ATP-γ-S

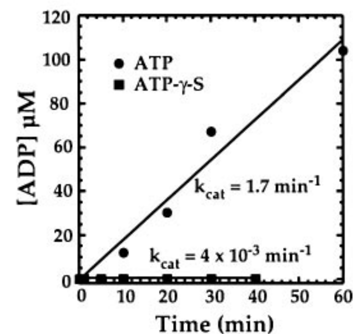


Fig. 1. γ complex opens the β clamp on binding ATP. The scheme indicates the position of a cysteine residue (S) within the dimer interface that is accessible for reaction only when the ring is opened. The structure of the [^{32}P]maleimide reagent used to label cysteine is also shown. (**A**) β (3 µM) incubated with [^{32}P]maleimide in the absence or presence of γ complex (3 µM), primed oligoDNA (3 µM) or ATP (2 mM), and analyzed by SDS–PAGE. ^{32}P-labeled β is indicated by an arrow, showing a 2- and 10-fold increase in β labeling in the presence of γ complex and γ complex + ATP, respectively. (**B**) γ complex-catalyzed ring opening assayed for varying times in the presence of ATP (left) or ATP-γ-S (right). (**C**) γ complex-catalyzed ATP hydrolysis compared with ATP-γ-S hydrolysis. γ complex (0.1 µM), incubated with β (0.2 µM), hydrolyzes ATP with a k_{cat} = 1.7/min (●) and hydrolyzes ATP-γ-S with a k_{cat} = 4×10^{-3}/min (■).

modification was made in L273C-β to ensure that only the interface cysteine residue would be labeled; the reactive Cys333 at the protein surface was changed to serine. The modified β is a dimer and is fully active in DNA replication assays with γ complex and core polymerase (data not shown).

The results of the ring-opening assay are shown in Figure 1A. The [^{32}P]maleimide reagent was incubated with β in the absence or presence of γ complex, ATP and DNA, and then the reactions were analyzed on an SDS–polyacrylamide gel. The autoradiogram of the gel shows

very low reactivity of β alone (Figure 1A, lane 1). When β is mixed with γ complex, there is a 2-fold increase in β labeling (Figure 1A, lane 2). However, when both γ complex and ATP are present in the reaction, the β is 10-fold more reactive with the [^{32}P]maleimide (Figure 1A, lane 4). The results indicate that in the presence of ATP, γ complex can open the β ring or at least change the ring conformation enough to expose the cysteine residue buried in the dimer interface. When DNA is present in the reaction, β is labeled to the same extent as in the absence of DNA (compare lanes 4 and 5), indicating that γ complex can open the β ring even in the absence of DNA.

Next, the assay was used to examine whether ATP binding or hydrolysis is required to stimulate β ring opening. To separate the effects of ATP binding from ATP hydrolysis, we substituted ATP with its analog, ATP-γ-S (Figure 1B). The turnover number for ATP hydrolysis catalyzed by γ complex (in the presence of β) is 1.5–2/min at 37°C (Figure 1C). Therefore, in a 10 min assay, each γ complex hydrolyzes 15–20 molecules of ATP. The γ complex binds ATP-γ-S with similar high affinity as it does ATP (K_d^{ATP} = 2 μM and $K_d^{ATP\text{-}\gamma\text{-}S}$ = 5 μM, respectively; Hingorani and O'Donnell, 1998), but the turnover number for ATP-γ-S hydrolysis is very low ($4–6\times10^{-3}$/min; see Figure 1C). Since γ complex catalyzes hydrolysis of only one ATP-γ-S every 2–3 h, ATP-γ-S can be considered a non-hydrolyzable analog of ATP within the time frame of the ring-opening assay.

The data shown in Figure 1B demonstrate that ATP and ATP-γ-S facilitate γ complex-catalyzed ring opening to the same extent. Therefore, ATP binding appears sufficient to drive γ complex-catalyzed opening of the β ring. Other non-hydrolyzable ATP analogs such as AMP-PNP and AMP-PCP were also tested in the assay, but ring opening was not detected (data not shown). However, this negative result may be explained by the fact that γ complex binds these analogs with much lower affinity than ATP (K_d ~1 mM versus 2 μM for ATP), and this binding energy may be insufficient to support ring opening (H.Xiao and M.O'Donnell, unpublished data).

Next, the ring-opening step was examined in greater detail to determine whether the clamp loader opens β at both interfaces (e.g. monomerizes the dimer to assemble it around DNA), or if opening the β dimer at only one interface is sufficient to allow entry of DNA into the ring.

The γ complex need open only one interface of the β dimer for clamp loading

To determine whether γ complex needs to open one or both interfaces of the β dimer for assembly of the clamp around DNA, we constructed a β dimer in which the monomers could be cross-linked at the dimer interface. Arg103 and Ile305, two residues close to each other but on opposing sides of the dimer interface (C_α atoms ~6 Å apart; β cyrstal structure; Kong *et al*., 1992), were substituted with cysteine. Due to the head-to-tail arrangement of the β dimer, the two interfaces are identical. It was expected that oxidation would result in formation of disulfide cross-links between the cysteine residues, and yield β dimers covalently linked at one or both interfaces.

Initially, the modified β ($\beta^{S–S}$) was ^{32}P-labeled at an N-terminal kinase recognition site and analyzed on a non-reducing SDS–polyacrylamide gel to determine if disulfide cross-links had formed. Figure 2A shows that in the absence of dithiothreitol (DTT), more than half of the $\beta^{S–S}$ migrates as an 80 kDa band of cross-linked dimers; the rest migrates as a 40 kDa band of monomers (lane 1). Native β migrates as a single band at 40 kDa (lane 2). Incubation of $\beta^{S–S}$ with DTT reduces the disulfide cross-links such that at 2 and 20 mM DTT essentially all $\beta^{S–S}$ migrates as monomers on the SDS–polyacrylamide gel (lanes 3 and 4, respectively).

Next we analyzed the cross-linked $\beta^{S–S}$ for assembly around a circular DNA substrate. The ^{32}P-labeled $\beta^{S–S}$ was incubated with γ complex and DNA in the presence of ATP, and then assayed by gel filtration over a Bio-Gel A-15m column (this large pore resin includes proteins but excludes the large DNA substrate as well as any protein bound to it). Figure 2B shows that about half of the $\beta^{S–S}$ is assembled on DNA in the absence of DTT (β on DNA, fractions 9–13; free β, fractions 18–27). Treatment of $\beta^{S–S}$ with DTT prior to the clamp loading reaction results in assembly of nearly all the $\beta^{S–S}$ on DNA (fractions 9–13), and is comparable with assembly of native β on DNA (Figure 2B). Presumably in the absence of DTT the disulfide cross-links at the interface prevent some (or all) of the $\beta^{S–S}$ dimers from being assembled on DNA.

To determine if the $\beta^{S–S}$ clamps assembled onto DNA in the absence of DTT contain any cross-links, the column fractions were analyzed on a non-reducing SDS–polyacrylamide gel (Figure 2C). As expected, non-cross-linked $\beta^{S–S}$ was assembled onto DNA (monomers in fractions 9–13). However, a large fraction of the cross-linked $\beta^{S–S}$ was also assembled onto DNA (dimers in fractions 9–13), while the remainder eluted as free protein (dimers in fractions 18–27). Presumably the $\beta^{S–S}$ dimers on DNA contain only one cross-link, and therefore can still be opened at one interface. The $\beta^{S–S}$ dimers that were not assembled on DNA may contain two disulfide cross-links that preclude ring opening and assembly onto DNA.

To confirm that cross-linked $\beta^{S–S}$ on DNA (dimers in fractions 9–13; Figure 2C) is cross-linked at only one interface, the experiment in the absence of DTT was repeated and split into two halves. One half was analyzed by gel filtration as described above (Figure 2C), to confirm that $\beta^{S–S}$ was assembled on DNA. The other half was analyzed by gel filtration in the presence of SDS (Figure 2D). If the $\beta^{S–S}$ on DNA contains one disulfide cross-link, then SDS denaturation will result in a dimer opened at the non-cross-linked interface that will dissociate from DNA during gel filtration. If the $\beta^{S–S}$ on DNA were to contain two disulfide cross-links, the protein ring will remain covalently sealed even after SDS denaturation, and thus remain topologically linked to DNA in the presence of SDS. The results show that the cross-linked $\beta^{S–S}$ dissociates from DNA during gel filtration in the presence of SDS, indicating that the rings were cross-linked at only one interface (dimers in fractions 18–27; Figure 2D). Thus, the γ complex can load clamps that are sealed at one interface, and does not need to monomerize the β dimer to load it onto DNA.

δ is the ring opener

We performed the Cys-labeling assay described in Figure 1 with individual subunits and subassemblies of the

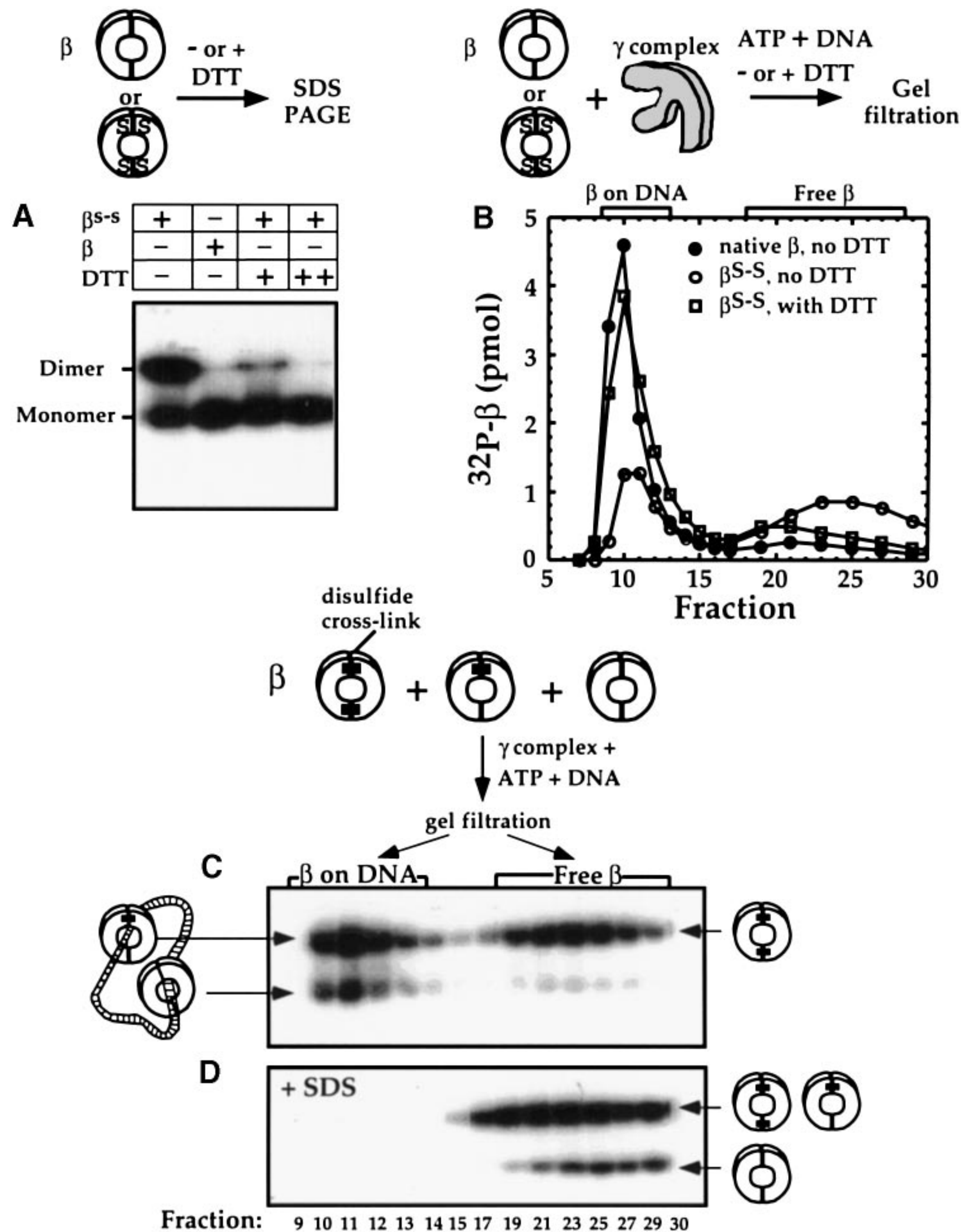


Fig. 2. γ complex opens the β clamp at one interface. Cysteine residues were placed at the β dimer interface to design a clamp with disulfide cross-links at the interface ($β^{S–S}$). (**A**) Native β and $β^{S–S}$ analyzed on a non-reducing SDS–polyacrylamide gel with no DTT, 2 mM DTT and 20 mM DTT in the reaction. (**B**) Gel filtration analysis of native [^{32}P]β (●) and [^{32}P]$β^{S–S}$ clamps assembled onto DNA by γ complex in the absence of DTT (○) and in the presence of DTT (□). The next scheme shows a mix of $β^{S–S}$ rings with two, one or zero cross-links at the interface, assembled on DNA and analyzed by gel filtration followed by non-reducing SDS–PAGE. (**C**) Analysis of $β^{S–S}$ from a clamp-loading reaction without DTT [as in (B)]. (**D**) The same reaction analyzed with SDS present during gel filtration. Fraction numbers are indicated beneath the gels. The positions of non-cross-linked $β^{S–S}$ (monomers) and cross-linked $β^{S–S}$ (dimers) are indicated by arrows.

γ complex, to determine the minimal subunit requirement for ring opening. The minimal subassembly that tested positive for ring opening was the three-subunit γδδ′ complex, which, like γ complex, needs ATP or ATP-γ-S to crack open the β dimer interface (data not shown). Individual subunits or combinations of two subunits did not give a detectable signal. This result implies that more than two proteins are needed to detect ring opening in the Cys-labeling assay, that γδδ′ is sufficient for ring opening and that the χ and ψ subunits are not absolutely required for the mechanics of this process. This conclusion is consistent with earlier data showing that efficient stimulation of processive DNA polymerase activity requires the simultaneous presence of all three subunits, γ, δ and δ′ (Onrust *et al*., 1991). However, the Cys-labeling assay for ring opening does not rigorously exclude the possibility that a smaller subassembly than γδδ′, or even just one subunit, can crack open the dimer interface transiently. For example, the assay requires time for the [^{32}P]maleimide to react with an exposed cysteine residue, and is likely to proceed more efficiently the longer the β dimer interface is held open. To investigate if any one subunit can open the β ring at least transiently, we designed a more direct assay to detect opening of the dimer interface. This assay is based on the following rationale: once β is assembled onto DNA, the β–DNA complex is very stable ($t_{1/2}$ >1 h

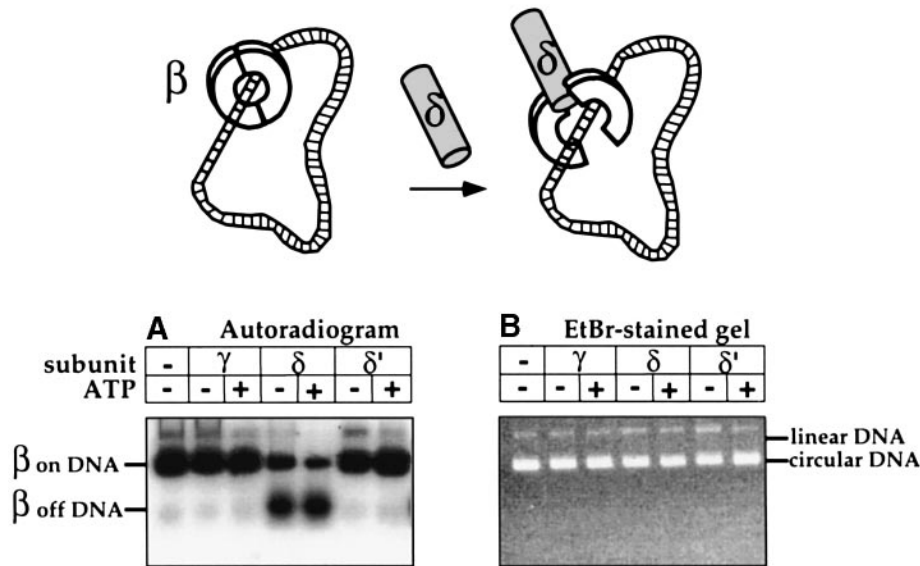


Fig. 3. A ring-unloading assay reveals that δ opens β. [^{32}P]β clamps were assembled onto circular DNA and incubated with γ, δ or δ′ in the absence or presence of ATP and analyzed by agarose gel electrophoresis (scheme). (**A**) An autoradiogram of the agarose gel shows the [^{32}P]β on DNA separated from the [^{32}P]β unloaded off DNA. (**B**) The gel stained with ethidium bromide shows that DNA is not degraded during the reaction.

at 37°C; Yao *et al*., 1996). However, if the dimer interface were to open even for a short time, the β ring could slip free of DNA. The experiment described below examines the ring-opening activity of the γ, δ and δ′ subunits by observing the unloading of circular clamps from a circular DNA substrate.

In this experiment, ^{32}P-labeled β clamps were assembled onto circular DNA by γ complex, and the [^{32}P]β on DNA was separated from free γ complex and free [^{32}P]β by gel filtration. The [^{32}P]β–DNA complex was incubated further with either γ, δ or δ′, in the absence or presence of ATP. Following this, the reactions were analyzed on an agarose gel to separate [^{32}P]β trapped on DNA from the [^{32}P]β released from DNA. The results in Figure 3A, show that the δ subunit by itself can release [^{32}P]β from DNA, and this process does not require ATP. The lack of ATP dependence is consistent with earlier evidence that δ binds β in the absence of ATP (Naktinis *et al*., 1995; note neither δ nor β binds ATP). It also verifies that the [^{32}P]β unloading (mediated by δ) observed in this assay is not a result of a γ complex contaminant in the δ protein preparation, since γ complex requires ATP to release β from DNA (Naktinis *et al*., 1996). The gel was also stained with ethidium bromide (Figure 3B), which showed that the circular DNA remained intact during incubation with each protein, including δ. The data confirm that β release from DNA is a result of ring opening and not due to the ring sliding off linearized DNA, as the circular DNA substrate is not degraded during the assay.

These results show that clamp opening is actually performed by only one subunit of the γ complex, and is the result of a simple protein–protein interaction. It should be noted that even though δ opens β enough to allow it to slip free from DNA, it cannot load β onto DNA. Thus, the γ and δ′ subunits play a critical role in assembly of the open clamp around DNA. The following experiments examine how the δ′ and γ subunits function in clamp loading.

δ′ and β compete for access to δ

Previous studies identified δ as the only subunit of γ complex with a detectable interaction with β (Naktinis *et al*., 1995). When δ is part of the γ complex, the interaction with β is not favorable in the absence of ATP (Naktinis *et al*., 1995). In the presence of ATP, however, γ complex undergoes a conformational change and binds β with similar affinity as δ alone (Naktinis *et al*., 1995; Hingorani and O'Donnell, 1998). These observations suggest that δ is partially buried in the γ complex and that the ATP-induced conformational change in γ complex results in exposure of δ for interaction with β. Which subunit of the γ complex is responsible for modulating the access of β to δ? Our previous studies have shown that δ′ binds stably to the δ subunit (Onrust and O'Donnell, 1993). Therefore, we examined whether δ′ might interfere with the interaction between δ and β.

In the experiment shown in Figure 4, we examined the interactions between δ, δ′ and β by gel filtration using a Superose 12 sizing column. Figure 4A, B and C shows the elution profiles of β (25 μM dimer), δ (5 μM) and δ′ (5 μM), respectively. Figure 4D shows that β (25 μM) mixed with the δ subunit (5 μM) forms a δβ complex, and Figure 4E shows that a 1:1 mix of δ (5 μM) and δ′ (5 μM) results in a stable δδ′ complex. Finally, Figure 4F shows that when β (25 μM) is added to purified δδ′ complex (5 μM), the δ subunit interacts preferentially with β (to form δβ), resulting in the displacement of δ′. The δβ complex is favored over δδ′ even when the reaction contains a 3-fold excess of δ′ over β (data not shown).

These protein–protein interaction studies indicate that β competes with δ′ for interaction with δ, and provide some insight into how the γ complex subunits may work during clamp loading. If, within the γ complex, δ′ binds δ and blocks access of β to δ, then the ATP-dependent change in γ complex conformation may help remove the δ′ block and favor δ–β interaction over the δ–δ′ interaction.

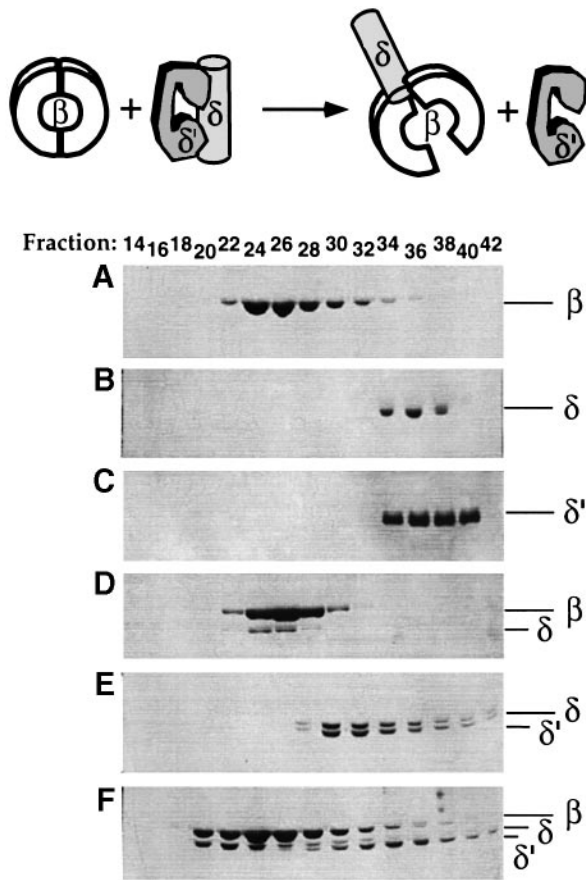


Fig. 4. β and δ′ compete for binding to δ. Protein–protein interactions were analyzed on a Superose 12 sizing column, and the column fractions were analyzed by SDS–PAGE. Fraction numbers are indicated above the gels, and the positions of β, δ and δ′ are indicated. (**A**) The elution profile of β alone (25 μM dimer), (**B**) δ (5 μM) and (**C**) δ′ (5 μM). Protein mixtures were incubated at 25°C for 15 min prior to analysis. (**D**) The elution profile of a mixture of δ (5 μM) and β (25 μM), (**E**) the δδ′ complex (5 μM) and (**F**) the profile of β (25 μM) mixed with purified δδ′ (5 μM).

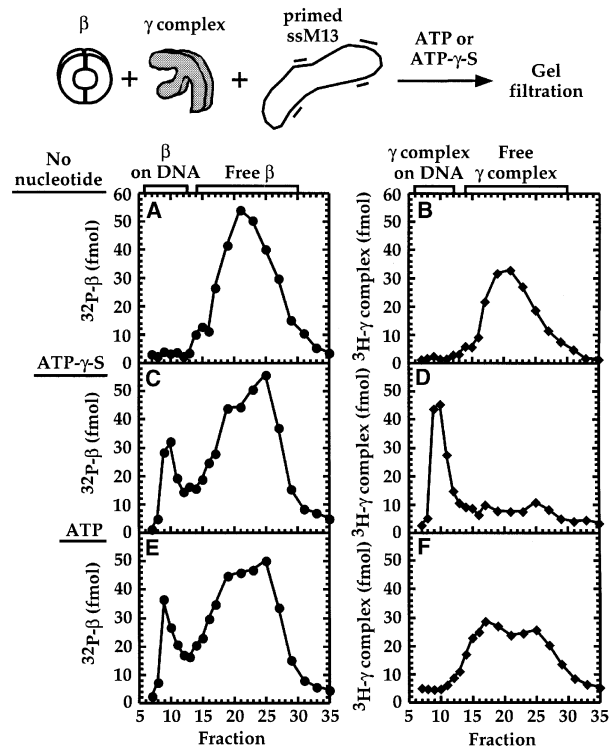


Fig. 5. ATP binding to γ complex facilitates interaction with DNA. Interactions of γ complex and β with DNA were assayed in the presence of ATP or ATP-γ-S. [^{3}H]γ complex (500 fmol) + β (500 fmol) or [^{32}P]β + γ complex were incubated with primed M13mp18 DNA (400 fmol) and 2 mM ATP or ATP-γ-S at 37°C, and analyzed by gel filtration. The fraction numbers are indicated beneath the elution profiles and the fractions corresponding to proteins 'on DNA' and 'free' protein are indicated above the profiles. (**A** and **B**) The elution profiles of [^{32}P]β (●) and [^{3}H]γ complex (◆), respectively, in the absence of nucleotide. (**C** and **D**) In the presence of ATP-γ-S, β (●) and γ complex (◆) are bound to DNA. (**E** and **F**) In the presence of hydrolyzable ATP, β is retained on DNA (●) and γ complex is released (◆).

Later in the reaction, when the γ complex binds DNA, the δ–β interaction may be severed to allow the β ring to close around DNA. Thus, δ′ may serve as a modulator during clamp assembly, first by facilitating access of δ to β, allowing it to open the ring, and then by facilitating the dissociation of δ from β, thereby allowing the ring to close around DNA (see Discussion; see Figure 7). Detailed studies with modified δ′ protein constructs are underway to explore further its role as a modulator in the mechanics of clamp loader activity.

No interaction between the γ subunit and δ or β has been detected. Therefore, if β were to completely severe the δ–δ′ contact in γδδ′, the δβ complex would be expected to dissociate from γδ′. Since this does not occur, the γ or δ′ subunits presumably maintain some contact with δ and/or β in the γδδ′–β complex.

ATP binding signals γ complex–β to bind DNA and ATP hydrolysis ejects γ complex from β on DNA

Clamp loading is a multistep process involving more than simple opening of the β ring. For example, after δ binds to β and opens the ring, β must be positioned at a primed DNA site and then closed to form a continuous protein ring around DNA. The experiment in Figure 1 demonstrated that energy from ATP binding to γ powers the events leading to ring opening. The next experiment examines interaction of γ complex and β with DNA, and the role of ATP binding and ATP hydrolysis in the clamp-loading reaction. Mixtures of [^{3}H]β and γ complex, as well as β and [^{3}H]γ complex, were used to follow their presence in complex with a primed DNA substrate. In the absence of ATP, no binding of [^{3}H]β or [^{3}H]γ complex to DNA is detectable (Figure 5A and B, respectively). In the presence of ATP-γ-S, both [^{3}H]β and [^{3}H]γ complex co-migrate with the DNA (Figure 5C and D, respectively). β appears essential for stable binding of γ complex to DNA, as no interaction of γ complex with DNA can be detected by gel filtration in the absence of β, whether or not ATP or ATP-γ-S is present in the reaction (Hingorani and O'Donnell, 1998). In the presence of ATP, only [^{3}H]β is observed on the DNA, whereas [^{3}H]γ complex elutes as free protein (Figure 5E and F, respectively; see also Stukenberg *et al.*, 1991). These data indicate that ATP binding to γ complex locks the clamp loader, clamp and DNA in a ternary complex that is stable to gel filtration, while ATP hydrolysis leads to release of the γ complex, leaving the β ring on DNA.

The next experiment was designed to measure the number of ATP molecules hydrolyzed by γ complex for

Table I. ATP hydrolyzed per clamp assembled on DNA

γ complex (nM)	β on DNA (fmol)	ATP hydrolyzed (fmol)	ATP/β ratio
25	446	850	1.9
50	748	1884	2.5
100	946	2928	3.1
200	1405	3640	2.6

[^{32}P]ATP hydrolysis and [^{3}H]β assembly onto DNA were measured in the same experiment, by TLC and gel filtration, respectively. Quantitation of ADP formed and β on DNA yielded the molar ratio of ATP hydrolyzed per clamp assembled on DNA.

each β clamp assembled on DNA. The clamp-loading reaction was performed with ^{3}H-labeled β in the presence of [^{32}P]ATP. The reactions were analyzed by thin-layer chromatography (TLC) to determine the amount of ATP hydrolyzed, and by gel filtration to quantitate the number of β clamps loaded on DNA. The experiment was repeated at four different concentrations of γ complex. In each case, ~2–3 ATP were hydrolyzed for each β clamp assembled onto DNA (Table I).

There is some ambiguity regarding the stoichiometry of γ subunits per γ complex. The free γ subunit can form tetramers (Tsuchihashi and Kornberg, 1989; Dallmann and McHenry, 1995; Onrust *et al*., 1995a); however, within γ complex, the estimates range from two to four γ subunits per complex (Maki and Kornberg, 1988; Dallmann and McHenry, 1995; Onrust *et al*., 1995a,b). A recent analysis of the molecular mass of γ complex by multi-angle laser light scattering indicates a molar ratio of 2.5 γ subunits per γ complex (unpublished data). Furthermore, quantitative ATP binding experiments yield a stoichiometry of two ATP molecules per γ complex (Hingorani and O'Donnell, 1998). These data suggest that even if there are more than two γ subunits per clamp loader, only two bind ATP, which is consistent with hydrolysis of 2–3 ATP per β clamp assembled on DNA as shown above. The results with the γ complex clamp loader are also consistent with an earlier study indicating that two ATP are hydrolyzed during formation of a processive DNA polymerase III holoenzyme complex on primed DNA (Burgers and Kornberg, 1982).

Coupling of ATP hydrolysis to clamp assembly depends on the DNA structure

The previous experiments demonstrated that ATP binding drives γ complex to open the β ring and to bind DNA, whereas ATP hydrolysis ejects γ complex from β–DNA. Next, we examined γ complex ATPase activity to determine the effects of clamp-loading substrates (β and DNA) on ATP hydrolysis. The ATPase rate was quantitated in the absence and presence of β at 37°C (Table II). The data show that the γ complex ATPase activity is markedly inhibited by β, dropping from a turnover rate of 12/min to 1.7/min, respectively (see also Figure 1C). The γ complex still binds ATP with high affinity in the presence of β (K_d = 2 μM; Hingorani and O'Donnell, 1998). Therefore, this result implies that binding of β to γ complex stabilizes it in the ATP-bound state, leading to inhibition of ATP hydrolysis. Presumably, inhibition of ATPase activity is important in the clamp-loading pathway

Table II. ATPase activity of the clamp loader

γ complex substrate	k_{cat} (per min)
None	12
β	1.7
Primed M13 DNA	55
ss M13 DNA	35
β + primed M13 DNA	110
β + ss M13 DNA	25
Primed oligoDNA	72
ss oligoDNA	108
β + primed oligoDNA	350
β + ss oligoDNA	120

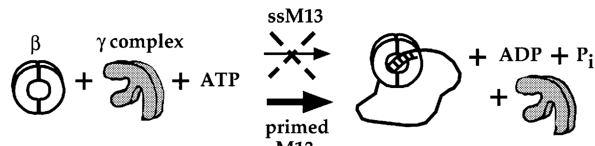
γ complex (0.1 μM)-catalyzed ATP (1 mM) hydrolysis was measured at 37°C, in the absence or presence of β (0.2 μM) and DNA (0.1–0.5 μM). The k_{cat} was determined by dividing the rate of formation of ADP by γ complex concentration.

to minimize futile cycles of ATP hydrolysis until γ complex interacts with DNA.

DNA substrates stimulate the ATP hydrolysis activity of γ complex (Onrust *et al*., 1991). In the absence of β, single-stranded DNA and primed DNA increase the γ complex ATPase rate from 12/min (γ complex alone) to 35 and 55/min, respectively (Table II). We have observed that γ complex dissociates from DNA after hydrolyzing ATP (Figure 5). Thus, DNA may hasten the turnover of γ complex, such that on binding DNA, the clamp loader rapidly hydrolyzes ATP and releases DNA to return to its original conformation.

Finally, the ATPase activity of γ complex was measured in the presence of both the β clamp and DNA. Addition of DNA to the reaction containing γ complex–β resulted in a large increase in γ complex ATPase activity (Figure 6A; Table II). Primed single-stranded DNA (ssDNA) is a better effector of ATP hydrolysis than ssDNA (k_{cat} = 110 and 25/min, respectively, at 37°C). The above experiments were performed with 1 mM ATP in the reaction to ensure maximum ATPase activity (γ complex K_m for ATP ranges from 10 to 30 μM with unprimed ssDNA and primed ssDNA substrates). Furthermore, to ensure saturating concentrations of the DNA substrates, we repeated the measurements in the presence of an excess of a synthetic ssDNA 100mer or a synthetic primed template DNA (28mer primer/100mer template), which serves as a template for β loading (Bloom *et al*., 1996). Again, the γ complex hydrolyzes ATP at a higher rate in the presence of primed oligoDNA (350/min) than in the presence of ss oligoDNA (120 min; Table II).

In Figure 6B, we investigated whether ATP hydrolysis stimulated by ssDNA leads to assembly of β clamps on ssDNA. The results show that no loading of [^{3}H]β clamps can be detected on unprimed, single-stranded M13DNA. Thus, γ complex ATPase activity is not coupled to clamp assembly on this substrate. The specificity of γ complex activity for clamp loading on primed DNA reflects the need for sliding clamps at primed sites on DNA that can be extended by the DNA polymerase. The uncoupled ATPase activity at unprimed ssDNA may in fact play an important role during lagging strand synthesis by helping γ complex locate primed sites on DNA by scanning long stretches of ssDNA via repeated DNA binding and release events.



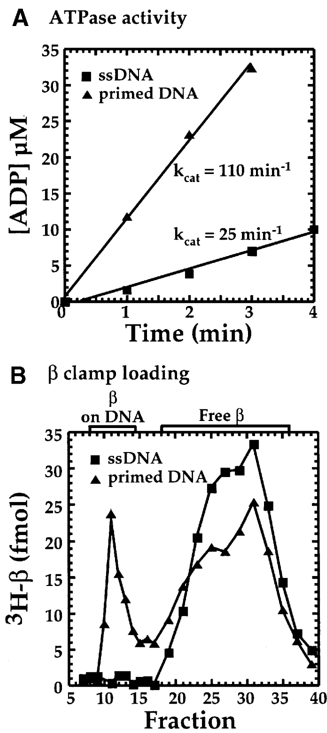
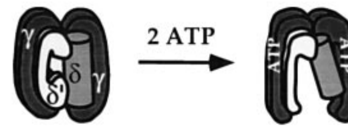
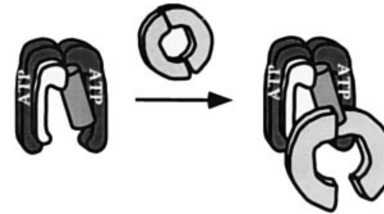


Fig. 6. ATP hydrolysis is coupled to clamp assembly at a primed site on DNA. (**A**) Primed M13mp18 ssDNA stimulates the ATPase activity of γ complex (in the presence of β) from 1.7/min (Table I) to 110/min (▲), while ssDNA stimulates the ATPase rate to 25/min (■). (**B**) A gel filtration analysis of γ complex-catalyzed assembly of [^{3}H]β on primed DNA (▲) compared with assembly on ssDNA (■). The fraction numbers are indicated beneath the elution profile, and the fractions corresponding to 'β on DNA' and 'free β' are indicated above the profile.

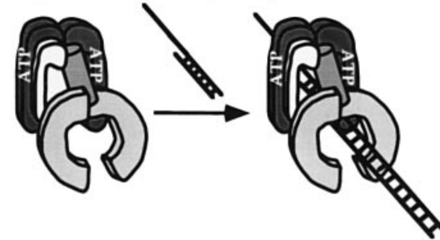
A On binding ATP, γ complex changes conformation to allow access to the δ subunit



B The δ subunit binds β and facilitates ring opening



C γ complex and β recognize primed DNA



D When all the correct components are assembled, the γ complex hydrolyzes ATP and changes conformation to release the ring around DNA

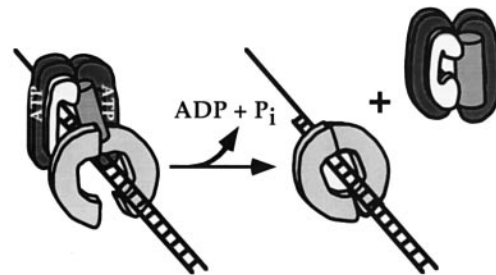


Fig. 7. A model of subunit dynamics during γ complex-catalyzed assembly of β onto DNA. (**A**) γ is the 'C'-shaped motor subunit of the clamp loader that harnesses the energy of ATP for assembly of β onto DNA. On binding ATP, a conformational change in γ affects δ′, another 'C'-shaped protein, which leads to exposure of the δ ring opener. (**B**) δ binds β and opens the ring at one interface. (**C**) The ATPase activity of this protein complex is suppressed until it binds DNA. (**D**) An encounter with primed DNA stimulates ATP hydrolysis, which is coupled to release of the β clamp and DNA. The open β dimer snaps shut to form a ring around DNA.

Discussion

Key elements of the clamp-loading mechanism

The γ complex catalyzes assembly of the β clamp onto a circular DNA molecule to form a protein–DNA catenane. In a process fueled by ATP, the clamp loader opens the clamp at the dimer interface, binds DNA, and then releases the clamp so that it forms a closed ring around DNA. The δ subunit is the key component of γ complex that opens the β ring. The interaction between δ and β yields sufficient energy to destabilize the β dimer interface. Therefore, ring-opening does not require ATP binding or hydrolysis, but is a result of a simple protein–protein interaction. Nevertheless, the δ 'ring opener' cannot facilitate assembly of the open β ring onto DNA by itself. This process requires the combined activities of γ, δ and δ′, indicating that clamp loading is a more complicated process than simply opening the ring. Thus, γ complex must perform several functions to complete the process of clamp assembly on DNA. For example, the open β ring and DNA must be positioned correctly for topological linkage, the ring must be closed and, finally, γ complex must dissociate from the ring on DNA.

Dissociation of γ complex from β is necessary for its catalytic activity and, more importantly, it is a prerequisite for processive DNA synthesis, as the clamp loader and polymerase compete for the same site on β (Naktinis *et al.*, 1996). Once γ complex releases β, the polymerase can bind the clamp and initiate DNA synthesis (Naktinis *et al.*, 1996). In DNA polymerase III holoenzyme, the γ complex and both leading and lagging strand polymerases are held together by a τ dimer (Onrust *et al.*, 1995a). The γ complex catalyzes rapid assembly of sliding clamps onto DNA for use by both polymerases (Yuzhakov *et al.*, 1996). This is particularly significant during lagging strand

synthesis. On the lagging strand, the polymerase releases its β clamp upon finishing an Okazaki fragment; it then rapidly targets to a new β that has been assembled at a fresh upstream primed site by the γ complex. The γ complex also recycles the used β clamps that have been abandoned on completed Okazaki fragments (Naktinis *et al*., 1996). Thus, γ complex and polymerase work in concert for clamp loading and primer extension, resulting in rapid and efficient duplication of *E.coli* genomic DNA.

A model for γ complex-catalyzed clamp loading

The γ complex can be described as a 'switch' protein that alternates between the 'on' state (ATP bound) and the 'off' state (either ADP or no nucleotide bound). It is, however, more than a simple switch protein because in each state, and in the transition between these states, the clamp loader machinery performs multistep functions that together result in clamp loading. A model summarizing the current information is presented in Figure 7 and the structure–function details are described below. The model shows only two γ subunits, although estimates of the stoichiometry of γ in γ complex range from two to four (Maki and Kornberg, 1988; Dallmann and McHenry, 1995; Onrust *et al*., 1995a,b).

The crystal structure of the δ′ subunit has been solved recently (Guenther *et al*., 1997). The protein is composed of three domains organized in the form of the letter 'C'. The top and bottom domains are barely connected through a small domain that may function as a hinge. The sequence identity between δ′ and γ predicts that γ also has a 'C' shape (Guenther *et al*., 1997). The structure of γ, modeled after δ′, predicts that the ATP-binding region lies very close to the hinge region between the top and bottom domains of the γ 'C'. This central position suggests that ATP binding to γ may perturb the 'C' shape, perhaps resulting in movement of the top and bottom domains, as speculated in Figure 7A. Recent reports of an ATP-induced conformational change in γ, and in γ complex, are consistent with perturbation of the γ 'C' shape (Naktinis *et al*., 1995; Hingorani and O'Donnell, 1998).

The ATP-induced changes in γ suggest that it functions as a 'motor', using the energy of ATP binding to power the conformational change in γ complex that takes δ from a buried position into one that is exposed and allows interaction with the β ring (Naktinis *et al*., 1996). The present study indicates that δ′ may be the subunit that prevents δ from binding β in the absence of ATP. Hence, ATP binding to γ may re-position δ′ and/or affect the ring opener δ, such that it prefers binding to β rather than δ′. In Figure 7A, this is depicted as a change in the δ′ subunit 'C' shape in response to the ATP-induced change in the γ subunit 'C' shape (i.e. the δ′ conformation is guided by γ). The resulting arrangement leaves γ complex in the 'on' state in which δ can bind the β clamp and open the ring at one interface (Figure 7B). The resulting γ complex–ATP–open β ring composite is stabilized by the decrease in ATPase activity of the γ complex (Table II). If ATP were hydrolyzed, the clamp loader would release the clamp and 'turn over'. Thus the inhibition of ATPase activity probably maintains the integrity of the γ complex–ATP–open β ring composite for an encounter with a DNA substrate.

The γ complex–ATP–open β ring composite binds primed DNA with high affinity to form a γ complex–ATP–open β–DNA composite (Figure 7C). The DNA stimulates γ complex ATPase activity which leads to deposition of the β clamp onto DNA, and ejection of γ complex from β on DNA (Figure 7D). Two to three molecules of ATP are hydrolyzed per clamp-loading event. Accordingly, the model in Figure 7 indicates that both γ subunits hydrolyze ATP and that after hydrolysis (or upon ADP + P_i dissociation), they return to their original 'C' shape. Presumably, at this point, the δ–β interaction is disrupted, possibly facilitated by the rebinding of δ′ to δ, thereby facilitating release of γ complex from β and closure of the ring around DNA (Figure 7D).

The energy from ATP hydrolysis may be used simply to orchestrate dissociation of the γ complex from the clamp and DNA. Alternatively, ATP hydrolysis may fuel a more active process such as closing of the clamp around DNA. At this time, we favor the former, 'clamp release' mechanism, keeping in mind that ATP binding powers assembly of all three components (γ complex, β and DNA) in close proximity, and that the lowest free energy state of the free clamp is a closed ring. Therefore, when β is released, it should be capable of closing around DNA without further input of energy from ATP hydrolysis. Pre-steady-state studies of clamp loading and γ complex ATPase activity are underway to define more precisely the role of ATP hydrolysis in clamp loading.

Clamp assembly in vivo

DNA polymerase III holoenzyme contains two copies each of γ and τ (Onrust *et al*., 1995a). The γ subunit is approximately the N-terminal two-thirds of the τ subunit, and it is created by a frameshift during protein expression from the *dnaX* gene (Tsuchihashi and Kornberg, 1989; Blinkova and Walker, 1990; Flower and McHenry, 1990). The frameshift allows addition of one unique amino acid to γ, followed by a stop codon. *In vitro*, both γ and τ subunits can be assembled into functional clamp loaders with the δ, δ′, χ and ψ subunits (Onrust *et al*., 1995b). In addition, τ has the unique ability to bind the core polymerase (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991) as well as the DnaB helicase with high affinity (Kim *et al*., 1996; Yuzhakov *et al*., 1996). These properties require the extra 213 amino acids in τ, which are absent in γ. Specifically a τ dimer, not γ, dimerizes the two core DNA polymerases in the holoenzyme and contacts the DnaB helicase during DNA replication.

Which protein, γ or τ, functions as the motor subunit for the clamp loader within the holoenzyme, or do both γ and τ form the clamp loader (i.e. a γτ complex)? This question has been addressed in a previous study of the γ and τ proteins. ATP-binding site mutants of γ and τ were constructed which, when constituted into either γ complex or τ complex with δ, δ′, χ and ψ, were unable to hydrolyze ATP and were inactive in DNA replication assays with core polymerase and β (Xiao *et al*., 1995). The mutant proteins were also assembled into DNA polymerase III holoenzymes. The holoenzyme containing mutant γ + wild-type τ was inactive in DNA replication. The holoenzyme containing mutant τ + wild-type γ was as active as the wild-type DNA polymerase III holoenzyme. These results demonstrate that the ATP site of γ is needed for

holoenzyme function, and thus γ is the clamp loading motor in the holoenzyme.

In normal cells, the holoenzyme contains both γ and τ (Maki *et al*., 1988). However, if the frameshift signal in *dnaX* is mutated such that γ is not produced, the cells remain viable (Blinkova *et al*., 1993). Presumably the holoenzyme in these γ-less cells contains four τ subunits, where two τ subunits dimerize the polymerases and the other two substitute for γ as the clamp loader motor. However, even though the τ subunit can substitute for γ, its ATPase activity and clamp-loading activity is not essential when the γ subunit is present in the holoenzyme, as demonstrated by the ATP site mutant study (Xiao *et al*., 1995).

The τ complex has the unique ability to utilize ATP-γ-S to provide the core polymerase with β-dependent processivity for DNA synthesis, although the level of synthesis is much lower than in the presence of ATP; the γ complex does not function in this capacity (Dallmann *et al*., 1995). This phenomenon is interesting since neither γ complex nor τ complex hydrolyze ATP-γ-S significantly, and ATP hydrolysis is required by both clamp loaders for the completion of clamp assembly onto DNA (Hingorani and O'Donnell, 1998; unpublished data). The results of this report offer a new insight into the molecular mechanism by which the τ complex plus core, or the holoenzyme, may use ATP-γ-S for processive DNA replication. In as much as τ and γ are closely related, and ATP-γ-S induces γ complex to bind β and open the ring, it may be presumed that ATP-γ-S has the same effect on τ complex. Since τ, unlike γ, can bind the core polymerase, it may form a bridge between the polymerase and the clamp, resulting in an enzyme assembly that can function processively. The inability of γ to bind core would prevent formation of such an assembly, and, therefore, γ complex cannot facilitate β-dependent DNA synthesis by the core in the presence of ATP-γ-S. Thus, even though the γ complex appears to be the primary clamp loader in DNA polymerase III holoenzyme, under artificial conditions, such as in the presence of ATP-γ-S (Dallmann *et al*., 1995) or when γ is absent from the holoenzyme (Blinkova *et al*., 1993), τ is eminently capable of supporting clamp assembly, in addition to dimerizing the polymerases and maintaining contact with the helicase during DNA replication

A common mechanism for clamp loading?

Clamp loaders of three other well studied systems include the gp44/62 complex from bacteriophage T4, yRF-C from *S.cerevisiae* and hRF-C from humans. These clamp loaders use ATP to assemble their respective circular clamps, T4 gp45, yeast proliferating cell nuclear antigen (PCNA) and human PCNA, onto DNA.

Like γ complex, both the yeast and human clamp loaders are five-protein machines (O'Donnell *et al*., 1993). Earlier studies have shown that ATP-γ-S leads to formation of an RF-C–PCNA–DNA composite, but this is not competent for extension by DNA polymerase δ in the absence of hydrolyzable ATP (Lee and Hurwitz, 1990; Burgers, 1991). A possible interpretation of this result is that RF-C functions similarly to γ complex, in that ATP binding is sufficient for ring opening and interaction with DNA, but ATP hydrolysis is needed to eject RF-C, allowing the PCNA ring to close around DNA. As in the *E.coli* system, DNA polymerase δ requires contact with the same face of PCNA as RF-C. Hence RF-C must release PCNA for the polymerase to gain access to the ring. Studies of human RF-C have shown that a three-subunit subassembly (36, 37 and 40 kDa subunits) as well as the 40 kDa subunit alone is capable of removing PCNA clamps from DNA in the absence of ATP (Cai *et al*., 1997). These observations indicate that the RF-C clamp loaders may utilize a similar modular design and mechanism for assembly of rings around DNA as the *E.coli* clamp loader. The γ complex subunits also provide ancillary functions; for example the χ subunit binds SSB and is necessary for displacement of the primase from RNA primers (Kelman *et al*., 1998; Yuzhakov *et al*., 1999). It is tempting to speculate that eukaryotic clamp loader subunits may also provide additional functions at the site of DNA synthesis, other than clamp assembly on DNA.

The bacteriophage T4 clamp loader contains two different subunits (gp44 and gp62), and a circular sliding clamp (gp45), which is a loosely associated trimer that lacks the stability of the β and PCNA rings (Yao *et al*., 1996). Recent studies on the role of ATP in gp45 assembly on DNA suggest that ATP hydrolysis is needed to open the gp45 ring (Latham *et al*., 1996; Sexton *et al*., 1996; Pietroni *et al*., 1997). In contrast, in *E.coli*, ATP binding is sufficient for ring opening, and ATP hydrolysis is used to eject γ complex from the β ring on DNA. The unstable nature of the trimeric gp45 ring compared with the β dimer may account for this significantly different role for ATP hydrolysis in the T4 system compared with *E.coli*. For example, gp44/62-catalyzed opening of the gp45 trimer may enhance dissociation of the protomers and preclude reclosure of the clamp around DNA. Therefore, it is possible that the T4 system has evolved to allow assembly of the clamp loader and closed clamp on DNA before ATP hydrolysis, which powers both ring opening and loading of the ring onto DNA at the same time.

Clamp loaders in cell cycle regulation

Recently, a number of clamp loader or clamp loader-like proteins have been implicated in cell cycle checkpoint pathways (reviewed in Elledge, 1996; Mossi and Hubscher, 1998). Classical checkpoint proteins sense particular aspects of a cell's environment or condition and 'decide' whether to restrict or promote cell cycle progression from one phase to the next. For example, some checkpoint proteins monitor the integrity of DNA prior to and after replication. If DNA is damaged, the cell cycle can be arrested in G_1 phase, slowed in S phase and arrested in G_2 phase, to allow repair of the DNA prior to, during and after DNA replication, respectively. Similarly, if DNA synthesis is incorrect or incomplete, DNA replication checkpoints can inhibit transition of the cell cycle (e.g. from G_2 to mitosis) until the specific problems are fixed.

Three of the five subunits of the *S.cerevisiae* clamp loader, RF-C1, RF-C2 and RF-C5, are now thought participate in cell cycle checkpoint pathways involving DNA metabolism (Cullmann *et al*., 1995; Sugimoto *et al*., 1996; Noskov *et al*., 1998). Although the underlying mechanism by which these proteins regulate the cell cycle is not yet understood, it is possible that the regulatory pathways involve loading of PCNA onto DNA. For example, a clamp loader is crucial at an early stage in DNA

replication for initiation of processive DNA synthesis; therefore, a shutdown of its clamp-loading activity can prevent entry into S phase. The large subunit of human RF-C (p140) has several putative phosphorylation sites for protein kinase A, protein kinase C and tyrosine kinases, and this has led to speculation that the PCNA loading activity of RF-C may be regulated by phosphorylation events in checkpoint pathways (Mossi and Hubscher, 1998). Alternatively, clamp loaders may serve as signal sensors in checkpoint pathways through recognition of specific DNA structures produced during DNA replication and/or repair processes. For example, recent evidence suggests that RF-C5 may interact with Spk1 (Rad53/Mec2/Sad1), an essential protein kinase for the transition of S phase to mitosis (Sugimoto *et al.*, 1996).

Structural homologs of clamp loader subunits are also known to participate in cell cycle regulation. RAD24 in *S.cerevisiae*, which is homologous to *E.coli* γ and δ′ proteins as well as all the RF-C subunits, is required for DNA damage checkpoint pathways, and its homolog in *Schizosaccharomyces pombe*, RAD17, is required for both DNA replication and damage checkpoints (Elledge, 1996). Perhaps RAD24 and RAD17 interact with RF-C and regulate its clamp-loading activity, or they may be subunits of other protein loaders that play a role in checkpoint pathways. A recent study has shown that Cdc6p, an *S.cerevisiae* protein essential for entry of cells into S phase, also shares homology with prokaryotic and eukaryotic clamp loader subunits (Perkins and Diffley, 1998). It has also been suggested that Cdc6p may be involved in assembly of Mcm proteins onto chromatin, and thereby serves a function analogous to that of a clamp loader in assembling proteins onto DNA (Perkins and Diffley, 1998). Although quite speculative in nature, these findings imply that the use of clamp loaders and their underlying mechanisms may generalize to other processes beyond loading sliding clamps onto DNA.

Studies of *E.coli* DNA replication have been a guide to understanding similar mechanisms in complex eukaryotes, including humans. This report provides a detailed mechanism for the function of the γ complex clamp loader, a multiprotein enzyme that assembles sliding clamps onto DNA for processive and rapid DNA replication. The structural and functional homology between γ complex, RF-C and other important cell cycle regulatory proteins suggests that insights gleaned from the molecular mechanism of the γ complex will generalize to eukaryotic clamp loaders, and perhaps to yet other critical processes in DNA metabolism.

Materials and methods

Materials

Radioactive nucleotides were purchased from Dupont-NEN. M13mp18 ssDNA was prepared by phenol extraction of purified M13mp18 phage that had been banded twice in CsCl gradients as described (Turner and O'Donnell, 1995). Nicked circular DNA was prepared by incubating 200 μg of doubly CsCl-banded supercoiled Bluescript plasmid (pBS) with 100 U of mung bean nuclease (NEB) for 15 min, followed by phenol extraction and ethanol precipitation. Mung bean nuclease is a single strand-specific nuclease that nicks supercoiled DNA once, and by relaxing the plasmid precludes further nicking. The DNA was ~90% nicked. Multiprimed DNA was prepared by incubating 0.6 μM DnaG primase and 0.6 μM DnaB helicase with 1 mM NTPs for 30 min at 37°C, followed by phenol extraction and ethanol precipitation. This treatment results in ~30 primers per DNA circle (assuming an average primer length of 25 nucleotides). Synthetic ssDNA 100mer and synthetic primed template DNA were prepared as described (Hingorani and O'Donnell, 1998).

Buffer A is 20 mM Tris–HCl pH 7.5, 0.1 mM EDTA and 10% glycerol. Superose 12 gel filtration buffer is 20 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 10% glycerol, 5 mM DTT and 100 mM NaCl. Reaction buffer is 20 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 4% glycerol and 8 mM $MgCl_2$. Bio-Gel A-15m gel filtration buffer is reaction buffer plus 100 mM NaCl. Loading buffer is 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol.

Proteins

The γ complex was constituted from pure γ, δ, δ′, χ and ψ, followed by isolation from the free subunits on a MonoQ column (Pharmacia) as described (Onrust *et al.*, 1995b). Purification of γ (Studwell and O'Donnell, 1990), δ and δ′ (Dong *et al.*, 1993), χ and ψ (Xiao *et al.*, 1993) was performed as described earlier. The δ′ and β proteins were ^{3}H-labeled by reductive methylation as described (Kelman *et al.*, 1995a). The catalytic subunit of cAMP-dependent protein kinase produced in *E.coli* was the gift of Dr S.Taylor (University of California, San Diego, CA).

The modified versions of β were constructed by PerImmune, Inc. using a DNA oligonucleotide site-directed method. The mutant *dnaN* genes were placed into vectors encoding an N-terminal hexa-histidine tag and a kinase recognition sequence (pHK vector; Kelman *et al.*, 1995b). The specific β modifications are described in detail later. Below is a summary of the purification protocol. Modified β was overexpressed in *E.coli* BL21(DE3) pLysS cells by inducing cultures at $OD_{600} = 0.6$ with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested, and heat lysed by the method of Wickner (1976). The cell lysate was precipitated sequentially with 35 and 70% ammonium sulfate and the pellet was resuspended in 20 mM Tris–HCl (pH 7.9) 500 mM NaCl and 5 mM imidazole. The protein was loaded onto a 5 ml chelating Sepharose resin (Pharmacia) charged with $NiSO_4$, and bound proteins were eluted with a linear gradient of imidazole (60–500 mM) in the above buffer. Fractions were mixed immediately with EDTA (50 mM final) to chelate any nickel in the eluate that tends to precipitate protein. β was identified by SDS–PAGE, and peak fractions were pooled and dialyzed against buffer A. The pool was loaded onto an 8 ml MonoQ column equilibrated in buffer A, and the bound protein was eluted with a linear NaCl gradient (0–500 mM) in buffer A. Peak fractions were pooled and frozen at –70°C. Each of the modified β subunits had replication activity levels comparable with wild-type β.

Ring-opening assay

The β clamp was modified by changing Leu273 to cysteine and Cys333 to serine. The resulting Cys273 residue is buried in the dimer interface (L273C), and the highly reactive surface Cys333 is removed. The remaining three cysteine residues in β were found unreactive to thiol-labeling reagents and were left unchanged. The reagent used to probe the interface cysteine was prepared by labeling 300 nmol of peptide (NH_3-LRRASVP-COOH; Chiron mimotopes) with 0.03 U of cAMP-dependent protein kinase catalytic subunit and [γ-^{32}P]ATP 200 μCi (3000 Ci/mmol) in 150 μl of reaction buffer (30 min at 37°C). Then, ATP was added to a final concentration of 10 mM and the mixture was incubated for an additional 30 min. The mixture was incubated with 5 mg of sulfosuccinimidylc 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce) at 25°C for 1 h, and the ^{32}P-labeled maleimide was purified on a 24 ml Superdex-Peptide column (Pharmacia) equilibrated in 0.1 M sodium phosphate (pH 7.2), 0.15 M NaCl, using the Pharmacia SMART system. After a 7 ml void volume, 500 μl fractions were collected. The peak (fractions 17–18) containing [^{32}P]maleimide was identified by the co-incidence of the radioactive peak and the peak of absorbance at 305nm (λ_{max} for *N*-ethylmaleimide). [^{32}P]maleimide concentration was determined at 305 nm, using a molar extension coefficient of 620/M/cm for maleimide, and was typically at 400 μM.

The ring-opening assay was performed in 15 μl of reaction buffer containing 3 μM β, 3 μM γ complex, 2 mM ATP and 3 μM primed oligoDNA. Proteins were mixed on ice and the reaction was initiated with 5 μl of [^{32}P]maleimide and a shift to 37°C. The reactions were quenched after 12 min with 5 μl of loading buffer + 5 μl of 1 M DTT. Samples were analyzed on a 10% SDS–polyacrylamide gel that subsequently was dried and analyzed on a PhosphorImager. The γ complex subunits exhibit some background labeling by [^{32}P]maleimide;

however, they are easily resolved from β during SDS–PAGE and do not interfere with the detection of β ring opening.

In experiments using ATP-γ-S, 5 μM each of γ complex and β were incubated in 30 μl of reaction buffer with either 200 μM ATP or ATP-γ-S. Reactions were initiated by adding 10 μl of [^{32}P]maleimide, and 4 μl aliquots were removed and quenched at 0, 0.5, 1, 2, 5 and 10 min, and analyzed as described above.

Cross-linked β loading assay

For this experiment, Arg103 and Ile305 in β were changed to cysteine to create a disulfide-linked β (β^{S-S}). Native β and β^{S-S} were radiolabeled with the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP as described (Kelman *et al*., 1995a), except that DTT was omitted in order to maintain the disulfide cross-links in β^{S-S}. The γ complex used in this assay was constituted in the absence of DTT.

The assembly reaction was performed in 50 μl of reaction buffer containing 500 fmol of pBS DNA, 19 pmol of β, 5.4 pmol of γ complex and 500 μM ATP, in the absence or presence of 20 mM DTT. The reaction was allowed to proceed for 10 min at 37°C and then loaded onto a 5 ml Bio-Gel A-15m gel filtration column (Bio-Rad), equilibrated in A-15m buffer in the presence or absence of 1% SDS. Fractions (200 μl) were collected and 40 μl aliquots were quantitated by liquid scintillation counting or analyzed on a 12% SDS–polyacrylamide gel under non-reducing conditions (no DTT). The gels were dried and exposed to film or analyzed on a PhosphorImager (Molecular Dynamics)

Clamp-unloading assay

β (1.6 μM), ^{32}P-labeled at a C-terminal kinase site (Naktinis *et al*., 1996), was incubated with 3.3 μM γ complex and 5 pmol of pBS DNA in 75 μl of reaction buffer containing 500 μM ATP for 10 min at 37°C. The reaction was applied to a 5 ml A-15m gel filtration column and eluted with A-15m buffer. Peak fractions, containing β on DNA, were pooled, and 25 μl aliquots (containing 0.2 μM β) were incubated with 1 μM γ, δ or δ′ with or without 500 μM ATP. After 10 min, each sample was mixed with glycerol (15% final) and analyzed on a 1% agarose gel (89 mM Tris–borate buffer with 10 ng/ml ethidium bromide). After electrophoresis for 2 h at 100 V, the agarose gel was photographed under UV light and exposed to film.

Analysis of β, δ and δ′ interactions

A δ′ complex was formed by mixing 2 mg of δ and 3 mg of δ′ in buffer A followed by purification on a 1 ml MonoQ column (with a linear gradient of 50–500 mM NaCl in buffer A). Then 5 μM of the pure δδ′ complex was mixed with 25 μM β (dimer) in 200 μl of buffer A for 15 min at 25°C, and analyzed by gel filtration on a Superose 12 column equilibrated with buffer A containing 100 mM NaCl. After a void volume of 7 ml, 180 μl fractions were collected, and 20 μl aliquots were analyzed by SDS–PAGE and visualized by Coomassie staining of the gels. The control gel filtration profiles of β alone, δ alone, δ′ alone, β with δ, and δ with δ′ were also obtained under the conditions described above for the Superose 12 column chromatography.

Interaction of γ complex and β with DNA

To monitor γ complex interaction with DNA, 500 fmol of γδ[^{3}H-δ′]χψ was incubated with 500 fmol of β and 400 fmol of multiply primed M13mp18 DNA for 10 min at 37°C in reaction buffer containing 2 mM ATP or ATP-γ-S (75 μl total volume). To monitor β assembly onto DNA, [^{3}H]β was incubated with unlabeled γ complex. The reactions were applied to 5 ml A-15m gel filtration columns equilibrated in reaction buffer containing 0.2 mM ATP or ATP-γ-S. Column fractions were quantitated by liquid scintillation counting, and the amount of γ complex and β in the fractions was determined from the known specific activity.

γ complex ATPase activity

The number of ATP molecules hydrolyzed by γ complex for each β assembled onto DNA was measured by incubating 25, 50, 100 or 200 nM γ complex with 1 μM [^{3}H]β at 25°C, in reaction buffer containing 40 mM glucose. At time zero, 1 μM [γ-^{32}P]ATP was added to the reaction and a 1 μl aliquot was removed and quenched with 1 μl of 0.5 M EDTA for quantitation of ATP hydrolysis. After 15 s, another 1 μl aliquot was removed to quantitate of the amount of ATP hydrolyzed, and 1 pmol of nicked DNA was added as substrate for γ complex-catalyzed loading of [^{3}H]β clamps. After an additional 15 s, a final 1 μl aliquot was removed for ATP analysis and the assembly reaction was quenched by addition of 0.5 U of hexokinase. The reaction was applied to an A-15m gel filtration column to quantitate the amount of [^{3}H]β assembled onto DNA. ATP hydrolysis was quantitated by TLC on PEI cellulose F sheets (EM Science), using 0.6 M potassium phosphate buffer, pH 3.4. The TLC sheet was dried and the molar amount of ADP + P_i formed was determined by analysis on a PhosphorImager.

ATPase assays (or ATP-γ-Sase assays) measuring γ complex turnover rate were performed at 37°C with 0.1 μM γ complex, with or without 0.2 μM β and with 1 mM ATP + [α-^{32}P]ATP (or 0.5 mM ATP-γ-S + [γ-^{35}S]ATP-γ-S). At various times, 5 μl aliquots of the reaction were quenched with equal volumes of 0.5 M EDTA and analyzed as described above. To compare the effect of primed and ssDNA on γ complex–β-catalyzed ATP hydrolysis, similar reactions were performed, except with 0.1 μM M13 ssDNA coated with 17 μM SSB or with 0.1 μM primed M13 DNA coated with SSB in the reaction (or 0.5 μM ss-oligoDNA and primed oligoDNA). The molar amounts of products formed were plotted versus time of reaction, and the slope of each time course was divided by γ complex concentration to yield the turnover number.

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