

# Clamp loader structure predicts the architecture of DNA polymerase III holoenzyme and RFC

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**Recent determinations of the crystal structure of the *Escherichia coli*  $\gamma$  complex and  $\delta$ - $\beta$  assembly have shed light on the bacterial clamp loading reaction. In this review, we discuss the structures of  $\delta$ - $\beta$  and the  $\gamma_3\delta\delta'$  complex and its mechanism of action as a clamp loader of the *E. coli*  $\beta$  sliding clamp. We also expand upon the implications of the structural findings to the structure and function of the eukaryotic clamp loader, RFC, and the structure of *E. coli* DNA polymerase III holoenzyme.**

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## Introduction

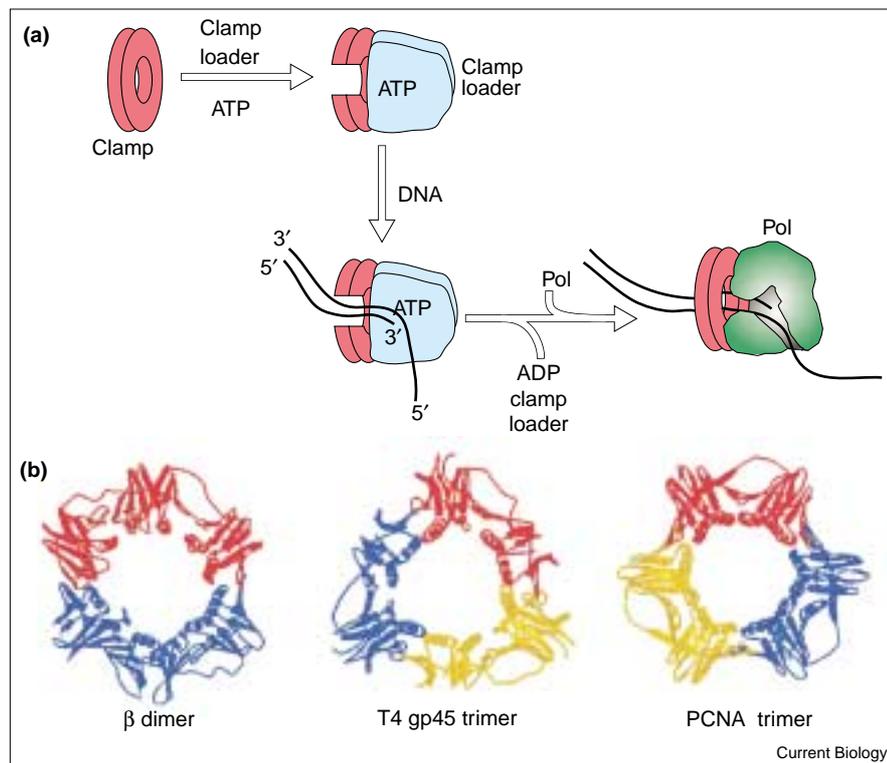
Cellular replicases, from prokaryotes to eukaryotes, are composed of three components: a DNA polymerase, a ring-shaped sliding DNA clamp, and a clamp loader complex (reviewed in [1–3]). The multisubunit clamp loader couples ATP binding and hydrolysis to assembly of the sliding clamp around DNA at a primed site. Association of the polymerase with the sliding clamp bound to DNA allows the polymerase to slide along the DNA duplex and perform highly processive synthesis (see Figure 1a) [4].

In the *E. coli* system, the  $\beta$  sliding clamp is composed of two identical crescent shaped protomers that assemble head-to-tail to form a ring with an inner diameter of approximately 35 Å (see Figure 1B) [5]. Each protomer consists of three domains with such similar structure that they practically superimpose, yet share no significant sequence identity. The six-fold appearance of the  $\beta$  dimer derives from this internal repeated domain structure. PCNA, the eukaryotic clamp, adopts the same fold except each monomer consists of only two of these domains [6,7]. Thus, PCNA trimerizes to form a six-domain ring that can almost be superimposed on *E. coli*  $\beta$ , yet the level of sequence identity between the internal domains of these proteins is very low. The T4 bacteriophage follows a similar strategy and the gp45 trimer assumes a similar ring shape to PCNA and  $\beta$  [8,9].

The  $\beta$  dimer appears to be a tightly closed ring, and once it is on DNA it slides until it finds an end whereupon it slides off due to insufficient chemical contacts to the DNA [4]. But on circular DNA, the  $\beta$  ring remains firmly attached, even though it is free to slide around the DNA circle. Spontaneous dissociation occurs with a half-life of over one hour at 37°C, showing that the interfaces remain tightly associated [10,11]. The clamp loader complex is required to rapidly open and close the  $\beta$  ring.

The *E. coli*  $\gamma$  complex clamp loader contains five different subunits,  $\gamma$ ,  $\delta$ ,  $\delta'$ ,  $\chi$ ,  $\Psi$  (reviewed in [3]). Clamp loading only requires the  $\gamma\delta\delta'$  complex;  $\chi$  and  $\Psi$  are involved in other interactions and will not be discussed further [12]. The  $\delta$  subunit alone binds to the  $\beta$  ring and has the intrinsic capability to open it up, as inferred from the ability of  $\delta$  to rapidly release  $\beta$  rings from circular DNA [13]. The energy for ring opening must be derived from the protein–protein interaction as ATP is not required; neither  $\beta$  nor  $\delta$  are ATP binding proteins. Mutation of two residues at the dimer interface of  $\beta$  result in a stable monomer,  $\beta_1$ , to which  $\delta$  binds with 50-fold higher affinity than to the  $\beta$  dimer [14].

Figure 1



Clamp loaders assemble clamps onto DNA for use by other enzymes. (a) The scheme illustrates the need for a clamp loader to open and close the circular clamp, placing it around DNA in an ATP dependent reaction. The clamp loader leaves, allowing other enzymes, such as polymerase, to target the clamp for processive synthesis. (b) Sliding clamps have similar architecture, comprising six domains arranged in a circle. *E. coli*  $\beta$  is a dimer (three domains per monomer), and T4gp45 and PCNA are trimers (two domains per monomer).

This result shows that  $\delta$  interacts with only one member of the  $\beta$  dimer. Furthermore, the observation that one  $\delta$  monomer binds to the  $\beta$  dimer and does not lead to complete dissociation of  $\beta$  dimer suggests that only one of the dimer interfaces is disrupted [14–15]. Indeed,  $\beta$  that has been cross-linked at one interface can be efficiently loaded onto DNA by the  $\gamma$  complex clamp loader, showing that the integrity of the dimer is maintained during clamp loading [13].

The tighter binding of  $\delta$  to  $\beta_1$  compared to  $\beta_2$  implies that the work that  $\delta$  normally exerts to part one dimer interface of  $\beta_2$  need not be performed on a  $\beta$  monomer for lack of a dimer interface to work upon. In other words, when  $\delta$  binds the  $\beta$  dimer it expends some of its binding energy to distort the structure of  $\beta$ , and these  $\delta$  induced conformational changes must work against the structure of the interface. However,  $\delta$  does not need to contend with a dimer interface in  $\beta_1$  and therefore  $\delta$  retains all of its binding energy in the  $\delta$ - $\beta_1$  complex instead of expending some of this energy to perform work in the  $\delta$ - $\beta_2$  complex.

### The $\delta$ - $\beta$ structure

The crystal structure of  $\delta$  in complex with  $\beta_1$  — a mutant of  $\beta$  that forms a stable monomer — gave far more information than anticipated [16]. First, the structure revealed

that  $\delta$  has the same chain fold as  $\delta'$ , a conclusion that could not be easily reached by comparison of proteins having only 6–8% sequence identity. Second,  $\delta$  subunit contacts  $\beta$  in two different places. One is a hydrophobic pocket, probably responsible for most of the binding energy, located between the middle and the carboxy-terminal domain of  $\beta$ , domains 2 and 3, respectively. The locale and nature of this interaction bears striking resemblance to the complex of PCNA with a carboxy-terminal peptide of p21, and to the structure of the RB69 gp45 clamp to the carboxy-terminal peptide of the polymerase [7,9].

The second site of interaction between  $\delta$  and  $\beta$  involves contacts between  $\delta$  helix  $\alpha_4$  and  $\beta$  loop  $\alpha_1'$ - $\beta_2'$ . We surmise that this contact which alters the conformation of the  $\beta$  loop with respect to the dimeric structure leads to the observed change of conformation of the interfacial helix  $\alpha_1'$  (see Figure 2a). In the  $\beta$  dimer, a portion of this  $\alpha$  helix adopts a distorted conformation relative to a canonical  $\alpha$  helix, in order to allow for proper packing of Ile272 and Leu273 with the second monomer. In the  $\delta$ - $\beta_1$  complex, this helix has straightened placing  $\beta$  in a conformation that is not competent to dimerize.

There are at least two different mechanisms by which  $\delta$  may open  $\beta_2$  as inferred from the structure. One is that  $\delta$

**Figure 2**

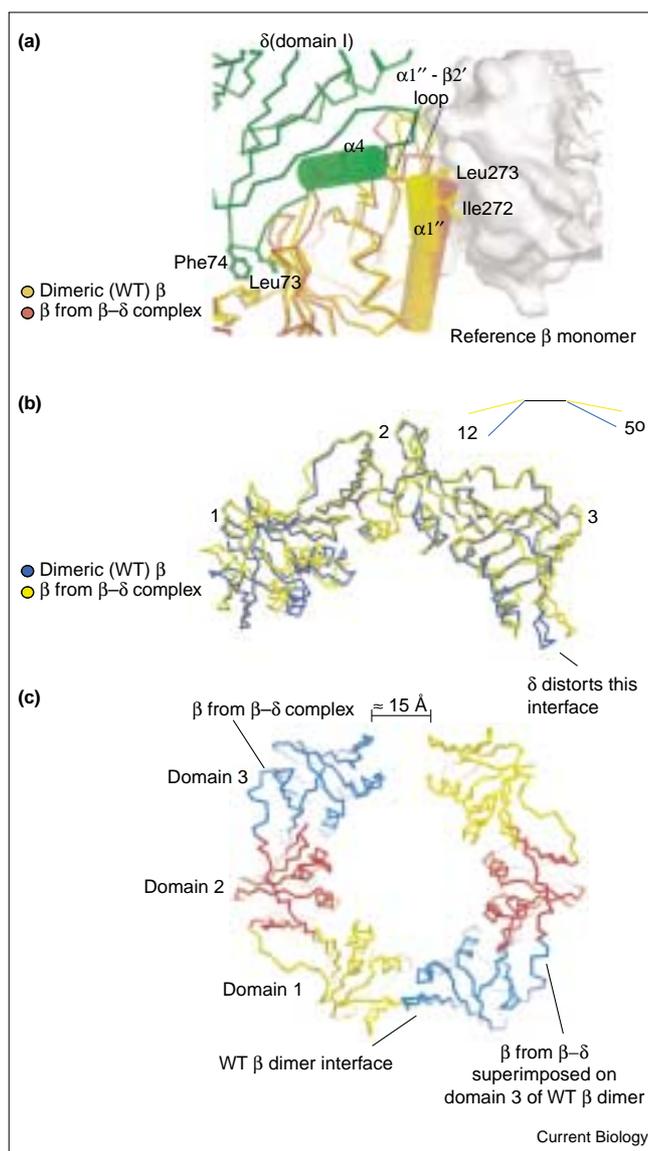
The  $\beta$  clamp is spring loaded. (a) Close up of  $\delta$  wrench acting on the interface of  $\beta$ . Residues Leu73 and Phe74 of  $\delta$  (green) participate in binding to  $\beta$ , while an  $\alpha$  helix on  $\delta$  ( $\alpha 4$ ) displaces a loop at the interface of  $\beta$  (red/yellow) resulting in altering the conformation of the  $\alpha 1'$  helix on  $\beta$  which is an essential element in forming the interface.  $\beta$  of  $\beta_2$  is colored yellow;  $\beta$  of  $\delta\text{-}\beta_1$  is colored red. Key residues Ile272, Leu273 critical for dimeric contacts, change locations in the two structures. The gray surface is the second  $\beta$  protomer in the dimer. (b)  $\beta$  monomers from  $\delta\text{-}\beta_1$  (yellow) and  $\beta_2$  (blue) are superimposed relative to the middle domain. The rigid body motions between domains result in a shallower crescent for  $\beta$  in  $\delta\text{-}\beta_1$ . The greatest motion is between domains 2 and 3 which are farthest from the site of  $\delta$  action. (c) Two  $\beta_1$  protomers from the  $\delta\text{-}\beta_1$  structure are set next to one another assuming that one dimer interface is similar in structure to  $\beta_2$ . This model results in a 14–16 Å gap. WT: wild type. This Figure was adapted from Figure 4 of [16].

forcibly breaks the interface. The second is that the  $\beta_2$  ring may rapidly alternate between the opened and closed states, and  $\delta$  binds the open state of  $\beta_2$  preventing ring closure. The stability of  $\beta_2$  on circular DNA implies that the ring is closed most of the time, suggesting that  $\delta$  actively opens  $\beta$ . However, formally the possibility exists that  $\delta$  may stabilize a conformation that, without  $\delta$ , would not rapidly dissociate from DNA. Regardless of how the interface of  $\beta$  is disrupted by  $\delta$ , an opening must be created to allow DNA to enter the ring. Remarkably, the  $\delta\text{-}\beta_1$  structure provided information on this process.

Perhaps the most striking feature of the  $\delta\text{-}\beta_1$  structure is the shape change in  $\beta_1$  relative to  $\beta_2$  (see Figure 2b). This feature may underlie how the ring actually opens. The three domains of  $\beta_1$  undergo rigid body motions which result in a monomer with a shallower crescent shape than the protomers of the  $\beta$  dimer. The largest of these rigid body motions is between the amino-terminal and middle domains, domains 1 and 2. The  $\delta$  subunit does not bind this region of  $\beta$ , most contacts are confined to the interface and the junction of domains 2 and 3. Hence, this rigid body motion appears to be directed internally by the architecture of  $\beta$ .

Why would the dimer accept the strain of bending these domains into a tighter crescent to form the  $\beta$  ring? Presumably the free energy of forming the interface is greater than the energy needed to bend  $\beta$  into a ring. This implies that when an interface is disrupted, the tension between domains will be allowed to relax with the consequence that the interface opens. Existence of this ‘spring tension’ is supported by molecular dynamics simulations that show a spontaneous relaxation of an isolated  $\beta$  monomer [16].

Only one interface should need to be cracked open in order for spring tension to be relaxed in both  $\beta$  protomers of the dimer. Modeling two relaxed  $\beta$  protomers with one remaining interface, using the interface structure of the



$\beta$  dimer, gives a dimer with a gap that is opened about 15 Å (see Figure 2c). An opening of this size may be sufficient for the passage of single-stranded DNA but is too small to slip a duplex through. If a duplex must pass in and out of  $\beta_2$ , the gap may perhaps ‘breathe,’ or maybe it is opened wider by interaction with other subunits of  $\gamma$  complex. Alternatively, the remaining interface formed by the last domain of one monomer and first domain of the other monomer may also be under spring tension, similar to the tension between intramolecular domains, and thus would relax upon cracking of the opposite interface. A slight change in the remaining interface would, of course, be leveraged to produce a much larger change at the gap that is clear across the other side of the molecule.

Spring tension in the  $\beta$  dimer has another very interesting consequence for the interface besides producing a gap once

one interface has been cracked. Namely, both interfaces should be somewhat destabilized by the spring tension between internal domains. In other words, these ‘springs’ would exert an opening force on the interface, weakening them. This should make it easier to open the first interface. Now consider the remaining interface after the first has opened. The spring tension is relieved when the ring opens and is no longer a destabilizing factor for this second interface, which would in effect tighten up. The above scenario has two biological consequences for clamp loading. One is that the job of opening the first interface is made easier, and second, retention of the dimer structure of the open ring is facilitated by the extra stability of the second interface after the spring tension in the ring has been released. In the closed state, both  $\beta$  dimer interfaces are equivalent. Therefore, whichever  $\beta$  half  $\delta$  binds to determines which interface will open and which will stay closed.

### The $\gamma$ complex

The crystal structure of  $\gamma_3\delta\delta'$ , combined with biochemical data, provides an exceedingly detailed view of how these subunits interdigitate during clamp loading [17]. Three  $\gamma$  subunits, and one each of  $\delta$  and  $\delta'$ , are arranged in a circle (see Figure 3a–d). Each subunit adopts the same chain topology, and folds into three domains. However, the relative orientation of these domains is different for each subunit, especially the degree of twist between domain 3 and domains 1, 2. The carboxy-terminal domains provide the major subunit contacts of the pentamer, although other intersubunit contacts are present. Consequently, a view of  $\gamma$  complex looking directly at the carboxy-terminal faces demonstrates an unbroken circle (Figure 3c). However, the amino-terminal domains do not form a continuous circle. These domains are arranged in a highly asymmetric fashion, and appear to dangle under the carboxy-terminal pentamer ‘umbrella’. A view looking at the amino-terminal face gives the appearance of a C, instead of a circle, where the break in the circle appears between  $\delta$  and  $\delta'$  (see Figure 3d).

Exactly how do the  $\gamma$  complex subunits function together to open and close the  $\beta$  ring around primed DNA? Biochemical studies show that  $\delta$  can open the  $\beta$  ring [13]. Ring opening doesn’t require ATP or any other  $\gamma$  complex subunit besides  $\delta$ . Yet  $\gamma$  complex requires ATP to open  $\beta$ . What is ATP doing if it is not needed for  $\delta$  to open  $\beta$ ? The  $\gamma$  subunit is the only one that interacts with ATP, and therefore  $\gamma$  can be thought of as the motor of this machine. In the absence of ATP,  $\gamma$  complex has only low affinity for  $\beta$ , less so than  $\delta$  subunit alone [15]. Hence,  $\delta$  would appear to be at least partially occluded in  $\gamma$  complex by other subunits. In the presence of ATP,  $\gamma$  complex undergoes a conformational change that facilitates interaction with  $\beta$  [15,18]. Study of  $\delta'$  shows it can prevent contact between  $\delta$  and  $\beta$ , suggesting that in  $\gamma$  complex,  $\delta'$  may prevent  $\delta$ – $\beta$  contact

in the absence of ATP [13]. ATP binding to  $\gamma$  may separate  $\delta'$  from  $\delta$ , allowing  $\delta$  to bind  $\beta$  and open the ring.

How do the above observations fit with the  $\gamma$  complex structure? The  $\beta$  interactive element site on the  $\delta$  subunit is contained within the amino-terminal domain (see Figure 3a). The  $\beta$  interactive element on  $\delta$  is fairly exposed in the  $\gamma_3\delta\delta'$  structure, and appears available to bind  $\beta$ . From the  $\delta$ – $\beta$  crystal structure, one can attempt to dock  $\beta$  onto  $\delta$  in the  $\gamma_3\delta\delta'$  structure. Only minor conformational changes are required to permit interaction of  $\delta$  with  $\beta$ . Hence, the  $\gamma$  complex appears to have crystallized in a conformation that perhaps resembles the ATP activated state, even though ATP was not present during crystallization. The inactivated state of  $\gamma$  complex in which  $\delta$  is less accessible to  $\beta$  may be one in which the amino-terminal domain of  $\delta'$  interacts more extensively with the amino-terminal,  $\beta$ -interactive, domain of  $\delta$ . Given very few modeling operations (explained in [17]) a hypothetical ‘closed’ structure can be formed in which the amino-terminal domains of all five subunits contact and form an unbroken circle (see Figure 3b). In this hypothetical closed form, the  $\beta$  interactive domain of  $\delta$  contacts  $\delta'$  in a way that would prevent binding to  $\beta$ .

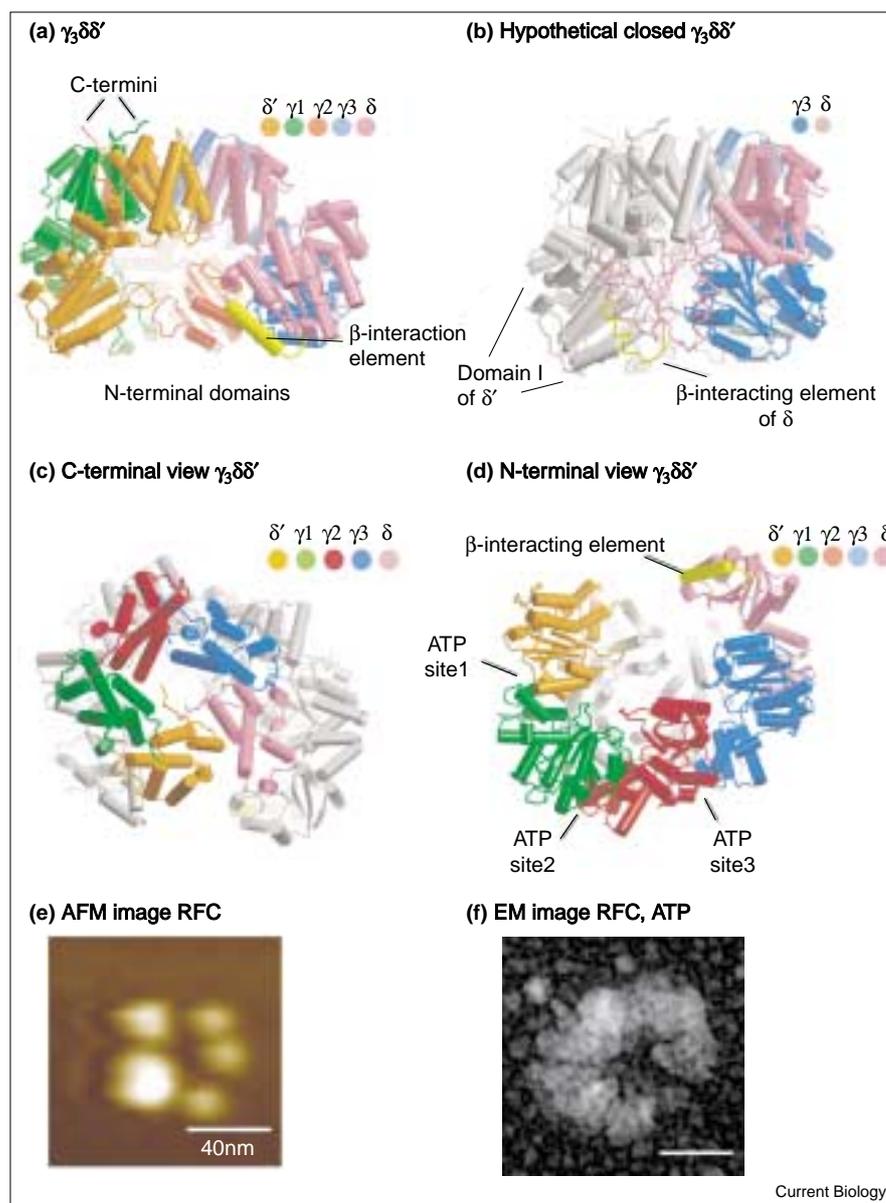
Modeling the  $\beta$  ring onto the activated  $\gamma$  complex suggests that  $\beta$  may fit on the bottom of  $\gamma$  complex, where it interacts with the amino-terminal domains of all five subunits, not just the  $\delta$  subunit. Guided by this suggestion, the  $\gamma$ – $\beta$  interaction has been observed biochemically, although it is considerably weaker than the  $\delta$ – $\beta$  interaction [19]. Perhaps interaction of  $\beta$  with multiple subunits of  $\gamma$  complex helps open the gap at the  $\beta$  interface wider than the 15 angstroms predicted from the  $\beta$ – $\delta$  structure.

The  $\delta'$  subunit was the first structure of a  $\gamma$  complex subunit to be determined [20]. The orientation of the three domains in  $\delta'$  within the  $\gamma$  complex structure are very similar to  $\delta'$  alone. However, the domains of  $\delta$ , especially domain 3 relative to domains 1 and 2, have different conformations in  $\delta$ – $\beta$  compared to  $\gamma_3\delta\delta'$ . And the three  $\gamma$  subunits each have unique conformations as a result of rigid body motion about the domains. The more rigid, or static, behavior of  $\delta'$  may be explained by the presence of a more extensive network of interdomain connections which, combined with a very short linker between domains 2 and 3, may hold  $\delta'$  in a fixed position. This unique configuration of domains in  $\delta'$  has earned it the title of ‘stator’. Perhaps the rigid  $\delta'$  stator serves as a backboard upon which other subunits push to attain their altered conformations, and as an anvil for  $\beta$  interactive elements to strike following ATP hydrolysis.

Why would  $\gamma$  complex crystallize in a nearly active form when ATP was not even present? At a high concentration

Figure 3

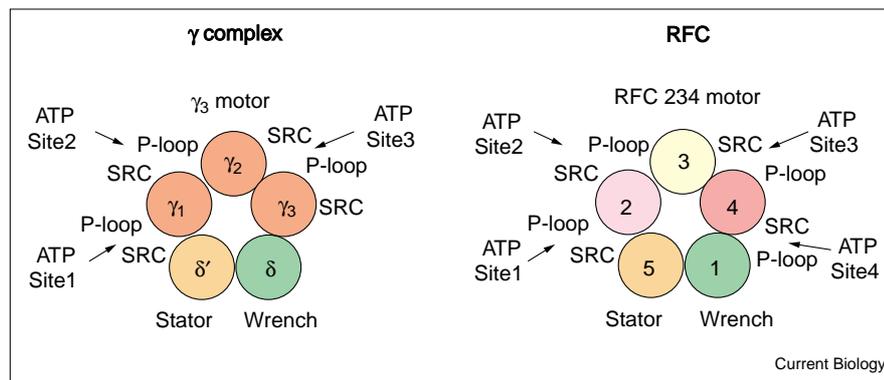
The *E. coli*  $\gamma$  complex. (a) Side-view of the  $\gamma_3\delta\delta'$  pentamer. The carboxy-terminal domains that form the pentameric contacts are at the top of the structure. The amino-terminal domains are at the bottom. The gap between the amino-terminal domains of  $\delta$  and  $\delta'$  is evident. The  $\beta$  interactive element in  $\delta$  (yellow) is exposed. (b) The hypothetical closed structure of  $\gamma_3\delta\delta'$  brings the amino-terminal domains of  $\delta$  and  $\delta'$  together, sequestering the  $\beta$  interactive element of  $\delta$  (yellow). (c) View looking down the top of  $\gamma_3\delta\delta'$  at the pentamer of carboxy-terminal domains. (d) View looking up the bottom at the amino-terminal domains of  $\gamma_3\delta\delta'$ . (e) Atomic force microscope image of RFC. (f) Electron microscope image of RFC in the presence of ATP. Panels (a,b,d) were adapted from Figure 5 of [17]. Panels (e,f) were from Figure 4e and 3r, respectively, of [32].



of  $\beta$ , the  $\gamma$  complex can bind  $\beta$  whether ATP is present or not, but ATP increases the affinity between them (V. Naktinis and M.O'D., unpublished). Thus, an excess of  $\beta$  appears to drive the same conformational change that ATP promotes. In the absence of ATP the  $\gamma$  complex probably flickers between the open and closed forms, and  $\beta$  can trap it in the open form provided sufficient amounts of  $\beta$  are present. The partially open form of the  $\gamma$  complex seen in the structure may be stabilized in the crystal by more favorable crystal contacts compared to the closed  $\gamma$  complex. And the elevated salt or presence of polyethylene glycol may have favored the partially open form during crystallization.

Structural studies on the ATPase *N*-ethylmaleimide-sensitive fusion protein (NSF), Ras, heterotrimeric G-proteins, and the  $F_1$  ATPase provide insight into how ATP may be employed in  $\gamma$  complex. The  $\gamma_3\delta\delta'$  structure reveals that the ATP binding sites in the amino-terminal domains of  $\gamma$  are positioned at intersubunit junctions: site 1,  $\delta'/\gamma_1$ ; site 2,  $\gamma_1/\gamma_2$ ; and site 3,  $\gamma_2/\gamma_3$  (Figure 3d; see also Figure 4). The  $\delta'$  and  $\gamma$  subunits contain a structure, sensor 1, that may participate in the ATP binding site of the adjacent subunit; this nucleotide binding element is referred to as switch 2 in G-proteins. The importance of these residues is established by studies of clamp loaders containing mutant  $\gamma$

Figure 4



Proposed arrangement of RFC subunits. The left diagram shows the arrangement of  $\gamma_3\delta\delta'$  subunits. The P-loop, and the SRC motif in sensor 1, are shown for those subunits that contain these sequences. ATP sites formed from these elements at subunit interfaces are indicated. At the right is a proposed arrangement of RFC subunits. SRC motifs and P-loops are shown on the RFC subunits that contain them, and proposed ATP sites formed from them are shown.

subunits at this position which are defective [21]. The structure suggests that sensor 1 can either block ATP binding to the site in the adjacent subunit, or allow it to bind to ATP. ATP sites 1 ( $\delta'/\gamma_1$ ) and 3 ( $\gamma_2/\gamma_3$ ) appear available to bind ATP, but site 2 ( $\gamma_1/\gamma_2$ ) is blocked by sensor 1 of the adjacent  $\gamma_1$ .

The sensor 1 motif contains within it the three residues, SRC. The SRC residues in  $\gamma$  and  $\delta'$  are highly conserved in bacteria and in eukaryotic clamp loader subunits [22,23]. The hypothetical closed state of  $\gamma$  complex contains only one open site, site 1 ( $\delta'/\gamma_1$ ). Sensor 1 of the  $\delta'$  stator does not block this site and the structural rigidity of  $\delta'$  probably holds it open at all times. ATP binding to site 1 may destabilize interaction among amino-terminal domains, thereby promoting the open conformation. Subsequent ATP binding to another site may prevent reclosure, maintaining the open form in which the amino-terminal domains are released and available to bind  $\beta$ .  $\beta$  binding to the  $\gamma$  complex probably stabilizes the open state further, preventing its transition to the closed form. In fact, it is also possible that  $\beta$  initially 'captures' the open  $\gamma$  complex, followed by ATP filling the open sites to stabilize this form.

Scrutiny of the two open ATP sites in the crystal structure shows that the arginine of the SRC motif is likely to play a catalytic role, as suggested for other AAA+ ATPases such as p97, but is positioned just beyond reach of ATP modeled into the site. This arrangement is reminiscent of the 'arginine finger' of GTPase activating proteins [24]. The catalytic arginine may be withheld until  $\gamma$  complex- $\beta$  bind primed DNA and the geometric requirements of DNA aligned through the  $\beta$  ring are met. Thus, the arginine could act as the trigger in a fidelity mechanism in which proper placement of the arginine, and consequent catalysis, requires both  $\beta$  and a primed site in the correct alignment. Hydrolysis at this point is consistent with the

fact that  $\beta$  stimulates  $\gamma$  complex ATPase activity specifically in the presence of primed DNA and not ssDNA [25].

How does  $\gamma$  complex close the  $\beta$  ring, and depart from  $\beta$  leaving it on DNA? Upon hydrolysis of ATP, ADP may be less tightly bound at the interfaces of  $\gamma$  complex subunits, allowing it to dissociate and letting the  $\gamma$  complex assume the closed configuration. In proceeding to the closed state,  $\delta$  will close into  $\delta'$ , obscuring contact between  $\beta$  and  $\delta/\gamma$  and severing the tie between  $\gamma$  complex and  $\beta$ . It is possible that as ATP is hydrolyzed, the  $\gamma$  complex guides the  $\beta$  ring closed. Whatever the case, upon losing contact with  $\gamma$  complex, the  $\beta$  ring should completely close as this is its lowest free energy state. The high affinity DNA binding site in  $\gamma$  complex requires both  $\beta$  and bound ATP [18]. Hence, after ATP hydrolysis,  $\gamma$  complex no longer has significant affinity for DNA, aiding its departure from the  $\beta$  ring on DNA [14,25,26]. Departure of  $\gamma$  complex from  $\beta$  is essential to the use of  $\beta$  by DNA polymerase, as polymerase and  $\gamma$  complex compete for binding to  $\beta$  due to the overlap of their binding sites on  $\beta$  [27].

### Implications for eukaryotic clamp loader

The five subunit  $\gamma_3\delta\delta'$  pentamer structure has been conserved in the eukaryotic clamp loader. The eukaryotic RFC clamp loader is a pentamer of nonidentical subunits [28,29] and all five subunits share sequence homology to  $\gamma$  and  $\delta'$  [22,23]. Four of the five subunits range from 36–40 kDa, similar to the masses of  $\gamma$  (47 kDa),  $\delta$ , (39 kDa) and  $\delta'$  (37 kDa). The largest subunit, yeast RFC1 (95 kDa) and its human homolog, p140 (128 kDa), have a region of homology to the motor domains of the other subunits, plus additional sequences of unknown function at the amino and carboxyl termini [23]. Biochemical experiments in the human system show that carboxy-terminal sequences of RFC subunits are needed to form the pentamer [30,31]. This is consistent with formation of a circular pentamer like  $\gamma_3\delta\delta'$  in which the major subunit contacts are formed

Table 1

Functional analogy of the three different gears of the *E. coli*  $\gamma_3\delta\delta'$  clamp loader to the subunits of yeast and human RFC.

Function	$\gamma$ complex component	RFC	Characteristics
Wrench	$\delta$	yRFC-1 (h-p140)	Only subunit that lacks SRC, binds to the clamp, and has conserved hydrophobic residues for clamp binding.
Stator	$\delta'$	yRFC-5 (h-p38)	Altered P-loop and contains SRC motif.
Motor	$\gamma_3$	yRFC-2 (h-p37), yRFC-3 (h-p36), yRFC-4 (h-p40)	Trimeric in solution, DNA dependent ATPase, and contains P-loop and SRC.

by the carboxy-terminal domains. In addition, atomic force microscope (AFM) and electron microscope (EM) studies of RFC provide images that are quite similar to  $\gamma_3\delta\delta'$  [32]. An AFM view of RFC is similar to the carboxy-terminal face of  $\gamma$  complex (see Figure 3e). An EM image of RFC in the presence of ATP shows a C shape similar to the amino-terminal face of  $\gamma$  complex (see Figure 3f).

The crystal structure of an RFC subunit from the archeon, *Pyrococcus furiosus*, has recently been solved and it has strong structural similarities to *E. coli*  $\gamma_3\delta\delta'$  [33]. Archae contain two RFC subunits, large and small, which have homology to eukaryotic RFC subunits. Multiple copies of these subunits assemble to form an active clamp loader [34]. The small subunit, RFCS, of *Pyrococcus furiosus* crystallized as a dimer of trimers, some of which contained bound ADP. The orientation of these subunits in the trimer is similar to that observed between the three  $\gamma$  subunits in  $\gamma_3\delta\delta'$ , and the ADP is located at subunit interfaces as predicted by the  $\gamma_3\delta\delta'$  structure. As in the  $\gamma$  complex, the major intersubunit contacts in the RFCS trimer are formed by the carboxy-terminal domains. Further, the chain-fold of RFCS is the same as that of  $\gamma_3\delta\delta'$  subunits despite remarkably low sequence identity between the carboxy-terminal domains of RFCS and either  $\gamma$ ,  $\delta$ , or  $\delta'$ . Finally, the differing orientation of domains 1 and 2, and domains 2 and 3 in the RFCS trimer is also consistent with the twisting between domains observed in each subunit of the  $\gamma$  trimer and in  $\delta$ .

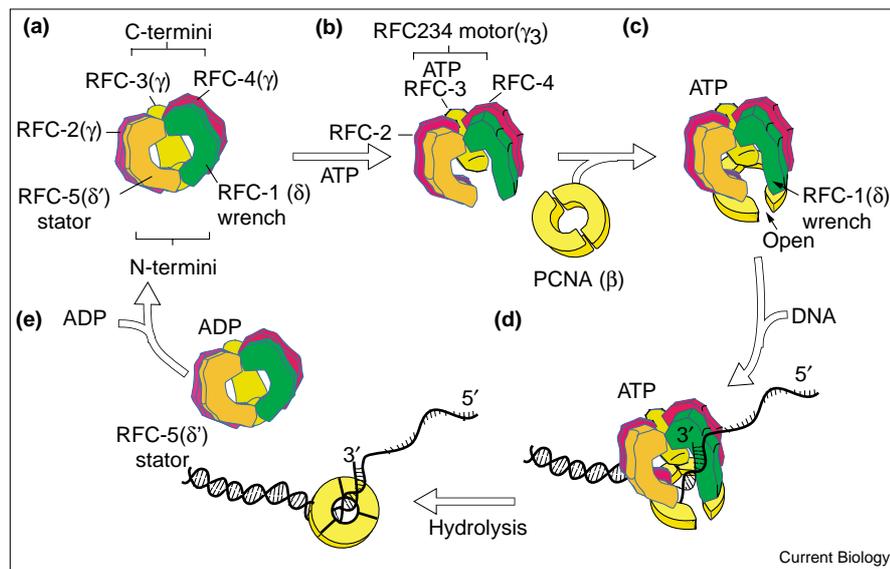
Given that RFC is a circular pentamer like  $\gamma_3\delta\delta'$ , how are the subunits arranged, and which subunits correspond functionally to the  $\gamma$ -trimer motor,  $\delta'$  stator and  $\delta$  wrench? The sequences of yeast and human RFC subunits, combined with experimental data, suggest the assignments given in Table 1. RFC-1 binds to the PCNA clamp [35], and like  $\delta$  it is the only subunit that lacks an SRC motif. These features suggest RFC-1 is the wrench, analogous to  $\delta$ . In addition, RFC-1 has two conserved adjacent hydrophobic residues between RFC boxes IV and V (Phe701,

Tyr702 in h-p140), a similar location to the two conserved hydrophobic residues in  $\delta$  that bind in the hydrophobic pocket of  $\beta$  (Leu73, Phe74). The other RFC subunits lack these residues. The yRFC-5 (h-p38) subunit may be the functional homolog of the  $\delta'$  stator as it is the only subunit that has a divergent P-loop yet contains an SRC motif. The human p40/p37/p36 subunits form a trimer and, like  $\gamma_3$ , they each contain a P-loop and SRC motif and have DNA dependent ATPase activity. Likewise, yRFC 2,3,4 form a trimer (O. Yurieva and M.O'D., unpublished), have DNA dependent ATPase activity (D. Zhang and M.O'D., unpublished), and each contain a P-loop and SRC motif. Hence, RFC 2,3,4 (h-p40,p37,p36) is the equivalent of the  $\gamma$  trimer motor.

How are these RFC subunits arranged in the pentamer? Placing the RFC subunits in the positions of their  $\gamma$  complex equivalents gives the arrangement in Figure 4. This arrangement is also consistent with experiments that have demonstrated the following subunit contacts: h-p36–h-p37, h-p37–h-p38, h-p140–h-p38, h-p140–h-p40 [36,37]. As discussed earlier, the three  $\gamma$  complex ATP binding sites are composed of two elements designated in Figure 4 as 'SRC' and the 'P-loop'. Using this notation, the first site in  $\gamma$  complex is composed of the SRC of the  $\delta'$  stator and the P-loop of  $\gamma_1$ . The comparable site in RFC is composed of the SRC of the yRFC-5 stator and P-loop of the yRFC-2 motor protein. Likewise, sites 2 and 3 are also formed at interfaces of adjacent proteins. An interesting difference between  $\gamma$  complex and RFC is the presence of a P-loop in yRFC-1 (h-p140), which may constitute a fourth ATP site along with the SRC of RFC-4. Indeed yRFC has recently been shown to bind four molecules of ATP [38]. Mutational studies in yeast indicate that the P-loop of RFC-1 is not required for clamp loading [39], but similar studies in the human system show that the fourth site contributes to the replication reaction [40].

A mechanism for RFC and  $\gamma$  complex is illustrated in Figure 5 based on the crystal structure of  $\gamma_3\delta\delta'$  and

Figure 5



Mechanism of RFC. (a) The clamp loader is in the closed form with amino-terminal domains closed upon the RFC-5 stator. (b) ATP binding to the RFC 234 motor releases the amino-terminal domains from RFC-5. (c) PCNA docks onto the amino-terminal domains and the RFC-1 wrench cracks one of the interfaces, allowing the PCNA ring to spring open like  $\beta$ . (d) The RFC complex binds DNA and places it through the open PCNA ring. (e) DNA binding results in ATP hydrolysis which closes the amino-terminal domains upon the RFC-5 stator, releasing the PCNA ring onto DNA. Protein subunits in the diagram are also labeled with the analogous proteins of the *E. coli* system. The analogous human subunits are given in Table 1.

biochemical analysis of both RFC and  $\gamma$  complex. Figure 5a shows the pentamer in the hypothetical closed form in which the amino-terminal domains occlude one another from binding the ring. We propose that ATP binding to the RFC234 motor results in the open form of the complex in which the amino-terminal domains swing down from the carboxy-terminal domains that form the pentameric contacts holding the complex together (Figure 5b). Consistent with a conformational change at this step,  $\gamma$  complex undergoes an ATP induced conformational change [15], and an ATP induced conformational change has also been seen in RFC [32].

The next step is the interaction of PCNA with the exposed amino-terminal domains of RFC (Figure 5c). Clamp binding to the ATP induced form of RFC is consistent with the finding that ATP strengthens RFC interaction with PCNA [41] this is similar to observations in the *E. coli* system [15]. Based on results of  $\gamma$  complex and  $\beta$  [13], we presume that PCNA is open at the step in Figure 5c. By analogy to  $\delta$ - $\beta$ , it seems likely that the RFC-1 wrench opens one interface of the PCNA ring, and that a gap is produced at the interface as the result of spring tension between the domains of PCNA. Although RFC-1-PCNA interaction has been documented, it has been noted that other RFC subunits, perhaps all of them, interact with PCNA [42,29]. Likewise, in  $\gamma$  complex the  $\delta$  and  $\gamma$  subunits, and possibly  $\delta'$  too, bind to  $\beta$  [15,19]. Multiple subunit attachment to the PCNA trimer may help to preserve the trimer, as it has been shown that the PCNA trimer disassembles at concentrations above that at which the  $\beta$  dimer dissociates [10]. Finally, the RFC-PCNA complex must recognize and bind a primed site (Figure 5d).

In *E. coli*, binding of the ATP analogue, ATP $\gamma$ S, to  $\gamma$  complex powers  $\beta$  ring opening and attachment of  $\gamma$  complex- $\beta$  to DNA [18]. Ring closure requires ATP hydrolysis [13]. Likewise, RFC-PCNA has been shown to bind DNA in the presence of ATP $\gamma$ S [29,38,43], and we presume that PCNA is open by analogy to the *E. coli* system [18]. The *E. coli*  $\gamma$  complex is ejected from the  $\beta$  clamp-DNA complex upon hydrolysis of ATP [4,13], likely due to closure of the amino-terminal domains of the clamp loader  $\delta$  and  $\gamma$  subunits upon the  $\delta'$  stator. Thus, the stator acts like an anvil upon which the amino-terminal domain of  $\delta$  strikes, thereby occluding the  $\beta$  interactive element and pinching off the connection to the  $\beta$  clamp. The ADP bound form of the clamp loader facilitates this final step [44]. These actions are shown in Figure 5e and are consistent with recent studies in the eukaryotic system showing that RFC dissociates after placing PCNA onto DNA [38].

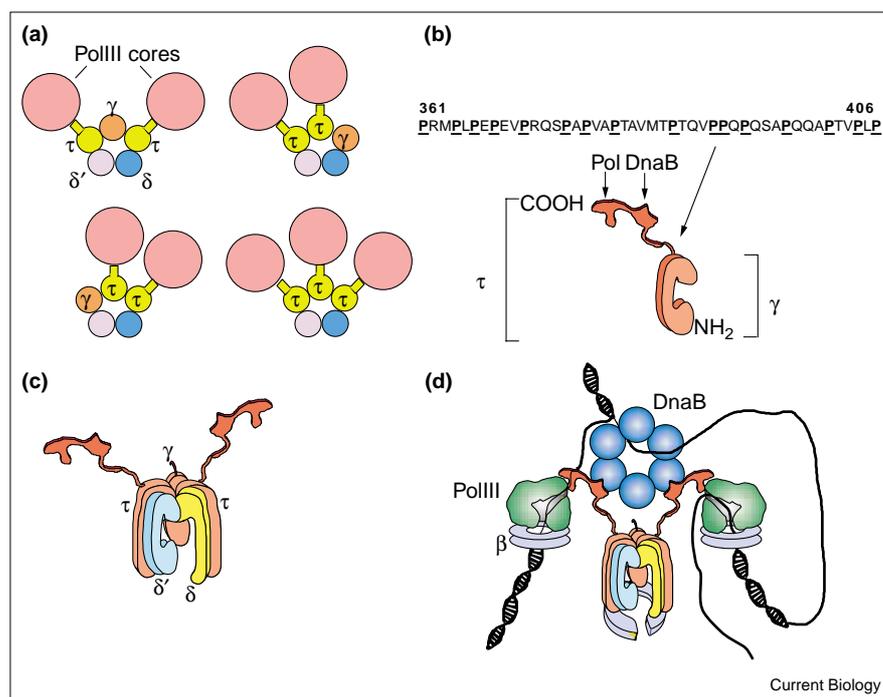
It is essential that  $\gamma$  complex dissociate from the  $\beta$  clamp on DNA as the  $\gamma$  complex and DNA polymerase III core compete for the same surface of  $\beta$  [15]. Similar competition is observed between RFC and DNA polymerase  $\delta$  [45]. In fact, numerous polymerases and other proteins bind PCNA [46] and recent studies in *E. coli* show that this is also the case for  $\beta$ . All five *E. coli* DNA polymerases function with  $\beta$ , and ligase and MutS also bind to  $\beta$ . These proteins all compete with one another for  $\beta$  implying a high degree of protein trafficking on these sliding clamps and placing them at the center of numerous DNA metabolic reactions [47].

### Implications for replisome structure

The *E. coli* replicase, DNA polymerase III holoenzyme, contains two molecules of DNA polymerase III core (Pol III

Figure 6

The  $\gamma$  complex organizes the replisome. The  $\gamma$  complex in the DNA polymerase III (Pol III) holoenzyme contains at least two  $\tau$  subunits in place of two  $\gamma$  subunits. The carboxyl terminal sequences of  $\tau$  contain a domain for binding Pol III core and therefore each  $\tau$  is shown with one core. The illustration shows three different  $\gamma$  complexes, each of which contain two  $\tau$  subunits; the structures only differ with respect to which  $\gamma$  subunits are replaced by  $\tau$ . Also shown is a holoenzyme form containing three  $\tau$  subunits (and cores) in place of the  $\gamma$  trimer. (b) The motor domains of  $\tau$  are separated from the Pol III and DnaB binding domains of  $\tau$  by a sequence which may be flexible. (c) The carboxyl termini of  $\gamma$  subunits in  $\gamma_3\delta\delta'$  protrude from the top, implying the approximate location of the protein binding domains in the  $\gamma_1\tau_2\delta\delta'$  complex. (d) Architecture of the *E. coli* replisome in which the DnaB hexamer attaches to carboxy-terminal sequences in  $\tau$  adjacent to the polymerases. Both cores are shown with their  $\beta$  clamps, and  $\gamma/\tau$  complex has hold of a  $\beta$  clamp for the repeated clamp loading events that occur on the lagging strand [63].



core) for concurrent replication of leading and lagging strands [18,48]. The two molecules of Pol III core are cross-linked via their connection to a single clamp loader [49]. Where do these Pol III molecules bind to the clamp loader? The  $\tau$  subunit holds the key to how the components of the holoenzyme are organized.

In *E. coli*, the *dnaX* gene encoding the  $\gamma$  motor also produces a second protein,  $\tau$ .  $\tau$  is the full length protein (71 kDa), and  $\gamma$  is smaller (47 kDa), truncated by a translational frameshift followed by a stop codon [50–52]. Hence,  $\tau$  contains the  $\gamma$  motor sequences plus extra carboxy-terminal sequences. Given the structural identity it is not surprising that  $\tau$  fully substitutes for  $\gamma$  in action as a clamp loader with  $\delta$  and  $\delta'$  [12]. Active  $\gamma/\tau$  complexes can also be formed that contain either two  $\gamma$  and one  $\tau$ , or one  $\gamma$  and two  $\tau$  subunits [53]. The carboxy-terminal sequences unique to  $\tau$  have been shown to be essential for cell viability [54]. *E. coli* cells survive in the absence of  $\gamma$ , but can not live without  $\tau$ . Indeed, many bacteria produce only  $\tau$  from their *dnaX* gene [55,56].

What is so special about the carboxy-terminal sequence that is unique to  $\tau$ ? The carboxyl terminus of  $\tau$  gives it the capacity to bind to the DNA polymerase III core, and DnaB helicase [57–59]. Sites of interaction between  $\tau$  and these proteins have been mapped to two separate domains within the carboxy-terminal sequence of  $\tau$  [60,61]. The most

carboxy-terminal domain binds Pol III core. As the holoenzyme contains two molecules of Pol III core, the  $\gamma$  complex within DNA polymerase III holoenzyme must contain at least two  $\tau$  subunits in place of two  $\gamma$  subunits. One  $\gamma$  subunit is thought to remain — along with two  $\tau$ , one  $\delta$  and one  $\delta'$  — as  $\gamma$  is present in holoenzyme preparations.

Figure 6a illustrates the three different subunit arrangements within the holoenzyme assuming two  $\tau$ , one  $\gamma$ , one  $\delta$  and one  $\delta'$  in the clamp loader. Due to the asymmetry of the  $\gamma/\tau$  complex, each subunit occupies a unique position. Thus each of the dimeric polymerase forms of holoenzyme in Figure 6a is structurally asymmetric. Asymmetric distribution of subunits within the  $\gamma/\tau$  complex may aid the two core polymerases in their distinct functions of continuous leading and discontinuous lagging strand synthesis (see below). Unless there is a mechanism that leads to only one of these forms, it seems likely that the holoenzyme within the cell is a mixture of the forms illustrated in Figure 6a.

A  $\tau$  subunit form of the holoenzyme may also be present *in vivo* (Figure 6a). Certainly, an *E. coli* mutant that lacks the *dnaX* frameshift site and makes only  $\tau$  [54] probably has a holoenzyme that contains three  $\tau$  subunits. Reconstitution studies indicate that this form contains three polymerases, and this trimeric polymerase appears fully capable of function at a replication fork *in vitro* (P. McInerney and M.O'D., unpublished). Moreover, many bacteria produce

only  $\tau$  and no  $\gamma$ , and therefore they may also contain a trimeric polymerase holoenzyme [55,56]. Presumably, one core polymerase functions on the leading strand, and the lagging strand may be serviced by one or even both of the other core polymerases. For the discussion to follow, it will be assumed that the holoenzyme contains only two DNA polymerases.

To assess where the polymerases are located relative to the clamp loader in the holoenzyme it is instructive to note the location of the carboxy-terminal ends of  $\gamma$  to which the polymerase and helicase binding domains of  $\tau$  are attached. The carboxyl termini of the  $\gamma$  subunits, from which the carboxyl terminal sequences of  $\tau$  originate, protrude out the top of the clamp loader (see Figure 3a). The sequence near the carboxyl terminus of  $\gamma$  contains several proline and hydrophilic residues suggesting it is unstructured (see Figure 6b). Consistent with this,  $\gamma$  is cleaved at least twice in this region by elastase (D.J. and J.K., unpublished). Thus the motor domains of  $\tau$  are connected to the Pol III/DnaB binding domains by a flexible region. A diagram of  $\gamma/\tau$  complex containing two  $\tau$  subunits in place of two  $\gamma$  subunits is shown in Figure 6c. Attachment of two molecules of Pol III core and one DnaB hexamer to the  $\gamma$  complex containing two  $\tau$  subunits is shown in Figure 6d. The diagram also includes replication fork DNA in which DnaB encircles the lagging strand, and each polymerase is clamped to daughter strands by  $\beta$  rings.

The arrangement of proteins at a replication fork shown in Figure 6d implies that the two polymerases are parallel. Duplex DNA is antiparallel, and therefore even though the lagging strand polymerase moves with the replication apparatus, it synthesises the lagging strand in the direction opposite fork progression. It is possible that the flexible linker connecting the motor domains of  $\tau$  with the carboxy-terminal protein binding sequences in  $\tau$  provides a range of motion between the two polymerases and the clamp loader. Thus, the lagging strand polymerase could possibly flip and point in the other direction to that shown in Figure 6d. Indeed it may be that the polymerases constantly shift their orientation to cope with the difficult geometry of spiral motions implied in making helical products, especially on the leading strand [62], and formation and dissolution of large DNA loops on the lagging strand, accompanied by polymerase hopping from completed fragments to new primed sites [63].

Flexibility between the  $\gamma/\tau$  complex and its connection point to the DNA polymerases may also aid the single  $\gamma/\tau$  complex in loading  $\beta$  onto DNA for both DNA polymerases [59]. A flexible tether may enable  $\gamma/\tau$  complex to swing close to both polymerase enzymes, even if the polymerases are distant from one another in the holoenzyme. The  $\gamma$  complex and Pol III core are known to bind the

same or overlapping site on  $\beta$  [15] and thus  $\gamma$  complex must move away from  $\beta$  after loading it on DNA so that Pol III core can take its position with the  $\beta$  clamp. A flexible region between Pol III and  $\gamma/\tau$  complex may be instrumental in facilitating a smooth transition between  $\gamma/\tau$  complex and Pol III core on the  $\beta$  clamp.

The anti-parallel structure of DNA results in two seemingly different processes for replicating the leading and lagging strands. The leading strand is continually synthesized whereas the lagging strand is synthesized as numerous fragments. However, these two processes are not as different as they may first seem to be. In each case, primase synthesizes the RNA primer — at the origin for the leading strand — and the holoenzyme must recruit the primer from primase, assemble a clamp on it, and then extend DNA processively until finished, whereupon the polymerase dissociates from DNA. This process is performed once, or only a few times, on the leading strand, and is repeated over and over on the lagging strand. Hence, except for the frequency in which these steps are repeated, these events are similar on the two strands.

Two major differences in replication of the leading and lagging strands are the need to cope with single strand binding protein (SSB) on the lagging strand, and the need for the leading polymerase to maintain its attachment to DNA while constantly bumping into what would appear to be completely replicated DNA at the fork. Continued attachment of the leading polymerase is solved by the helicase, which is contacted by  $\tau$ , and this contact prevents the holoenzyme from parting from the fork [59]. McHenry and coworkers [64] propose that the asymmetric structure of the clamp loader in the holoenzyme provides different properties to the two polymerases. Recent results using ATP $\gamma$ S show that the two polymerases respond differently to this nucleotide, which is most likely the result of ATP $\gamma$ S binding to  $\gamma$  complex. The structural asymmetry of  $\gamma$  complex is also likely to impart functional asymmetry onto the lagging strand polymerase by helping it function with SSB, as the single  $\chi$  subunit of the clamp loader contacts SSB. Study of the function of this contact shows, among other things, that it aids polymerase extension on SSB coated ssDNA [65,66].

## Conclusions

The strategy of using clamps and clamp loaders as a way of achieving high processivity is conserved across the evolutionary spectrum of all free living cells. In fact, clamps are used by numerous other polymerases and proteins that function in diverse pathways of replication, repair and recombination. Thus it is more appropriate to think of the clamp and clamp loader as being at the center of many DNA metabolic pathways; their use in replication was simply the first cellular application to be discovered.

Although there are important differences between eukaryotic RFC/PCNA and prokaryotic  $\gamma$  complex/ $\beta$ , the striking similarities between the systems are much more extensive than the differences. The new structures in the *E. coli* system give a highly detailed look into the inner workings of these machines, both prokaryotic and eukaryotic. Indeed, the studies suggest that the circular clamp itself is a finely tuned machine. No longer is it simply a beautiful but relatively unintelligent ring that is acted upon by other proteins. The new findings show that it carries within its structure its own mechanical action that directs ring opening to produce a gap. The structures also address how the clamp loader organizes the polymerases and helicase at the replication fork. Perhaps the eukaryotic RFC, like the *E. coli* clamp loader, organizes the higher order architecture of the replication machinery. It will be most interesting in the future to bring these higher order replisome structures into sharper focus.

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