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# Comparative Studies of Processivity Clamp Loader ATP Site Function.

Aaron M. Johnson

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**COMPARATIVE STUDIES OF  
PROCESSIVITY  
CLAMP LOADER ATP SITE FUNCTION**

A Thesis Presented to the Faculty of  
The Rockefeller University  
in Partial Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy

by

Aaron M. Johnson

June 2007



# COMPARATIVE STUDIES OF PROCESSIONITY CLAMP LOADER ATP SITE FUNCTION

Aaron M. Johnson, Ph.D.  
The Rockefeller University, 2007

DNA replicases utilize ring-shaped sliding clamps to ensure polymerase processivity. An ATP-dependent clamp loader topologically links the clamp around DNA in a multi-step mechanism. Clamp loaders are ring-shaped pentamers of AAA+ subunits. The pentamer complex contains 3-4 ATP sites, each located at the interface of two subunits. In each ATP site, an arginine residue from one subunit is located near the  $\gamma$ -phosphate of ATP bound to the adjacent subunit. These arginines act as “arginine fingers” that can potentially perform two functions: sensing that ATP is bound and catalyzing ATP hydrolysis. This thesis utilizes mutations in the arginine fingers of the *E. coli* and *S. cerevisiae* clamp loaders in order to examine steps in the clamp loading mechanism after the clamp loader binds ATP.

The *E. coli*  $\gamma$  complex couples ATP hydrolysis to the loading of  $\beta$  sliding clamps onto DNA. We demonstrate that the  $\delta'$  subunit of  $\gamma$  complex contributes an arginine finger into ATP site D. Hydrolysis in site D is inhibited by mutation of the  $\gamma$  subunit arginine finger, which affects sites B and C. The  $\delta'$  arginine finger also mediates  $\beta$  binding and the  $\gamma$  arginine fingers are important for DNA binding. Utilizing a three-subunit fusion construct, we determined that a single arginine finger mutation in either site B or C causes inactivation of  $\gamma$  complex.

Replication factor C (RFC) loads the processivity clamp, proliferating cell nuclear antigen (PCNA), onto DNA. ATP binding to RFC activates a  $\gamma$ -phosphate



sensor in ATP site C that promotes DNA association by RFC-PCNA. DNA binding to the RFC-PCNA complex then triggers the ATP hydrolysis cycle which starts in site C and ends with ATP site D. ATP hydrolysis in site D is uniquely stimulated by PCNA and we propose that this site is coupled to closure of PCNA around DNA. PCNA closure severs contact to RFC subunits D and E, and the  $\gamma$ -phosphate sensor of ATP site C is switched off, leading to ejection of RFC from the site of PCNA loading. This work sheds light on conserved aspects of ATP site function that may extend to all clamp loaders.

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Aaron Johnson, June 27<sup>th</sup>, 2006

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## **Introduction: DNA Replicases: Components and Dynamics at the Replication Fork<sup>1</sup>**

### **ABSTRACT**

Chromosomal replicases are multicomponent machines that have evolved clever strategies to perform their function. Although the structure of DNA is elegant in its simplicity, the job of duplicating it is far from simple. At the heart of the replicase machinery is a heteropentameric AAA+ machine that couples ATP hydrolysis to load circular clamp proteins onto DNA. The clamps encircle DNA and hold polymerases to the template for processive action. The circular sliding clamp protein structures have been solved, in both prokaryotic and eukaryotic systems as have those of the multisubunit clamp loaders. The heteropentameric clamp loaders are circular oligomers, reflecting the circular shape of their respective clamp substrate. Clamps and clamp loaders also function in other DNA metabolic processes including repair, checkpoint mechanisms, and cell cycle progression.

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<sup>1</sup> Adapted with permission, from the Annual Review of Biochemistry, Volume 74  
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## INTRODUCTION

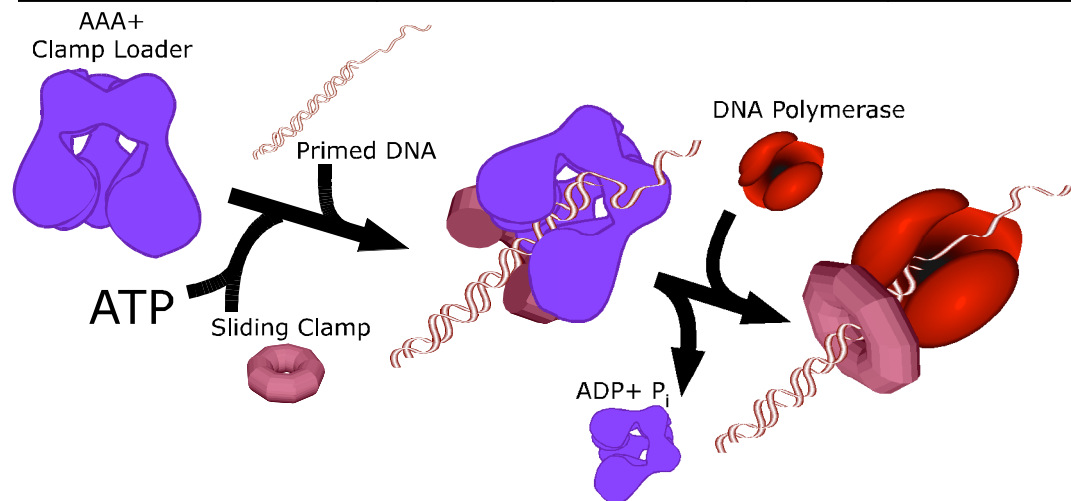
Chromosomal DNAs are exceedingly large molecules and the vast repository of information they hold must be duplicated with precision (see (Kornberg and Baker, 1992) for overview). The sheer size of these molecules may explain the use across all kingdoms of ring-shaped sliding clamp proteins that encircle duplex DNA and serve to hold the DNA polymerases to their long substrates for highly processive synthesis (See Figure 1.1). The eukaryotic (PCNA) and prokaryotic ( $\beta$ ) clamp proteins have unrelated sequences, yet they have strikingly similar structures and almost certainly share a common ancestor (Kong et al., 1992; Krishna et al., 1994). The clamps must be opened and closed around DNA, and this job is performed by a five-subunit oligomer composed of proteins in the large AAA+ family (reviewed in (O'Donnell et al., 2001)). These subunits are arranged in a circular fashion, and as with the clamps, the eukaryotic (RFC) and the prokaryotic ( $\gamma$  complex) clamp loaders are highly related structures. The relatedness of structure in the bacterial replicase has led to comparative analysis of the two systems which suggests that their mechanisms are also related.

Archaeobacteria also utilize a similar clamp and clamp loader strategy, as does the T4 bacteriophage (see Table I and end of chapter). It should be noted that the replicative polymerases of most bacteriophage and viruses do not utilize a paired clamp and clamp loader, but generally require one or two accessory factors that confer processivity. The exact means by which they do so is not entirely clear (see: T7 phage (Benkovic et al., 2001; Huber et al., 1987; Tabor et al.,

**Figure 1.1 Components of cellular replicases.** The table lists the three replicase components in the well-defined systems of *Escherichia coli*, eukaryotes, Archaea, and T4 phage. The scheme below is a generalized mechanism of replicase action. A multiprotein clamp-loader couples ATP binding and hydrolysis to loading of a ring-shaped processivity clamp that is then used by the replicative polymerase as a tether to the DNA template.

**Figure 1.1 Components of cellular replicases.**

<b>Replicase Component</b>	<b><i>E. coli</i></b>	<b>Eukaryotes</b>	<b>Archaea</b>	<b>T4 Phage</b>
Polymerase	Core ( $\alpha\epsilon\theta$ )	Pol $\delta$ /Pol $\epsilon$	Pol $\delta$	gp43
Sliding Clamp	$\beta$ Clamp	PCNA	PCNA	gp45
Clamp Loader	$\gamma$ Complex	RFC	RFC	gp44/62



1987), vaccinia virus (Klemperer et al., 2001; McDonald et al., 1997), herpes virus (Gottlieb et al., 1990; Hernandez and Lehman, 1990), and Pol  $\gamma$  (Kaguni, 2004)).

The replicases of all cells function within a greater context involving many other proteins that together replicate the genome. This larger machinery is termed the “replisome” and is fairly well defined in prokaryotes (Benkovic et al., 2001; Kelman and O'Donnell, 1995; Kornberg and Baker, 1992). For example, two other basic activities at a replication fork include a helicase to separate the duplex DNA and a primase which produces short RNA primers that are required to initiate DNA synthesis. In *E. coli*, and presumably in other prokaryotes, these activities reside in two proteins: the homohexameric DnaB helicase and DnaG primase. The leading and lagging strand polymerases are connected to the clamp loader which also binds the helicase. The replicase also makes a specific functional connection to single-strand DNA binding protein (SSB) which is present at the fork to protect ssDNA and melt hairpins.

The eukaryotic replisome, in contrast to its prokaryotic counterpart, involves more proteins and remains somewhat less concretely defined at the current time. In eukaryotes the helicase, primase, and SSB are all heterooligomers. The helicase is thought to be the heterohexameric MCM complex and the primase is the four-subunit DNA polymerase  $\alpha$ /primase that makes a hybrid RNA/DNA primer (Waga and Stillman, 1998)(Table II). The heterotrimeric replication protein A (RPA) functions as the SSB (Wold, 1997). In addition to these basic activities that have simpler prokaryotic counterparts, there are a number of other proteins that are thought to act in fork progression and thus are candidates for replisome components (GIN5 complex (Takayama et

al., 2003), Cdc45, Dpb11, Sld2 (Kamimura et al., 1998), Sld3(Kamimura et al., 2001)). The functions of these components are presently unknown. In addition, the leading and lagging strand polymerases are thought to be different enzymes, Pol  $\delta$  (3-4 subunits) and Pol  $\epsilon$  (4 subunits). It is still unclear which strands these polymerases act upon. Finally many of these proteins are regulated by post-translational modification, including the PCNA clamp which is ubiquitinated and SUMO-ylated (Haracska et al., 2004; Hoege et al., 2002; Kannouche et al., 2004).

The clamp and clamp loaders of all cellular replicases also function in several other processes besides replication. For example, PCNA and  $\beta$  bind DNA ligase, mismatch repair, and excision repair proteins, although the exact role of these interactions is still not entirely clear (Lopez de Saro and O'Donnell, 2001; Maga and Hubscher, 2003; Warbrick, 2000). A weak consensus sequence for proteins that bind PCNA reveals a broad array of additional proteins that bind this clamp and are generally involved in repair or cell cycle control (Warbrick, 2000). The clamps also function with other DNA polymerases (Lopez de Saro et al., 2003a; Maga and Hubscher, 2003) probably helping to target them to sites where their action is required, and in one case (*E. coli* Pol IV), even conferring the ability to bind dNTPs at the intracellular concentration (Bertram et al., 2004). Eukaryotes also make several alternative RFC clamp loaders for diverse jobs in which one subunit is replaced by a unique protein that presumably specializes the complex for the alternative function (Bellaoui et al., 2003; Lindsey-Boltz et al., 2001; Naiki et al., 2001). These alternative clamp

loaders are presumed to load PCNA clamps at specific target sites, or in one case an alternate clamp is used.

The first cellular DNA replicase to be studied, *E. coli* DNA polymerase III holoenzyme, was isolated as an intact particle from *E. coli* extracts in the early 1980s and its structure and function have served as a suitable paradigm for the eukaryotic counterpart (Kornberg and Baker, 1992). This review begins with the prokaryotic replicase machinery, then builds off of the well-defined prokaryotic replicase system to comparatively analyze its counterpart in eukaryotes.

## **THE *E. COLI* REPLICASE**

The DNA polymerase III holoenzyme has been well-characterized since its discovery over thirty years ago, yet new insights into DNA replication machines are still made with this invaluable model system. Many sources have compiled the established activities of the holoenzyme and its subcomplexes (see (Kelman and O'Donnell, 1995; Kornberg and Baker, 1992)). This review will highlight certain key points of previous work to put into context the most current findings.

### **Pol III Core**

The DNA Polymerase III (Pol III) core was originally identified in a mutant strain of *E. coli*, *polA*-(Kornberg and Gefter, 1971). This strain lacked the comparatively strong DNA Polymerase I activity, unmasking the replicative polymerase. The identity of this activity was likely the core polymerase subcomplex, acting non-processively. The specific activity of Pol III core is similar to Pol I, but, as will be described later, Pol III core functions with accessory proteins that convert it to an exceedingly efficient enzyme having the



**Table I** *E. coli* replisome components and associated functions

Replisome component (stoichiometry <sup>a</sup> )	Gene	Mol. Wt. (kDa)	Function
<b>Pol III holoenzyme</b>		<b>791.5</b>	<b>Dimeric, ATP-dependent, processive polymerase/clamp loader</b>
Pol III*		629.1	Dimeric polymerase / clamp loader
Core		166.0	Monomeric polymerase/exonuclease
α (2)	<i>dnaE</i>	129.9	DNA polymerase
ε (2)	<i>dnaQ</i>	27.5	3'-5' Exonuclease
θ (2)	<i>holE</i>	8.6	Stimulates ε exonuclease
γ/τ complex		297.1	ATP-dependent clamp loader
γ/τ (1/2)	<i>dnaX</i>	47.5/ 71.1	ATPase, τ organizes Pol III* and binds DnaB
δ (1)	<i>holA</i>	38.7	Binds β clamp
δ' (1)	<i>holB</i>	36.9	Stator, stimulates γ ATPase in ATP site D
χ (1)	<i>holC</i>	16.6	Binds SSB <sup>b</sup>
ψ (1)	<i>holD</i>	15.2	Connects χ to clamp loader
β (2 dimers)	<i>dnaN</i>	40.6	Homodimeric processivity sliding clamp
Primase (1)	<i>dnaG</i>	64	Generates RNA primers for Pol III holoenzyme
DnaB helicase (6)	<i>dnaB</i>	52.4	Unwinds duplex DNA 5'-3' ahead of the replication fork
SSB (4)	<i>ssb</i>	18.8	Melts secondary structure in ssDNA, binds clamp

<sup>a</sup> Refers to the stoichiometry in the holoenzyme and replisome.

<sup>b</sup> Abbreviations include: SSB, single-stranded DNA-binding protein; and ssDNA, single-stranded DNA

highest specific activity of any *E. coli* DNA polymerase (Kornberg and Baker, 1992).

Purification and characterization of Pol III core (McHenry and Crow, 1979) revealed it to be a heterotrimer of the  $\alpha$  polymerase,  $\epsilon$  3'-5' proofreading exonuclease (Scheuermann and Echols, 1984), and  $\theta$  subunits in a 1:1:1 complex (Studwell-Vaughan and O'Donnell, 1993)(see Table I). The  $\alpha$  subunit (gene product of *dnaE*) contains the template-dependent DNA polymerase activity, incorporating 8 nucleotides/second, similar to Pol III core (20 nt/sec) (Maki and Kornberg, 1985). The  $\epsilon$  (*dnaQ*) 3'-5' exonuclease is the proofreader and is capable of removing misincorporated nucleotides that  $\alpha$  would otherwise have a difficult time extending. It is interesting to note that without  $\epsilon$  the processivity of holoenzyme is severely reduced to 1-3 kilobases (Studwell and O'Donnell, 1990), thereby ensuring that the proofreading  $\epsilon$  subunit is present during genome duplication. In contrast the small  $\theta$  subunit (*holE*) has no known function besides a slight stimulation of  $\epsilon$  (Taft-Benz and Schaaper, 2004) and the *holE* gene can be deleted with little consequence (Slater et al., 1994).

Little structural information is available for Pol III core. The  $\alpha$  subunit is a member of the C family of DNA polymerases (Braithwaite and Ito, 1993). Members of this family are present only in bacteria and it remains the only major family of DNA polymerases for which no crystal structure has been solved. Conservation of the active site structure across the other DNA polymerase families suggests that  $\alpha$  also possesses a similar two-metal active site for primer/template recognition with the characteristic "right hand" shape with "palm," "thumb," and "fingers" domains (Steitz, 1999). However, the peripheral

regions of  $\alpha$  are likely to be structurally unique. Deletion mutagenesis suggests that the active site is located in the N-terminal two-thirds of  $\alpha$  while contacts to other holoenzyme components are made through the C-terminal region (Kim and McHenry, 1996a; Kim and McHenry, 1996b).

Atomic resolution structures of portions of  $\epsilon$  and  $\theta$  are available (DeRose et al., 2003; Hamdan et al., 2002; Keniry et al., 2000). The  $\epsilon$  subunit of core is composed of two distinct domains (Perrino et al., 1999). The N-terminal domain (186 residues out of 243) contains the exonuclease active site and the  $\theta$  binding site. The crystal structure of this active fragment has been solved (Hamdan et al., 2002) and it is structurally similar to other polymerase-associated exonucleases, in particular the exonuclease domain of DNA polymerase I. This partial structure also suggests how  $\epsilon$  might interact with a DNA template. The preferred DNA substrate for  $\epsilon$  is a single-strand 3' terminus (Brenowitz et al., 1991; Miller and Perrino, 1996). Double-stranded primer/template (p/t) DNA with a recessed 3' terminus is not a good substrate for  $\epsilon$  alone, especially when fully base-paired, but  $\alpha$  tightly associates with the C-terminus of  $\epsilon$  and stimulates  $\epsilon$  activity on a recessed 3' terminus, probably by bringing  $\epsilon$  to the primer site.  $\epsilon$  preferentially degrades a 3' mismatched p/t DNA alone and as a component of core. In addition,  $\alpha$  polymerization is inhibited by a mismatch, which provides  $\epsilon$  more time to complete the excision.

Structural studies of  $\theta$  (DeRose et al., 2003; Keniry et al., 2000) have revealed its general tertiary structure and a detailed mapping of the main interactions with  $\epsilon$ . The structure of  $\theta$  bears a resemblance to a DNA-interacting domain of eukaryotic DNA polymerase  $\beta$ . This structural similarity may speak

to  $\theta$ 's role in slight stimulation of  $\epsilon$  and the increased mutator phenotype in a *holE*-null mutant with an  $\epsilon$  mutant background (Slater et al., 1994).

### **The $\beta$ Sliding Clamp**

Pol III core by itself is slow, incorporating 20 nt/s, and weakly processive, extending only 1-10 bases per binding event (Maki and Kornberg, 1985). In fact no matter how much time or enzyme, Pol III core can not extend a unique primer full-circle around an M13 ssDNA genome (Fay et al., 1981; LaDuca et al., 1983).

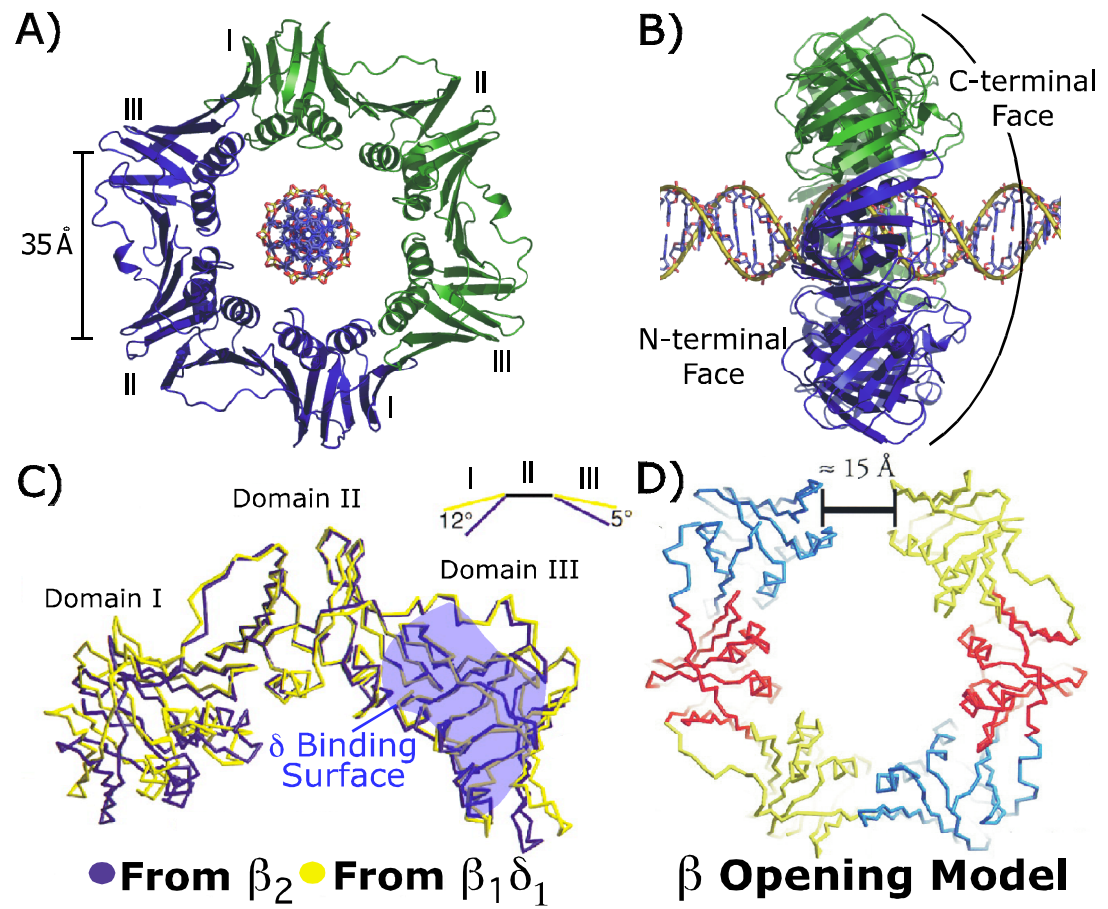
To become an efficient replicase, the core polymerase requires the  $\beta$  processivity sliding clamp. In the case of Pol III core: $\beta$ , the processive polymerase also becomes the fastest enzyme of its kind, incorporating ~750 nucleotides/sec for many kilobases. Biochemical studies initially revealed that  $\beta$  binds DNA topologically (Stukenberg et al., 1991), implying it has a ring shape and encircles the duplex whereupon it freely slides along it. This hypothesis was quickly proven by structure analysis (Kong et al., 1992)(Figure 1.2). Core polymerase directly associates with  $\beta$  (Kuwabara and Uchida, 1981; LaDuca et al., 1986; Stukenberg et al., 1991), initially occupying roughly 22 bp of primer (Reems et al., 1995; Yao et al., 2000), and as it extends DNA it pulls the clamp along behind it.

The crystal structure of the  $\beta$  clamp shows that it is a homodimer (Kong et al., 1992), arranged in a head-to-tail manner, creating two distinct “faces” of the clamp and a large central channel of ~35 Å diameter that easily fits a B-form double-stranded DNA modeled inside (Figure 1.2). In fact, one or two layers of water molecules can also fit between the modeled DNA, suggesting that  $\beta$  may

**Figure 1.2 Structure and dynamics of the  $\beta$  sliding clamp.** *Panel A and B*

Crystal structure of the  $\beta$  dimer with modeled double-stranded DNA through the central channel (courtesy of D. Jeruzalmi and J. Kuriyan). *Panel B* highlights the two “faces” of the ring. The C-terminal face (*right*) is implicated in many of the interactions of  $\beta$  with other proteins. *Panel C* Superposition of one  $\beta$  subunit from the  $\beta$  dimer structure (*purple*) and the  $\beta$  monomer from the  $\beta_1\delta_1$  crystal structure (*yellow*), using domain II as a reference. *Panel D* Model of an open  $\beta$  clamp made by arranging two  $\beta$  monomer structures from  $\beta_1\delta_1$  to create one dimer interface (described in (Jeruzalmi et al., 2001b)). *Panels C and D* adapted from (Jeruzalmi et al., 2001b), copyright 2001, with permission from Elsevier.

**Figure 1.2 Structure and dynamics of the  $\beta$  sliding clamp.**



“skate” along duplex DNA. The center of the  $\beta$  ring is lined with 12  $\alpha$  helices, pairs of which are supported by an outer  $\beta$  sheet. This helix pair and sheet motif is repeated six times around the ring, creating a six-fold pseudosymmetry. The “faces” of the ring are structurally distinct, with one having several loops and the C-termini of the protomers protruding. This is the face of the ring that interacts with other proteins as discussed below (Naktinis et al., 1995; Naktinis et al., 1996).

The  $\beta$  clamp is a tight dimer and its half-life on DNA is over one hour at 37° C (Leu et al., 2000; Stewart et al., 2001). The  $\delta$  subunit of  $\gamma$  complex can open  $\beta$  by itself, as determined by its ability to rapidly remove the  $\beta$  clamp from DNA (Leu et al., 2000; Turner et al., 1999). It appears that only one  $\delta$  binds  $\beta_2$  and it does not dissociate the  $\beta$  dimer into monomers even though it must destabilize one interface (Naktinis et al., 1995; Stewart et al., 2001; Turner et al., 1999). This idea is consistent with the ability of  $\gamma$  complex to load  $\beta$  that is cross-linked across one dimer interface (Turner et al., 1999). A co-crystal structure of  $\delta$  bound to a monomeric mutant of  $\beta$  has been solved (Jeruzalmi et al., 2001b). This structure shows two distinct points of contact between  $\delta$  and  $\beta$ . The contact points are located on the opposite ends of the same  $\alpha$  helix which is located in the N-terminal domain of  $\delta$ . One end of the helix binds a hydrophobic pocket between domains II and III of  $\beta$ . The other end appears to push on a loop at the dimer interface leading to a distorted interface that can no longer close. It is proposed that  $\delta$  gets a grip on  $\beta$  via the hydrophobic pocket and pushes on the loop to crack the interface or to hold the interface open. Mutational analysis of these

contact points has confirmed their roles in clamp loader interaction and clamp opening (Indiani and O'Donnell, 2003).

The  $\delta$ - $\beta$  structure explains how the interface is cracked but not how the ring opens to accommodate DNA in the central channel. Comparison of the  $\beta$  dimer and  $\beta$  monomer (from the  $\delta$ - $\beta$  structure) shows significant rigid body motions between the domains leading to a shallower crescent shape in the monomer (Jeruzalmi et al., 2001b) (see Figure 1.2C). This implies that the  $\beta$  ring is under spring tension in the closed state until  $\delta$  disrupts one interface, allowing tension between the domains to relax and producing a gap for DNA strand passage (see Figure 1.2D). The strong interactions at the dimer interfaces likely maintain this tension.

### **The $\gamma$ Complex Clamp Loader**

The tight interfaces of the  $\beta$  ring and its long half-life on DNA suggest that an active process is necessary to pry open the ring for assembly onto DNA. This process is performed by the third component of Pol III holoenzyme: the clamp loader. The *E. coli* clamp loader is a complex composed of five different subunits in a defined stoichiometry:  $\gamma_3\delta_1\delta'_1\chi_1\psi_1$  (Jeruzalmi et al., 2001; Pritchard et al., 2000). The  $\gamma$  complex harnesses the energy of ATP binding and hydrolysis to topologically link  $\beta$  to a primed DNA, then ejects from DNA leaving the closed clamp behind (Hingorani and O'Donnell, 1998; Turner et al., 1999). It is convenient to dissect the clamp loader by the primary function of each subunit (O'Donnell et al., 2001). The three  $\gamma$  subunits are the only subunits that bind ATP and have thus been termed the “motor” of the complex. The  $\delta$  subunit is called



the “wrench” because it is the main  $\beta$  clamp-interacting subunit and it can open the dimer interface by itself. The  $\delta'$  subunit modulates  $\delta$ - $\beta$  contact (Turner et al., 1999).  $\delta'$  appears to be a rigid protein alone (Guenther et al., 1997) and within  $\gamma$  complex and has been termed the “stator” of the complex, which is the stationary part of a machine upon which other parts move. The  $\gamma_3\delta_1\delta'_1$  complex is termed the “minimal” clamp loader, as it is sufficient to place  $\beta$  on a DNA template (Onrust and O'Donnell, 1993). The  $\chi$  and  $\psi$  subunits are not essential for the clamp loading mechanism (Xiao et al., 1993), but  $\chi$  links the clamp loader to SSB and primase (Glover and McHenry, 1998; Kelman et al., 1998), which will be discussed later.  $\psi$  serves as a connector to  $\chi$  and also strengthens the  $\gamma\delta\delta'$  complex (Olson et al., 1995).

A broad outline of the clamp loading mechanism has been determined from biochemical studies (O'Donnell et al., 2001). The nucleotide-free clamp loader has a low affinity for the clamp. Upon ATP binding, the  $\gamma$  complex undergoes a conformational change that allows tight binding to the clamp, whereupon  $\delta$  opens one dimer interface of the clamp. The clamp-clamp loader complex has a strong affinity for DNA, particularly a primed template. The DNA, presumably threaded through the clamp, stimulates ATPase activity in  $\gamma$  complex and this allows the ring to close around the DNA and the clamp loader to be ejected, recycling to catalyze another loading reaction.

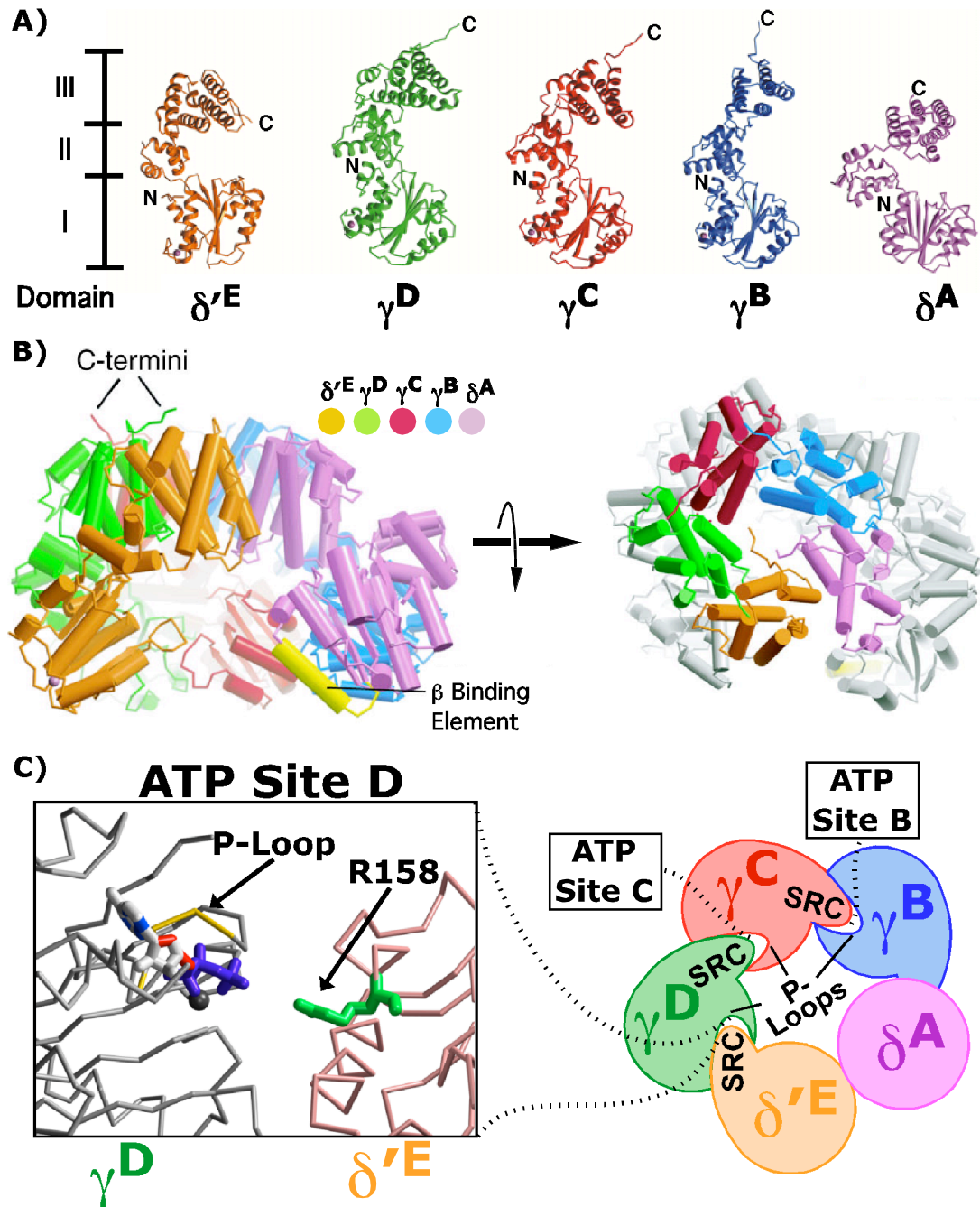
The structure of the  $\gamma_3\delta\delta'$  complex provided deeper insight into clamp loader action (Jeruzalmi et al., 2001). This accomplishment was followed two years later by the structure of the eukaryotic clamp loader bound to its clamp (Bowman et al., 2004), which has filled in many details that will be discussed

later. The analogous positions in both the  $\gamma_3\delta\delta'$  and RFC pentamers have been given an alphabetical nomenclature (for  $\gamma$  complex, see Figure 1.3). The five subunits in the minimal *E. coli* clamp loader structure are arranged in a circular fashion (Jeruzalmi et al., 2001)(Figure 1.3B). The three  $\gamma$  subunits are adjacent to each other, flanked on one side by  $\delta$  and the other by  $\delta'$ , which in turn interact with each other to complete the ring and close it at one end. Each subunit consists of three domains (I-III), and oligomerization is mediated by the C-terminal domain III (Figure 1.3A, 1.3B). The N-terminal domains I and II of all five subunits adopt the chain fold of the AAA+ (ATPases associated with a variety of cellular activities) family (Figure 1.3A) (reviewed in (Davey et al., 2002b; Neuwald et al., 1999)). Although only the  $\gamma$  subunits bind ATP (Tsuchihashi and Kornberg, 1989),  $\delta'$  has conserved many sequence elements of the AAA+ family (Dong et al., 1993). In contrast, the  $\delta$  sequence has diverged to a larger extent, making its AAA+ fold more surprising. The three  $\gamma$  subunits provide consensus ATP binding sequences to contribute to the ATP sites of  $\gamma$  complex.

The ATP sites of  $\gamma$  complex are located at subunit interfaces and are supplemented with residues from the adjacent subunit (see Figure 1.3D). The interfacial location of ATP sites is typical of AAA+ oligomers and is presumed to promote communication among the subunits.  $\delta'$  contributes a key arginine to “ATP Site D” of  $\gamma$  complex (containing the P-loop of  $\gamma^D$ ), as seen in the left panel of Figure 1.3C (green residue) with an ATP molecule modeled along the phosphate-binding loop (yellow). This arginine is located in a conserved serine-arginine-cysteine (SRC) motif that is present in all known clamp loaders.

**Figure 1.3 Crystal structure of the *E. coli*  $\gamma_3\delta_1\delta'_1$  complex.** *Panel A* Isolated subunits from the structure of the complex, domains I--III noted (adapted from (Davey et al., 2002), copyright 2002 Nature Publishing Group (<http://www.nature.com>)). *Panel B* Side view of  $\gamma_3\delta\delta'$  (*left*). The  $\beta$ -interacting helix of  $\delta$  is marked in yellow. View from the C termini of  $\gamma_3\delta\delta'$  (*right*) (adapted from (Jeruzalmi et al., 2001), copyright 2001, with permission from Elsevier). *Panel C* The ATP sites of the ring-shaped  $\gamma$  complex are located at subunit interfaces, as illustrated in the cartoon (*right*) and taken from the structure of ATP Site D (*left*) with ATP modeled against the P-loop (*yellow*) of  $\gamma^D$ . The Ser-Arg-Cys (SRC) motif of  $\delta'^E$ , conserved in all clamp loaders, points its arginine (*green*) toward the  $\gamma$ -phosphate of ATP.

Figure 1.3 Crystal structure of the E. coli  $\gamma_3\delta_1\delta'_1$  complex.



Mutation of the  $\delta'$  Arg and the homologous residue in  $\gamma$ , causes a severe defect in clamp loading and ATPase activity and also disrupts interaction with DNA ( $\gamma$  mutants) or  $\beta$  ( $\delta'$  mutants) (Chapter 2; (Johnson and O'Donnell, 2003; Snyder et al., 2004)). Mutation of the P-loop of  $\gamma$  has more severe consequences that result in complete loss of activity (Xiao et al., 1995). These observations highlight the coordination of the ATP cycle with the clamp loading mechanism, suggesting regulation of substrate binding by conformational changes in distinct ATP sites during nucleotide binding and hydrolysis.

The C-termini (Domains III) of the five subunits of the minimal  $\gamma$  complex form a tight ring (Song and McHenry, 2001), but the N-terminal AAA+ domains (I and II) are more loosely associated, with a total lack of contact between  $\delta$  and  $\delta'$  in these domains, creating a gap in the N-terminal portion of the ring (Jeruzalmi et al., 2001) (Figure 1.3B). Both ATP and  $\beta$  clamp binding occur in these N-terminal areas of the complex (Jeruzalmi et al., 2001b; Podobnik et al., 2003). The loose connections of these domains has led to the suggestion that some conformational freedom of the N-termini is essential for function (Goedken et al., 2004). The lack of an interaction between the  $\delta$  and  $\delta'$  N-termini in the crystal structure underscores the potential modulation of the  $\beta$ -interacting element of  $\delta$ , located in Domain I. This modulation is characterized biochemically by the ability of free  $\delta$  and  $\delta'$  to interact, independently of ATP (Onrust and O'Donnell, 1993; Turner et al., 1999). However, the crystal structure of the  $\gamma\delta\delta'$  complex lacks ATP and thus is in the inactive state. Consistent with this, a  $\beta$  dimer cannot be docked onto the  $\gamma$  complex (replacing  $\delta$  with  $\delta$ - $\beta$ ) without significant clashes, even with the substantial gap between  $\delta$  and  $\delta'$  (Jeruzalmi et al., 2001).

Biophysical experiments in solution indicate that the distance between  $\delta$  and  $\delta'$  does not change dramatically during clamp association (Goedken et al., 2004). It is possible that the gap in the  $\gamma$  complex structure allows the N-termini of all five subunits the conformational freedom required to properly dock the clamp loader onto the  $\beta$  dimer. The  $\gamma$  subunit also binds  $\beta$  (Leu and O'Donnell, 2001), as do analogous RFC subunits to PCNA (Mossi et al., 1997; Yao et al., 2003; Yao et al., 2006), implying that after initial recruitment of  $\beta$  by  $\delta$ , a more extensive surface of interaction is formed between clamp and clamp loader.

Using pre-steady state kinetics and non-hydrolyzable analogs of ATP, it has been shown that  $\gamma$  complex binds preferentially to primer/template DNA that offers a recessed 3' terminus at a double-strand/single-strand junction (Ason et al., 2000; Ason et al., 2003; Williams et al., 2004). Hydrolysis of ATP upon DNA binding leaves the clamp loader in a low-affinity DNA binding state, presumably until ADP dissociates and  $\gamma$  complex can be recharged with triphosphate nucleotides. Structural evidence of clamp loader DNA recognition is provided by the recent structure of RFC which displays a helical conformation similar to double-strand DNA (Bowman et al., 2004), which has been confirmed by mutagenesis in RFC (Yao et al., 2006) and *E. coli* (Goedken et al., 2005). This relationship will be discussed in greater detail in the eukaryotic replicase section.

### **The Holoenzyme Particle**

Based on intracellular Pol III subunit concentrations, a portion of clamp loader is associated with the Pol III holoenzyme, but a majority of the clamp loader is free in solution (Leu et al., 2000; Wu et al., 1984). The clamp loader

associated with the holoenzyme contains a different form of the *dnaX* gene product. The  $\gamma$  subunit is produced through a ribosomal frameshift in the *dnaX* gene which causes almost immediate termination of translation to produce a 47.5 kDa protein (Flower and McHenry, 1986; Tsuchihashi and Kornberg, 1990). The full-length product of *dnaX* is the  $\tau$  subunit (71.1 kDa) which contains the  $\gamma$  sequence plus a unique 23.6 kDa C-terminal region ( $\tau_c$ ) (Figure 1.4A). The 23.6 kDa  $\tau_c$  is comprised of two domains, IV and V, that bind DnaB and Pol III core (through  $\alpha$ ), respectively (Gao and McHenry, 2001a; Gao and McHenry, 2001b). The  $\tau_c$  region is not required for clamp loading, but is essential for cell viability (Blinkova et al., 1993), probably due to its ability to organize the replisome as discussed below.

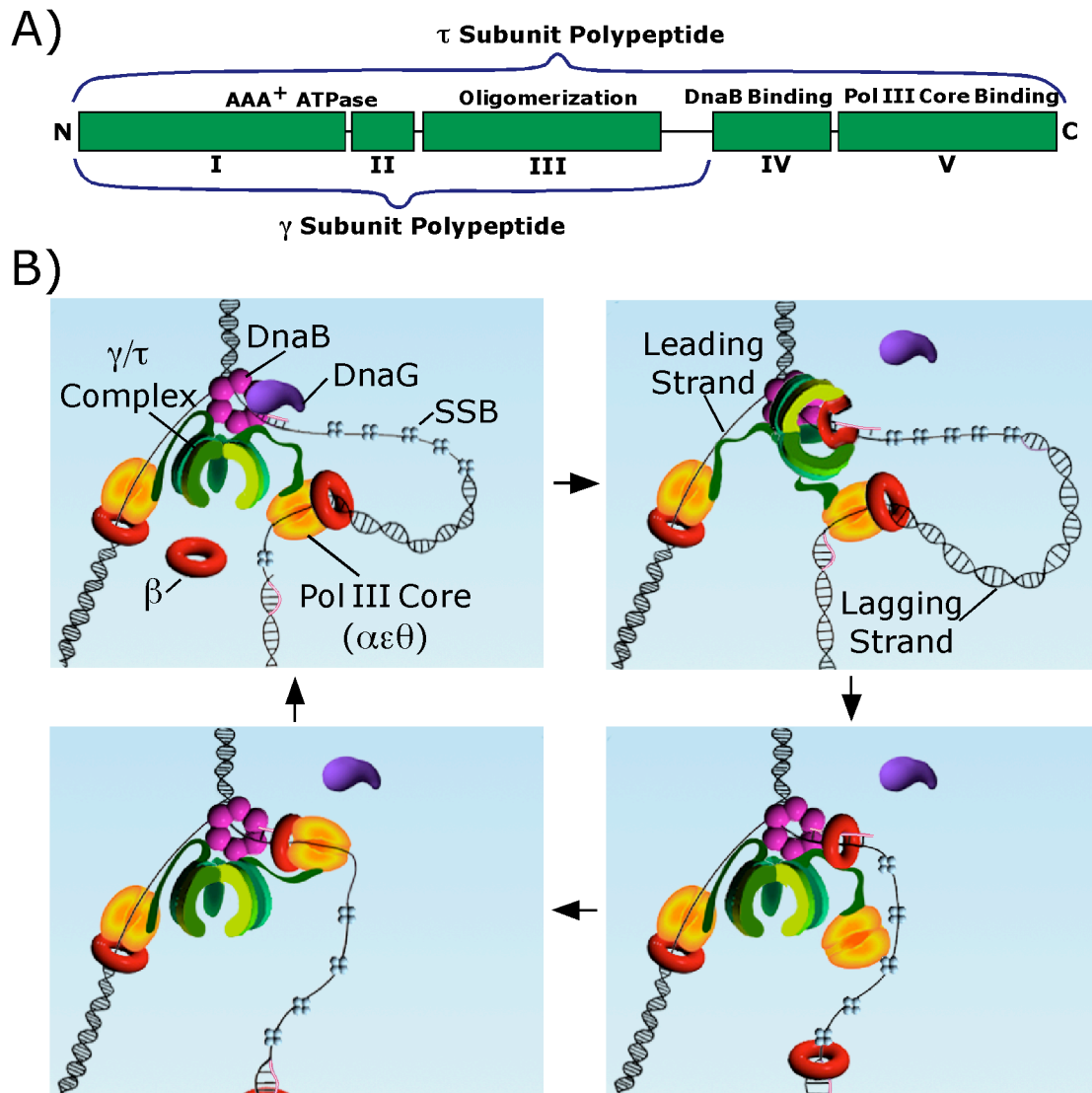
The DnaX protein is present in three copies in the clamp loader complex, and thus various species may assemble in stoichiometries of  $\gamma_3$ ,  $\gamma_2\tau_1$ ,  $\gamma_1\tau_2$ , and  $\tau_3$  (Pritchard et al., 2000). Each  $\tau$  C-terminus will recruit one polymerase and therefore the more  $\tau$  present in the clamp loader, the more core polymerase molecules it may bind. It is thought that holoenzyme contains two Pol III cores attached to a  $\tau_2\gamma_1\delta\delta'\chi\psi$  clamp loader and that a specific order of assembly leads to this particle (Glover and McHenry, 2000; Onrust et al., 1995; Pritchard et al., 2000). At least two polymerases are required for concurrent synthesis of leading and lagging strands (Figure 1.4B).

The single copy subunits of the clamp loader define an inherent asymmetry, and thus by definition the two cores attached to the two  $\tau$  subunits are in somewhat different environments (McHenry, 2003). The consequence of this asymmetric structure might be minimal, because a proline-rich segment of  $\tau$

**Figure 1.4 Organization and dynamics of the *E. coli* replisome.** *Panel A* The  $\tau$  subunit of DNA polymerase III holoenzyme is comprised of the clamp-loader domains I--III ( $\gamma$  sequence) and the replisome organization domains IV and V that bind the DnaB helicase and Pol III core, respectively. *Panel B* Polymerase cycling at the replication fork. As the replisome advances, the clamp loader loads a  $\beta$  clamp on an RNA primer (*pink*) synthesized by DnaG (*upper right*). When the lagging-strand polymerase replicates to a nick, it dissociates from DNA and  $\beta$  (*lower right*) and cycles to the newly loaded  $\beta$  clamp (*lower left*).



Figure 1.4 Organization and dynamics of the *E. coli* replisome.



separates the clamp loader and polymerase-interacting domains, suggesting a flexible connection. However, DnaB or other holoenzyme subunits may hold the cores in defined asymmetric positions (Yuzhakov et al., 1996). It has been proposed that the asymmetric structure imposes distinct properties onto the two polymerases, modeling their behavior to fit the different needs of replicating the leading and lagging strands (Dallmann et al., 1995; Glover and McHenry, 2000; Glover and McHenry, 2001; Johanson and McHenry, 1984; McHenry, 1988; McHenry, 2003; McHenry and Johanson, 1984; Wu et al., 1992a).

The  $\chi$  and  $\psi$  subunits are accessory to the clamp loading mechanism (Xiao et al., 1993).  $\chi$  is responsible for the association of  $\gamma$  complex with SSB (Glover and McHenry, 1998; Kelman et al., 1998). This contact is relevant in physiological salt concentrations where  $\gamma$  complex cannot load  $\beta$  onto DNA as efficiently. The  $\chi$ -SSB connection helps recruit the clamp loader to the DNA.

### **Replisome Dynamics**

Many bacteria have doubling times under one hour and must replicate their genome in this time, initiating from a single origin. To accomplish this goal, the replication fork moves at an average speed of 1000 nt/sec, synthesizing a continuous leading strand and many Okazaki fragments (Kornberg and Baker, 1992). To maintain this speed, the replicative polymerase (Pol III holoenzyme), helicase (DnaB), and primase (DnaG) that make up the replisome, along with SSB, act in a coordinated manner. The  $\tau$  subunit contacts to Pol III core and DnaB promote the fast progression of the replication fork (Dallmann et al., 2000; Gao and McHenry, 2001a; Gao and McHenry, 2001b; Kim et al., 1996a; Kim et al.,

1996b; Yuzhakov et al., 1996). In the absence of  $\tau$ , a processive Pol III core: $\beta$  complex replicates behind DnaB at a speed of  $\sim 35$  nt/sec (Kim et al., 1996a). In the presence of  $\tau$ , or just  $\tau_c$ , this polymerase complex is stimulated to near-holoenzyme speed (Dallmann et al., 2000; Kim et al., 1996b). The  $\tau_c$  results indicate that a 1:1:1 DnaB: $\tau_c$ :core is minimally required for this stimulation (Dallmann et al., 2000).

As the replisome advances, the polymerase on the leading strand simply extends DNA in a continuous fashion. Presumed impediments to leading strand extension include sites of DNA damage and the consequent collapse of the fork (see below). Repair and restart of synthesis is an elaborate process detailed in recent reviews (Kowalczykowski, 2000; Marians, 2004). Pol III holoenzyme has been implicated in mediating the restart of DNA replication after fork stalling (Grompone et al., 2002).

Lagging strand replication is a discontinuous process of fits and starts that repeats in a cycle time of 1-3 s. An overview of the lagging strand cycle is illustrated in Figure 1.4B. Each Okazaki fragment is initiated by primase which synthesizes an RNA primer of about 10-12 nucleotides (Zechner et al., 1992a; Zechner et al., 1992b). Primase action requires interaction with DnaB which involves a C-terminal region of primase (Tougu and Marians, 1996a; Tougu and Marians, 1996b). Primase extends the RNA in the opposite direction of helicase unwinding and is presumed to separate from DnaB, which may account for its observed distributive action (Wu et al., 1992c). Primase remains attached to the RNA primed site through its interaction with SSB (Stayton and Kornberg, 1983; Sun and Godson, 1996; Sun and Godson, 1998). Although primase eventually

dissociates, release of primase is accelerated allosterically by the  $\chi$  subunit of the clamp loader which binds SSB in a competitive fashion, recruiting the clamp loader to the DNA template to compete with primase (Yuzhakov et al., 1999b). The clamp loader then places  $\beta$  onto the primer for the lagging strand polymerase.

As the lagging polymerase extends a fragment, a loop is generated due to the fact that it is connected to the leading polymerase (via the clamp loader), yet extends DNA in the opposite direction (see top left of diagram), as originally proposed (Sinha et al., 1980) and recently confirmed (Chastain et al., 2003) in the T4 system. The 1-3 kb Okazaki fragment will be completed within a few seconds (Figure 1.4B, top right diagram) and at this point the core must rapidly release from DNA to start the next fragment (bottom right diagram). The highly processive Pol III requires a specific mechanism for this release step, which disengages core from  $\beta$ , leaving the  $\beta$  clamp behind on the finished fragment. The release step occurs only at a nick, thus ensuring completion of the fragment, and requires the  $\tau$  subunit (Leu et al., 2003; Li and Marians, 2000; O'Donnell, 1987; Stukenberg et al., 1994). The lagging strand core is now free to bind a new  $\beta$  clamp placed on the next RNA primer by the clamp loader (bottom, left).

Replication fork progression has been studied in *E. coli* using rolling circle DNA templates (Higuchi et al., 2003; McNerney and O'Donnell, 2004; Wu et al., 1992b; Yuzhakov et al., 1996). These circular DNAs provide a tail that the DnaB helicase and the Pol III holoenzyme can load onto, generating a leading strand product that subsequently acts as the template for the lagging strand. This technique has been employed for multi-kilobase single-strand circular phage

genome templates (Wu et al., 1992b) and also artificial minicircle templates where base composition allows specific labeling of leading and lagging strand products (McInerney and O'Donnell, 2004). Rolling circle studies in *E. coli* have confirmed that a single holoenzyme particle can replicate both leading and lagging strands efficiently (Wu et al., 1992a) and discovered the functional uncoupling of the two holoenzyme polymerases in response to a lagging strand replication block allowing unabated fork progression (Higuchi et al., 2003; McInerney and O'Donnell, 2004).

### ***Protein Trafficking on DNA Sliding Clamps***

Many different proteins function with sliding clamps. An understanding of the way proteins coordinate their traffic flow on clamps is now emerging. To illustrate how proteins may switch positions to utilize the clamp, we briefly describe three important switches on  $\beta$  that occur during the progression of the *E. coli* replication fork.

The  $\gamma/\tau$  clamp loader and Pol III core are known to bind the same face of  $\beta$  in essentially the same spot and therefore these two factors compete for the clamp (Lopez de Saro et al., 2003b; Naktinis et al., 1996). Yet both the core and  $\gamma/\tau$  complex must function with  $\beta$  at the start of each DNA chain. This protein trafficking event is facilitated by the fact that ATP hydrolysis ejects the clamp loader from  $\beta$  (Figure 1.5A) (Hingorani and O'Donnell, 1998). In addition, the clamp loader remains in a reduced activity state for a short interval, presumably due to slow ADP release (Ason et al., 2003; Bertram et al., 2000). The core

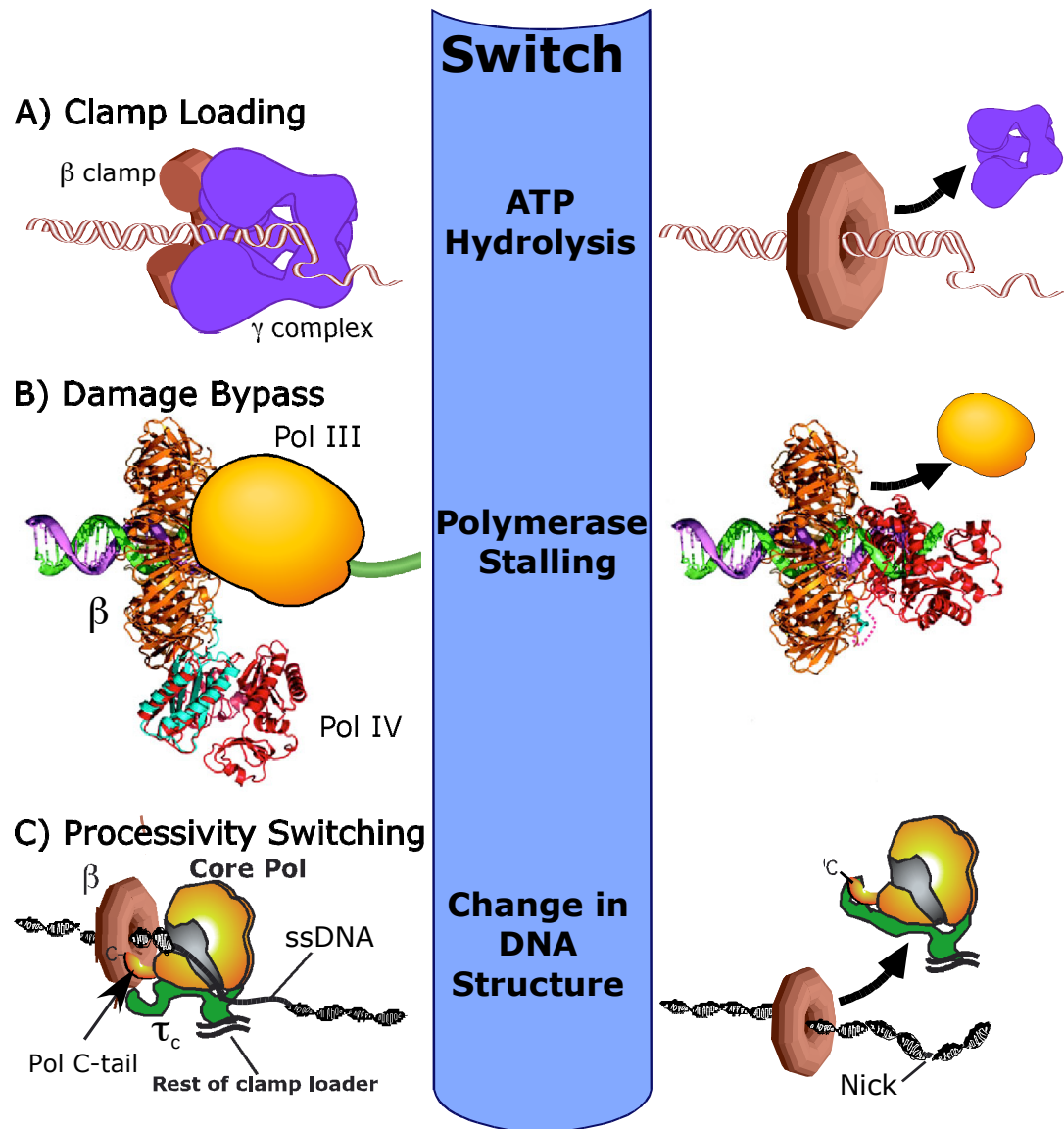
**Figure 1.5 Three examples of protein trafficking on sliding clamps.**

**Interactions with the  $\beta$  clamp from *E. coli*.** *Panel A* The  $\gamma$  complex clamp loader associates tightly with  $\beta$  when bound to ATP. DNA triggers ATP hydrolysis,

resulting in low affinity for  $\beta$  and DNA. *Panel B* When Pol III, the replicative polymerase, encounters a lesion in the DNA template, it stalls, unable to overcome its inherent fidelity to incorporate opposite a damaged base. Stalling allows an error-prone polymerase, such as Pol IV (*red*) passively traveling on  $\beta$ , an opportunity to trade places with Pol III on  $\beta$  to replicate past the lesion.

(adapted with permission from (Bunting et al., 2003)). *Panel C* Pol III maintains a tight grip on  $\beta$  via the polymerase C terminus. However, when it completely replicates its substrate DNA, the polymerase must release from  $\beta$  to recycle to the next primed site. The  $\tau$  subunit modulates this interaction, binding the polymerase C tail only when no more single-stranded template is present. This severs the connection between the polymerase and the clamp (adapted with permission from (Lopez de Saro et al., 2003), copyright 2003, National Academy of Sciences, U.S.A.).

**Figure 1.5 Three examples of protein trafficking on sliding clamps.**  
Interactions with the  $\beta$  clamp from *E. coli*.



polymerase is then free to bind the abandoned  $\beta$  clamp and in fact binds tighter on DNA than off (Naktinis et al., 1996).

Switching on  $\beta$  is also thought to occur when the leading polymerase stalls. In this case it is thought that a bypass polymerase, either Pol IV or Pol V, takes possession of the clamp to extend DNA through the stall site. Recent structural analysis suggests that Pol IV can associate in two ways with  $\beta$  (Bunting et al., 2003; Burnouf et al., 2004). Pol IV binds the edge of the  $\beta$  ring and one hydrophobic pocket, but is angled off the DNA (Bunting et al., 2003). Recently this relationship has been studied biochemically in detail. It was concluded that Pol IV can take control of  $\beta$  when Pol III stalls, but not when Pol III is actively incorporating nucleotides (Indiani et al., 2005) (see Figure 1.5B). Upon stalling of Pol III, Pol IV must break its interaction with the side of the  $\beta$  ring and swing down to the DNA, presumably maintaining its hold on the hydrophobic pocket of  $\beta$ . This action displaces Pol III from DNA, and perhaps disrupts the Pol III- $\beta$  contact, as well. Pol IV is distributive, even with  $\beta$ , allowing Pol III to regain the clamp after the lesion is bypassed. Recent evidence with Pol III, Pol V, and  $\beta$  suggests that a similar trade-off may occur (Duzen et al., 2004).

At the end of an Okazaki fragment the normally highly processive Pol III rapidly dissociates from  $\beta$  (Leu et al., 2003; Li and Marians, 2000; O'Donnell, 1987; Stukenberg et al., 1994), freeing the polymerase to extend the next Okazaki fragment. This  $\beta$  trafficking event is mediated by the  $\tau_c$  portion of  $\tau$ . The  $\alpha$  subunit binds  $\beta$  via the extreme  $\alpha$  C-terminal residues (Lopez de Saro et al., 2003b).  $\tau_c$  also binds the  $\alpha$  C-terminus and disrupts core- $\beta$  interaction. However,  $\tau_c$  binds ssDNA and this prevents  $\tau$  from binding the C-terminal residues of  $\alpha$ .



Hence, so long as there is ssDNA template,  $\tau_c$  is turned “off” and core functions with  $\beta$ . But when all available ssDNA is converted to duplex,  $\tau_c$  turns “on” and separates core from  $\beta$  (see Figure 1.5C).

These switch processes explain how interactions with the clamp are modulated by ATP or DNA structure to promote the trafficking of different proteins on sliding clamps to ensure progression of replication.

## THE EUKARYOTIC REPLICASE

Although the complexity of architecture, interaction, and regulation of DNA replication in eukaryotes is far greater than in bacteria, the core replicase components are structurally and functionally more similar than different. However, beyond this basic machinery lies a much larger network of proteins required for propagation of the replication fork and regulation of its advance as well as coordinating its activity with other DNA metabolic machineries (see Table II). Many of these details are only now coming into focus.

### Proliferating Cell Nuclear Antigen (PCNA)

PCNA derives its name from the early finding that the protein is abundant in proliferating cells (Miyachi et al., 1978). PCNA was shown to be directly involved in DNA replication through its ability to stimulate DNA Polymerase  $\delta$  in replicating long stretches of primed DNA (Prelich et al., 1987a; Prelich et al., 1987b; Tan et al., 1986). This characteristic suggested it may be analogous to the *E. coli*  $\beta$  subunit, although neither were known to be sliding clamps at the time. Subsequent work showed the importance of PCNA for SV40 DNA replication

**Table II. Eukaryotic replisome components.**

	<i>S. cerevisiae</i> (kDa)	Human (kDa)	Function and Remarks ( <i>S.p.</i> :pombe Name)
<b>RFC</b>	<b><i>RFC</i>(277.7)<sup>a</sup></b>	<b><i>RFC</i>(314.9)</b>	<b><i>Pentameric Clamp Loader</i></b>
	RFC-A(94.9)	p140(128.2)	Aka RFC1; Binds ATP; Phosphorylated
	RFC-B(36.1)	p40(39.7)	Aka RFC4; Binds ATP
	RFC-C(38.2)	p36(40.6)	Aka RFC3; Binds ATP
	RFC-D(39.7)	p37(39.2)	Aka RFC2; Binds ATP
	RFC-E(39.9)	p38(38.5)	Aka RFC5; Binds ATP or ADP
<b>PCNA</b>	<b><i>PCNA</i>(28.9)</b>	<b><i>PCNA</i>(28.7)</b>	<b><i>~87 kDa Homotrimeric Processivity Sliding Clamp</i></b>
<b>Pol <math>\delta</math></b>	<b><i>Pol <math>\delta</math></i>(220.2)</b>	<b><i>Pol <math>\delta</math></i>(238.7)</b>	<b><i>Replicative DNA Polymerase</i></b>
	Pol3(124.6)	p125(123.6)	DNA polymerase, 3'-5' exonuclease, Binds PCNA; Subunit A ( <i>S. p.</i> Pol3)
	Pol31(55.3)	p50(51.3)	Structural Subunit; Subunit B ( <i>S. p.</i> Cdc1)
	Pol32(40.3)	p66(51.4)	Binds PCNA; Subunit C( <i>S. p.</i> Cdc27); Binds Pol $\alpha$ large subunit
	-----	p12(12.4)	Structural, Stimulates processivity; Subunit D ( <i>S. p.</i> Cdm1)
<b>Pol <math>\epsilon</math></b>	<b><i>Pol <math>\epsilon</math></i>(378.7)</b>	<b><i>Pol <math>\epsilon</math></i>(350.3)</b>	<b><i>Replicative DNA Polymerase</i></b>
	Pol2(255.7)	p261(261.5)	DNA polymerase, 3'-5' exonuclease( <i>S.p.</i> Pol2 / cdc20)
	Dpb2(78.3)	p59(59.5)	Binds polymerase subunit( <i>S.p.</i> Dpb2)
	Dpb3(22.7)	p17(17.0)	Binds Dpb4
	Dpb4(22.0)	p12(12.3)	Present in ISW2/ $\gamma$ CHRAC chromatin-remodeling complex ( <i>S.p.</i> Dpb4)
<b>Pol <math>\alpha</math></b>	<b><i>Pol <math>\alpha</math></i>(355.6)</b>	<b><i>Pol <math>\alpha</math></i>(340.6)</b>	<b><i>DNA Polymerase/Primase</i></b>
	Pol1(166.8)	p180(165.9)	DNA Polymerase
	Pol12(78.8)	p68(66.0)	Structural Subunit
	Pri2(62.3)	p55(58.8)	Interacts tightly w / p48
	Pri1(47.7)	p48(49.9)	RNA primase catalytic subunit
<b>MCM</b>	<b><i>MCM</i>(605.6)</b>	<b><i>MCM</i>(535)</b>	<b><i>Putative 3'-5' Replicative Helicase</i></b>
	Mcm2(98.8)	Mcm2(91.5)	Phosphorylated by DDK
	Mcm3(107.5)	Mcm3(91.0)	Ubiquitinated, Acetylated
	Mcm4(105.0)	Mcm4(96.6)	Helicase w / MCM6,7; Phosphorylated by CDK; Aka Cdc54
	Mcm5(86.4)	Mcm5(82.3)	Aka Cdc46; Bob1 is a mutant form
	Mcm6(113.0)	Mcm6(92.3)	Helicase w / MCM4,7
	Mcm7(94.9)	Mcm7(81.3)	Helicase w / MCM4,6; Ubiquitinated
<b>RPA</b>	<b><i>RPA</i>(114)</b>	<b><i>RPA</i>(100.5)</b>	<b><i>Single-Strand DNA Binding Protein</i></b>
	RPA70(70.3)	RPA70(70.3)	Binds DNA, Stimulates Pol $\alpha$
	RPA30(29.9)	RPA30(29)	Binds RPA70 and 14, Phosphorylated
	RPA14(13.8)	RPA14(13.5)	Binds RPA30

<sup>a</sup> Bold and italic indicates information regarding a protein complex.

and its enhanced activity in the presence of RFC and ATP (Tsurimoto and Stillman, 1990).

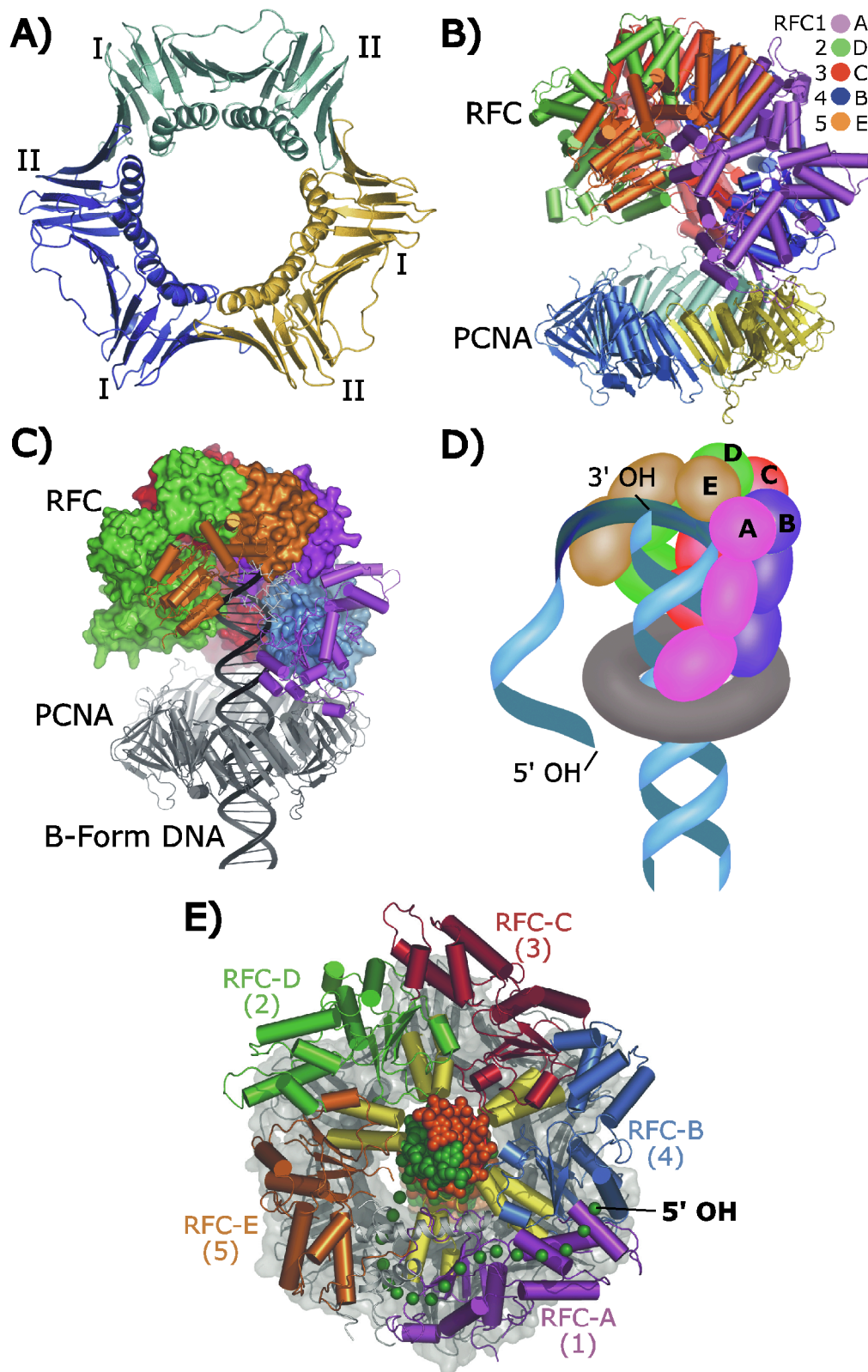
As a monomeric unit, PCNA is about 2/3 the size of  $\beta$ , and accordingly, each protomer contains two structurally similar domains instead of the three domains found in  $\beta$  (Krishna et al., 1994). The chain folds of the two PCNA domains are the same as found in the domains of  $\beta$ . PCNA and  $\beta$  form very similar ring-shaped structures except PCNA must trimerize to form a six-domain ring (Figure 1.6A). Like  $\beta$ , the PCNA ring is quite stably attached to DNA ( $t_{1/2} = 24$  min) (Yao et al., 1996). The PCNA protomers are also arranged head-to-tail to create two distinct “faces” of the ring, mirroring  $\beta$ . Interestingly, a recent report has suggested that PCNA, when open, may adopt a right-handed spiral helix (Kazmirski et al., 2005), similar to B-form DNA and the ATPase modules of RFC (see below).

As in the *E. coli* system, the eukaryotic clamp loader and polymerase compete for binding the same face of the PCNA ring, the face from which the C-termini project (Mossi et al., 1997; Oku et al., 1998). A variety of proteins involved in DNA repair and cell cycle control interact with PCNA, but this topic is beyond the scope of this article (reviewed in (Maga and Hubscher, 2003; Warbrick, 2000)). Relevant to the current review, a weak PCNA binding consensus sequence has emerged: Q-x-x-h-x-x-a-a; where x=any residue; h=L,I,M; a=F,Y (Maga and Hubscher, 2003; Warbrick, 2000). The crystal structure of human PCNA in complex with a peptide derived from the cell-cycle regulator p21<sup>WAF1/Cip1</sup> was the first to demonstrate that these clamp-binding proteins interact with PCNA at a hydrophobic pocket located between the two

**Figure 1.6 Structures of the eukaryotic clamp and clamp loader from**

*Saccharomyces cerevisiae*. *Panel A* View of the C-terminal face of PCNA. The ring-shaped PCNA is a head-to-tail trimer of a two-domain monomer. The six-fold pseudosymmetry of the  $\beta$  clamp is evident in PCNA as well. *Panel B* The structure of replication factor C (RFC) bound to PCNA reveals the structural similarity between RFC and  $\gamma$  complex. RFC binds to the C-terminal face of PCNA. (*Panels C-E*) The RFC subunits are arranged in a helix that tracks the minor groove of B-form DNA modeled through the PCNA ring. *Panel D* In this cartoon, the 5' terminus of a recessed primer template is positioned to exit the central channel of the clamp and clamp loader through the gap between RFC-A and RFC-E. *Panel E* N-terminal regions of the five RFC subunits and the PCNA ring from the RFC-PCNA structure. Two conserved helices in each RFC subunit (*yellow*) are in position to interact with DNA (*orange/green*) that passes through the central channel of PCNA (*gray*) with the 5' terminus (*green spheres*) exiting between RFC1 and RFC5. (adapted with permission from (Bowman et al., 2004), copyright 2004 Nature Publishing Group, <http://www.nature.com>)).

**Figure 1.6 Structures of the eukaryotic clamp and clamp loader from *Saccharomyces cerevisiae*.**



domains of a protomer (Gulbis et al., 1996). The finding generalizes to the position at which DNA polymerase binds the T4 gp45 clamp (Franklin et al., 2001; Hingorani and O'Donnell, 2000; Shamoo and Steitz, 1999). Also, the  $\alpha$  subunit of Pol III core and  $\delta$  subunit of  $\gamma$  complex bind *E. coli*  $\beta$  at a spot between domains II and III (Dalrymple et al., 2001; Jeruzalmi et al., 2001b; Lopez de Saro et al., 2003a).

### ***The Replication Factor C (RFC) Clamp Loader***

The eukaryotic clamp loader was first isolated based on its requirement in the *in vitro* Simian Virus 40 genome replication system (Fairman et al., 1988)(reviewed in (Waga and Stillman, 1994)). The activity was originally named replication factor-C (RFC) (Fairman et al., 1988) or activator-1 (Lee et al., 1989). RFC is a DNA-dependent ATPase that functions with PCNA to confer processivity on DNA polymerase  $\delta$  (Tsurimoto and Stillman, 1990)(reviewed in (Majka and Burgers, 2004; Mossi and Hubscher, 1998)). The similarity between RFC and *E. coli*  $\gamma$  complex was apparent early on. RFC is comprised of five different proteins that are homologous to each other and to  $\gamma$  and  $\delta'$  of *E. coli*  $\gamma$  complex (Cullmann et al., 1995; O'Donnell et al., 1993). Common sequence motifs among these clamp loader AAA+ proteins have been termed RFC boxes.

In overview, RFC acts similarly to  $\gamma$  complex. In an ATP-dependent reaction RFC loads PCNA onto a recessed 3' primer/template junction and then dissociates allowing PCNA to function with Pol  $\delta$  (Podust et al., 1998b; Tsurimoto and Stillman, 1991a). The RFC subunits in *S. cerevisiae* were numbered RFC1-5 as they were cloned (Cullmann et al., 1995) (see Table III for

human RFC nomenclature). More recently, structural information has led to the adoption of an alphabetical nomenclature (RFC-A, -B, -C, -D, -E), to facilitate comparison to the analogous positions in  $\gamma$  complex (Figure 1.6B,D,E). RFC-B, -C, -D, and -E share a similar molecular weight and three-domain architecture characteristic of *E. coli*  $\gamma$  subunit (Bowman et al., 2004; Uhlmann et al., 1997b). RFC-A also contains these three domains, along with sizable N- and C-terminal extensions (Bunz et al., 1993). The N-terminal region (residues 1-283 in *S. cerevisiae*) has clear homology to DNA ligases, although there is no evidence of ligase activity. The removal of the RFC-A N-terminus results in sensitivity to DNA-damaging agents (Gomes et al., 2000), but this region is not necessary for *in vitro* clamp loading (Uhlmann et al., 1997a) or cell viability (Gomes et al., 2000). The C-terminal domain (residues 660-861 in *S. c.*) has not yet been characterized genetically or biochemically.

The five-subunit composition of RFC bears a close similarity to the minimal *E. coli*  $\gamma_3\delta\delta'$  complex (Bowman et al., 2004). Based on subunit interactions and sequence similarity to  $\gamma$  complex subunits, the subunit arrangement of the RFC pentamer was proposed (O'Donnell et al., 2001; Yao et al., 2003) and has since been proven by structure analysis (Bowman et al., 2004). RFC-A shares characteristics of the  $\delta$  subunit in its conserved clamp-interacting residues and position in the pentamer (Fotedar et al., 1996; Uhlmann et al., 1997a). RFC-B/C/D are considered  $\gamma$ -like in their ability to form a trimeric ATPase subassembly (Cai et al., 1997). RFC-E is in the position of  $\delta'$ , and like  $\delta'$  it contains an SRC motif but lacks a consensus P-loop. Like the  $\gamma_3\delta\delta'$  complex, the ATP sites of RFC are located at subunit interfaces. However, unlike  $\gamma_3\delta\delta'$ , RFC

contains four competent ATPase sites because RFC-A also binds ATP where  $\delta$  does not. In fact, RFC-E also binds a nucleotide in the crystal structure.

During the RFC ATPase cycle in *S. cerevisiae* the complex initially binds 2 ATP, then a third upon PCNA binding, and a fourth when it locates the primer/template DNA, which triggers ATP hydrolysis causing RFC to eject and leaving the closed PCNA ring on DNA (Gomes et al., 2001). This mechanism excludes the route of RFC first encountering DNA and subsequently recruiting PCNA. ATP site mutational studies have demonstrated that, in general, disrupting any one of the four consensus ATP sites has a significant effect on the activity of RFC (Cai et al., 1998; Podust et al., 1998a; Schmidt et al., 2001), though ATP binding may suffice to rescue this defect in at least one ATP site (in RFC-B for *S. cerevisiae*). These single P-loop mutants still bind PCNA readily, but are deficient in DNA binding (Schmidt et al., 2001).

The RFC-ATP $\gamma$ S-PCNA crystal structure (Bowman et al., 2004) has provided a detailed view of how a clamp loader interacts with its cognate clamp (see Figure 1.6B). In addition, the structure has revealed how DNA binds to a clamp loader. The main PCNA contacts occur through RFC-A and RFC-C which bind to the hydrophobic pocket between domains of two different PCNA protomers. RFC-A interacts extensively with PCNA, whereas RFC-C seems to be only “partially engaged” with PCNA and the ring is closed. In the RFC-ATP $\gamma$ S-PCNA structure RFC-D and RFC-E do not bind PCNA at all. This sub-optimal interaction of RFC with PCNA may explain why the clamp remains closed and only slightly perturbed from its unbound structure. Alternatively, the closed PCNA may be due to crystal packing forces, instability of an open-ring complex,



or the arginine to glutamine mutation in the four SRC motifs. Although this mutation should ensure that no ATP $\gamma$ S becomes hydrolyzed, it may have prevented or perturbed some necessary RFC subunit-PCNA interaction needed for clamp opening.

The way RFC binds DNA is suggested by the helical arrangement of RFC subunits in the structure, with the helical axis passing through the central channel of the closed PCNA (see Figure 1.6C-E). The helix begins at RFC-A, the subunit that is fully engaged with the hydrophobic pocket on one PCNA protomer. Adjacent to RFC-A is RFC-B, displaced by the helical operator of 61° rotation and 5.5 Å translation. Overall, the helical operations that relate all five subunits have a pitch of ~5.6 Å per 60° rotation, ending with RFC-E which lies ~25 Å above PCNA and significantly separated from the ATPase domain of RFC-A. This right-handed helix and pitch mimics that of duplex B-form DNA. Furthermore, there is sufficient space in the center of RFC to model a DNA duplex which passes right through the center of PCNA. Each RFC subunit has two  $\alpha$  helices that are oriented so their positive dipole tracks the minor groove phosphate backbone of DNA modeled into the structure (Figure 1.6E). Several basic residues on these helices are conserved in *E. coli* and eukaryotic clamp loaders, consistent with the idea that DNA binds within this central area of RFC. Modeling a 3'-recessed primed-template from another crystal structure into the center of the ring shows that the specific recognition of a primer/template by RFC might occur by a simple clash of the primed-template junction with the Domain III cap of the RFC subunits. A stiff duplex DNA could not proceed through the RFC structure, because it would hit the cap and could not bend to

exit out the side of RFC. The flexible ssDNA of a primed junction may bend to exit through the gap between RFC-A and 5, perhaps assisted by a positive patch on the RFC-A surface (see Figure 1.6E). This hypothesis of specific primer/template recognition is in agreement with recent biophysical evidence of RFC/DNA association (Gomes and Burgers, 2001; Hingorani and Coman, 2002).

### **Eukaryotic DNA Polymerases**

It is now understood that many DNA polymerases function in the eukaryotic cell. These polymerases all share a common catalytic mechanism (reviewed in (Hubscher et al., 2002)), but most serve a specific function outside of basic genome duplication. DNA Polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$  are the established replicative polymerases that are thought to function at the replication fork to copy genomic DNA in a semi-discontinuous manner. Pol  $\alpha$ ,  $\delta$ , and  $\epsilon$  are all members of the B-family of DNA polymerases (Braithwaite and Ito, 1993). Structural studies of bacteriophage RB69 DNA polymerase, a homologue of the B-family, have revealed its atomic resolution architecture along with its interactions with DNA and its sliding clamp (Brautigam and Steitz, 1998; Shamoo and Steitz, 1999). This structural information can be taken as a guide to the basic structure of the eukaryotic replicative polymerases, and also suggests a mode of interaction with PCNA.

#### **DNA Polymerase $\alpha$**

DNA polymerase  $\alpha$  (Pol  $\alpha$ ) was initially thought to be the main replicase before Pol  $\delta$  was discovered. Pol  $\alpha$  is unique in its ability to initiate DNA synthesis by first synthesizing its own ~12 nucleotide RNA primer and then

extending it with about 20 bases of DNA (Conaway and Lehman, 1982a; Conaway and Lehman, 1982b)(reviewed in (Lehman and Kaguni, 1989)). Pol  $\alpha$  is now thought to be the eukaryotic primase, making a hybrid RNA/DNA primer, followed by a polymerase switch allowing the replicase to take over elongation (Tsurimoto and Stillman, 1991b). The switch is mediated by RFC which displaces Pol  $\alpha$  from the primer via competition for RPA (Maga and Hubscher, 1996; Yuzhakov et al., 1999a).

Pol  $\alpha$  consists of four subunits (Lehman and Kaguni, 1989). The DNA polymerase activity is found in the largest subunit (p180) and primase activity is located in the smallest subunit, p48. The exact functions of the middle two subunits are not clear, but all four subunits are present in Pol  $\alpha$  isolated from yeast, human, *Xenopus*, and *Drosophila*.

#### *DNA Polymerase $\delta$*

DNA polymerase  $\delta$  (Pol  $\delta$ ) in fission yeast, humans, and other eukaryotic organisms is composed of four essential subunits (Podust et al., 2002; Zuo et al., 2000). Interestingly, *S. cerevisiae* Pol  $\delta$  has only three subunits and no apparent homologue exists for the fourth subunit (Burgers and Gerik, 1998). Furthermore, the third subunit can be deleted from budding yeast, although cell growth is compromised (Gerik et al., 1998).

A unified subunit nomenclature for Pol  $\delta$  has recently been proposed (MacNeill et al., 2001). Based on the *S. pombe* Pol  $\delta$  subunits and their homologues in other eukaryotes the subunits have been renamed A-D for the Pol3(A), Cdc1(B), Cdc27(C), and Cdm1(D) polypeptides. Much debate has

centered on the possibility of the Pol  $\delta$  complex self-associating into a dimer. The most recent evidence in multiple systems indicates that Pol  $\delta$  complex contains only one copy of each subunit across a wide range of concentrations and has an elongated shape that resulted in the earlier confusion over whether it was a dimeric polymerase particle (Bermudez et al., 2002; Johansson et al., 2001). Pol  $\delta$  subcomplexes can also be isolated as a core A/B dimer (Burgers and Gerik, 1998; Lee et al., 1984). A zinc finger module in subunit A interacts with subunit B, which acts as a bridge to subunits C and D (when present) (Bermudez et al., 2002; MacNeill et al., 1996; Sanchez Garcia et al., 2004). The polymerase and 3'-5' exonuclease activities of Pol  $\delta$  are present in the large A subunit. The Pol  $\delta$  polymerase extends DNA with high fidelity, but the exonuclease seems surprisingly ineffective *in vitro* in *S. pombe* (Chen et al., 2000) and may be most important when Pol  $\delta$  is clamped onto DNA by PCNA (Hashimoto et al., 2003; Mozzherin et al., 1996).

Pol  $\delta$  associates with PCNA via interactions with at least two of its subunits. The clamp is positioned behind the polymerase (Mozzherin et al., 1999), preventing polymerase dissociation as it extends a primer (Einolf and Guengerich, 2000). The strongest interaction is between PCNA and subunit C (Reynolds et al., 2000). Subunit C contains the consensus PCNA-interaction motif and studies with yeast Pol  $\delta$  show that this PCNA-interacting subunit stimulates Pol  $\delta$  activity (Bermudez et al., 2002; Johansson et al., 2004). However, stimulation is not dependent upon the PCNA-interacting motif, but mainly on the domain involved in connection to the A/B complex. The A/B heterodimer alone can also associate with PCNA, presumably through subunit A (Burgers

and Gerik, 1998). The stimulation of subunit C on Pol  $\delta$  activity might be merely structural. Even the small D-subunit from the human four-subunit Pol  $\delta$  complex significantly stimulated the A/B/C subcomplex in assays with RFC and PCNA (Podust et al., 2002).

#### *DNA Polymerase $\epsilon$ (Pol $\epsilon$ )*

Studies showing Pol  $\epsilon$  is essential in yeast placed it at the replication fork (Morrison et al., 1990). Indeed, chromatin immunoprecipitation studies in yeast indicate that Pol  $\epsilon$  is located at origins prior to S phase (Feng et al., 2003) and moves away from origins upon releasing an S phase block (Aparicio et al., 1997), consistent with a role in chromosome replication. However, there are some conflicting genetic studies on whether the intrinsic DNA polymerase activity is required for replication or if the protein may instead serve another role, either as a DNA sensor or checkpoint protein (D'Urso and Nurse, 1997; Navas et al., 1996; Navas et al., 1995), or perhaps holding together other proteins that are essential in the replisomal particle (Kesti et al., 1999). Although further work will be required to fully understand the exact role of Pol  $\epsilon$ , it is widely believed to be directly involved in DNA synthesis at the replication fork.

A recent report on Pol  $\epsilon$  concludes that it is a heterotetramer with a stoichiometry of 1:1:1:1, presumably in all eukaryotes (Chilkova et al., 2003). The DNA polymerase and 3'-5' exonuclease of Pol  $\epsilon$  reside in the largest subunit, and appear to have a higher fidelity than Pol  $\delta$ /PCNA (Shimizu et al., 2002). The third-largest subunit, Dpb4, is also a member of a complex that appears to be involved in chromatin remodeling (Iida and Araki, 2004; Tackett et al., 2005). Pol

$\epsilon$  activity does not absolutely require PCNA, but PCNA stimulation of Pol  $\epsilon$  activity increases as the ionic strength is raised (Eissenberg et al., 1997; Lee et al., 1991; Maga and Hubscher, 1995). The PCNA-interaction motif on the large subunit of Pol  $\epsilon$  is not essential for cell viability, but mutational analysis of this sequence suggests a role in DNA repair (Dua et al., 2002).

### *The Eukaryotic Replisome*

The essential nature of both Pol  $\delta$  and Pol  $\epsilon$  and the fact that they both function with PCNA as monomeric polymerase particles have led to renewed proposals that they function together to replicate the leading and lagging strands. *Xenopus* extracts have shown that depletion of either polymerase results in a dramatic drop in replication (Fukui et al., 2004; Waga et al., 2001). Depletion of Pol  $\delta$  gave rise to many short nascent strands that may have been improperly elongated Okazaki fragments, whereas Pol  $\epsilon$  depletion merely slowed down the replication machinery. Viability of Pol  $\epsilon$ -deleted strains of yeast suggests that Pol  $\epsilon$  is not essential for cell viability (Kesti et al., 1999; Navas et al., 1995; Ohya et al., 2002). However, an inactive point mutant of Pol  $\epsilon$  results in cell death (Dua et al., 1999). One possible explanation for this apparent contradiction is that another polymerase, presumably Pol  $\delta$ , can carry out DNA replication in the complete absence of Pol  $\epsilon$ , but that the Pol  $\epsilon$  point mutant acts as a dominant negative to stop the fork.

Intensive studies of *in vitro* replication from the SV40 origin has provided great insight into eukaryotic fork function, but the SV40 T-antigen fills many roles ordinarily performed by host proteins, including helicase function

(Fanning, 1992). Study of polymerases in this system show that Pol  $\delta$  and Pol  $\alpha$ , and even Pol  $\alpha$  alone under some conditions, are sufficient for replication. However, the small size of the genome and use of T-antigen may minimize the requirements for replication (reviewed in (Waga and Stillman, 1994)). Reconstitution of a eukaryotic replisome on a rolling circle template may greatly facilitate our understanding of the roles of Pol  $\epsilon$  and Pol  $\delta$  and which strand(s) they operate on.

The ten-fold smaller size of eukaryotic lagging strand fragments (~200 bp) is counterbalanced by the ten-fold slower rate of fork movement. Stoichiometric use of PCNA clamps during lagging strand replication has not been demonstrated but is presumed to occur as it does with the *E. coli*  $\beta$  clamp. That PCNA clamps are left behind on lagging strand fragments is implied by the fact that PCNA interacts with, and in some cases stimulates, the factors necessary for Okazaki fragment maturation (Ayyagari et al., 2003; Jin et al., 2003; Kao and Bambara, 2003). There is also an interesting observation that PCNA-DNA complexes may persist through mitosis, marking chromosomes for epigenetic inheritance (Shibahara and Stillman, 1999; Zhang et al., 2000b).

The eukaryotic replisome factors that contain helicase, primase, and SSB are each comprised of multi-protein assemblies in eukaryotes (reviewed in (Waga and Stillman, 1998)). This complexity is in contrast to the single subunit factors in *E. coli*. For example, even the SSB in eukaryotes, termed RPA, is composed of three subunits in a 1:1:1 heterotrimer (reviewed in (Wold, 1997)). It is widely believed that the eukaryotic helicase is the heterohexameric MCM2-7 complex (reviewed in (Forsburg, 2004)), although this conclusion is not yet firm.

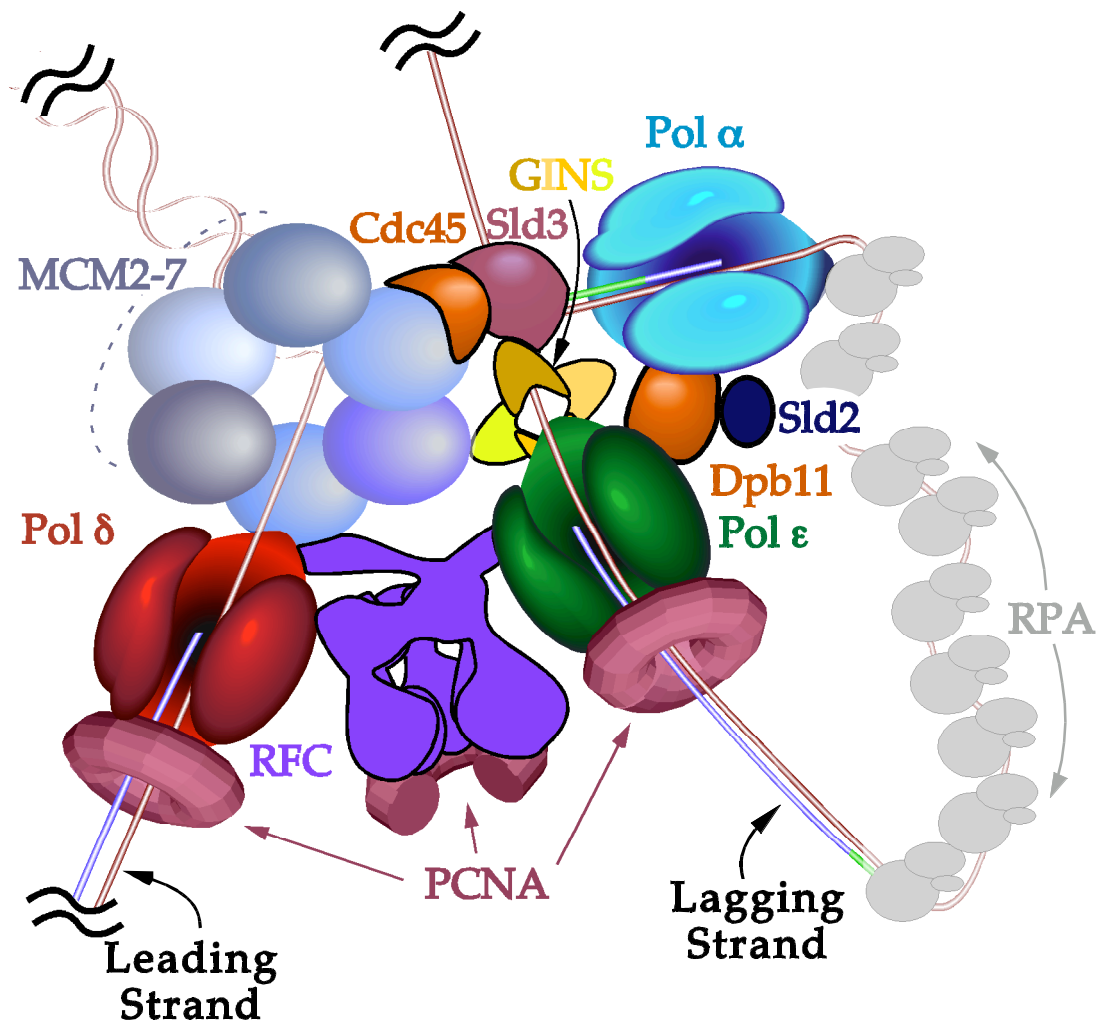
Like *E. coli* DnaB, the hexameric MCM complex is ring-shaped, but each MCM subunit is a different polypeptide. Subunit arrangements for the MCM2-7 complex have been proposed (Davey et al., 2003; Schwacha and Bell, 2001). Helicase activity has been observed only for the MCM4/6/7 subcomplex (Ishimi, 1997; Kaplan et al., 2003; Lee and Hurwitz, 2001), yet all six MCM genes are essential in a variety of systems (Labib et al., 2000)(also, see references within (Forsburg, 2004)). These findings have led to the suggestion that one or more of the MCM2,3,5 subunits act as regulators (Ishimi et al., 1998; Lee and Hurwitz, 2001; Schwacha and Bell, 2001). Consistent with this view, MCM2 inhibits the MCM4/6/7 helicase. The MCM complex is also a target of phosphorylation and ubiquitination and is thought to require activation for helicase action after assembly on DNA. MCM subunits are AAA+ proteins and thus are thought to have a distinct evolutionary origin from DnaB which is constructed from the RecA module. Furthermore, these helicases translocate on DNA with opposite polarities (Ishimi, 1997; Kaplan et al., 2003; Lee and Hurwitz, 2001), thereby placing the MCMs on the leading strand (see Figure 1.7). However, like DnaB, the MCMs have been shown to be capable of encircling two DNA strands (Kaplan et al., 2003; Kaplan and O'Donnell, 2002) and evidence that they may form a double hexamer exists in both Archaea (Chong et al., 2000; Fletcher et al., 2003) and *S. pombe* (Shin et al., 2003) systems. One line of evidence for this comes from an archaeal single-gene MCM that produces a circular double hexamer with helicase activity (Chong et al., 2000).

MCM helicase activity is rather weak, somewhat reminiscent of the relatively weak helicase activity of *E. coli* DnaB. As described earlier, DnaB becomes highly active when coupled to Pol III holoenzyme and this coupling



**Figure 1.7 Hypothetical arrangement of proteins at the eukaryotic replication fork.** The hexameric MCM complex encircles the leading strand. In this cartoon, Pol  $\delta$  is placed on the leading strand and Pol  $\epsilon$  on the lagging, with RFC bridging the two polymerases and helicase. Pol  $\alpha$ /primase action places it on the lagging strand along with RPA bound to the looping single-stranded DNA. Other factors involved in replication and known to bind certain proteins at the replication fork include Cdc45, Sld2, Sld3, Dpb11, and the heterotetrameric GINS complex.

**Figure 1.7 Hypothetical arrangement of proteins at the eukaryotic replication fork.**



occurs through the  $\tau$  subunit of the clamp loader (Kim et al., 1996a). It seems likely that a similar arrangement may exist in eukaryotes. In the scheme of Figure 1.7, RFC is proposed to act as a scaffold like the  $\gamma/\tau$  complex in *E. coli*, bridging the two polymerases and coupling them to the helicase. It should be pointed out that the proposed connections are simply inferred as only scant evidence exists for an RFC-Pol  $\delta$  interaction (Maga and Hubscher, 1996; Yuzhakov et al., 1999a) and none as yet exists for RFC binding either Pol  $\epsilon$  or the MCM complex. However, evidence exists for the presence of many other protein actors required for DNA synthesis and presumed to act at the eukaryotic replication fork. Although the individual functions of these factors are largely unknown, protein interaction studies indicate a network as illustrated schematically in Figure 1.7. Cdc45 has been known for some time to be required for replication, and interaction between Cdc45 and MCMs has been documented (Hopwood and Dalton, 1996; Zou et al., 1997). Newer actors include Sld3, which binds Cdc45 (Kamimura et al., 2001), and the heterotetramer GINS complex that appears to have a ring shape (Kubota et al., 2003; Takayama et al., 2003). The GINS complex also appears to bind Pol  $\epsilon$ , and like Sld3, assembles at origins just prior to DNA synthesis (Takayama et al., 2003). Dpb11 is thought to bind both Pol  $\alpha$  and Pol  $\epsilon$  (Masumoto et al., 2000), and it also forms a complex with Sld2 (Kamimura et al., 1998). Biochemical study of these various factors, alone and in combinations, will be required to understand their individual roles in chromosome replication and whether they all function together at each replication fork.

### *Alternate RFC Complexes and Other Roles for Clamp Loaders*

Sliding clamps are used in a variety of DNA metabolic processes (Maga and Hubscher, 2003; Warbrick, 2000) and one may presume that their respective clamp loader is also involved in most of these processes. In addition, the subunit composition of the eukaryotic clamp loader is altered to perform novel functions in DNA metabolism. This alteration involves the use of an alternate clamp loader subunit, in place of RFC-A. In fission yeast and human the Rad17 subunit (Rad24 in *S. cerevisiae*) replaces p140 (RFC-A) in complex with RFC-B/C/D/E (Green et al., 2000; Lindsey-Boltz et al., 2001). The Rad17-RFC complex is involved in the DNA damage checkpoint response, along with a novel PCNA-like sliding clamp formed from the trimer Rad9/Rad1/Hus1 (Rad17/Mec3/Ddc1 in *S.c.*) (reviewed in (Sancar et al., 2004)). This clamp loader loads the “911” clamp onto primed DNA in an RPA-stimulated reaction (Bermudez et al., 2003; Ellison and Stillman, 2003; Majka and Burgers, 2003; Zou et al., 2003), and does so with opposite polarity to RFC (Ellison and Stillman, 2003). The function of the 911 clamp is not clear, but it presumably recruits other factors when loaded on DNA. There may be a 3'-5' exonuclease activity in Rad9 or Hus1, thus providing a biochemical activity for the ring itself (Bessho and Sancar, 2000). RFC-A can also be replaced by Ctf18/Chl12 (Mayer et al., 2001; Naiki et al., 2001) or Elg1 (Bellaoui et al., 2003; Ben-Aroya et al., 2003; Kanellis et al., 2003) which are involved in cohesion and genome stability, respectively, although the specific roles of these complexes are not understood.

## ARCHAEAL REPLICASES

The archaeal replicase components form a unique bridge between bacterial and eukaryotic replicases (reviewed in (Grabowski and Kelman, 2003)). It is important to make the distinction between euryarchaeotes and crenarchaeotes because their replication machinery is quite different. Euryarchaeotes have one monomeric B-family polymerase and one dimeric polymerase (Pol II) whose large subunit has no known homologues in any other organism. Crenarchaeotes have multiple B-type polymerases, but lack the unique Pol II polymerase.

All archaea have adopted a trimeric processivity clamp for their replicative polymerase, similar to the phage and eukaryotic clamp. This clamp is referred to as archaeal PCNA, named after the eukaryotic equivalent, proliferating cell nuclear antigen. The euryarchaeal PCNA is a homotrimer, with structural similarity to all other homooligomeric clamps (Matsumiya et al., 2001). In contrast, crenarchaea have three distinct PCNA genes whose proteins preferentially associate into a heterotrimer (Dionne et al., 2003). Each subunit of this heterotrimeric PCNA forms a strong interaction with either a specific polymerase, flap endonuclease, or ligase, suggesting that the PCNA may maintain connection with all three throughout replication.

The mechanism of archaeal DNA replication is not yet well understood. All archaea have two clamp loader subunits, called RFC-L and RFC-S, with sequence and structural homology to other clamp loader complexes. They work in an ATP-dependent manner to load their PCNA for processive replication by the polymerase (Cann et al., 2001; Henneke et al., 2002; Seybert et al., 2002; Seybert and Wigley, 2004). The architecture of the clamp loader complex is

under debate with evidence for pentameric and hexameric complexes composed of some combination of large and small RFC subunits.

## **T4 REPLICASE**

Not only cellular organisms employ a DNA polymerase holoenzyme for genome duplication. Bacteriophages such as T4 have especially similar holoenzymes compared to their hosts and higher organisms. The T4 replicase has a dimeric polymerase, a ring-shaped processivity factor, and a pentameric clamp loader with sequence homology to  $\gamma$  complex (Benkovic et al., 2001).

The mechanism of T4 replicase action shares many general characteristics with the *E. coli* system, with specifics that are unique to the phage. The T4 replicase has only four unique polypeptides associated with it: gene product 43 (gp43) has polymerase and both 3'-5' and 5'-3' exonuclease activity, gp45 is the processivity clamp subunit, and gp44/62 is the clamp loader complex. These proteins work together for processive DNA replication, but do not form a stable holoenzyme particle as their *E. coli* counterparts do.

The gp43 polymerase associates as a dimer on a DNA substrate (Ishmael et al., 2003), independently of other proteins and this precludes the need for an organizer such as the *E. coli*  $\tau$  protein to work coordinately at the replication fork. The gp44/62 clamp loader does not associate with the polymerase, but acts in a handoff between loaded gp45 clamp and polymerase. The gp45 clamp is a trimeric ring (Moarefi et al., 2000). Its overall structure resembles the *E. coli*  $\beta$  clamp, adopting the same six-fold symmetry. Biochemical and biophysical studies indicate that the gp45 ring is less stable, both in solution and when

loaded on DNA, than the  $\beta$  clamp and will dissociate passively with the polymerase (Yao et al., 1996).

The T4 clamp loader, gp44/62, is a 4:1 complex like the archaeal RFC, except that only gp44 binds ATP and thus gp44/62 complex may only contain three competent ATP sites. However, ATP turnover studies indicate that all four ATP are hydrolyzed (Trakselis et al., 2003). The gp45 clamp is unstable in solution and dissociates into monomers, and can also assume an open form on its own (Alley et al., 1999; Yao et al., 1996). Two ATP are hydrolyzed by gp44/62 to stabilize the gp45 clamp in the open form, and the remaining two ATP are hydrolyzed upon linking the gp45 clamp to DNA. Structural information on the gp44/62 complex is needed for further insight on how individual ATP sites are utilized in DNA binding, ring opening, and ring closing.

## CONCLUSION

A deep foundation has been laid by the studies of *E. coli* and eukaryotic replicases. Both biochemical and structural studies have illuminated key aspects of the mechanism of DNA replication. With this wealth of information, comes many new questions to be answered. This thesis undertakes a comparative analysis of the clamp loaders of both the *E. coli* and *S. cerevisiae* systems in an effort to understand the degree of conservation in ATP-coupled processivity clamp loading. The findings point towards conserved aspects of these two systems and suggest that all clamp loaders may share similarities in harnessing ATP to drive the multi-step clamp loading mechanism.

### **The Arginine Fingers of *E. coli* $\gamma$ Complex Direct ATP Hydrolysis and Loading of the $\beta$ Clamp**

#### **ABSTRACT**

The  $\gamma$  complex couples ATP hydrolysis to the loading of  $\beta$  sliding clamps onto DNA for processive replication. The  $\gamma$  complex structure shows that the clamp loader subunits are arranged as a circular heteropentamer. The three  $\gamma$  motor subunits bind ATP, the  $\delta$  wrench opens the  $\beta$  ring, and the  $\delta'$  stator modulates the  $\delta$ - $\beta$  interaction. Neither  $\delta$  nor  $\delta'$  bind ATP. This report demonstrates that the  $\delta'$  stator contributes a catalytic arginine for hydrolysis of ATP bound to the adjacent  $\gamma^D$  subunit. Thus, the  $\delta'$  stator contributes to the motor function of the  $\gamma$  trimer. Mutation of arginine 169 of  $\gamma$ , which removes the catalytic arginines from only the B and C ATP sites, abolishes ATPase activity even though ATP site D is intact and all three sites are filled. These results suggest a model in which hydrolysis of the three ATP molecules occurs in a particular order, where ATP in site D is not hydrolyzed until ATP in sites B and/or C is hydrolyzed.



## INTRODUCTION

DNA polymerase III holoenzyme, the chromosomal replicase of *E. coli*, contains a clamp loading machine within its multicomponent structure (reviewed in (Kelman and O'Donnell, 1995)). The clamp loader couples ATP hydrolysis to the assembly of circular  $\beta$  clamps onto primed DNA sites. The circular  $\beta$  clamp, formed from two crescent shaped protomers, binds to the DNA polymerase III core ( $\alpha\epsilon\theta$ ), tethering it to template DNA for highly processive synthesis. There are at least two molecules of DNA polymerase III core within the holoenzyme architecture, held together by one clamp loader.

The  $\gamma$  complex clamp loader consists of several different subunits; three  $\gamma$  ( $\tau$ ) subunits, and one each of  $\delta$ ,  $\delta'$ ,  $\chi$  and  $\psi$  (Jeruzalmi et al., 2001; Onrust et al., 1995; Pritchard et al., 2000). The  $\chi$  and  $\psi$  subunits play roles in the primase-to-polymerase switching process (Yuzhakov et al., 1999b) and they also interact with SSB (Glover and McHenry, 1998; O'Donnell et al., 2001), but are not essential for clamp loading. The crystal structure of the minimal clamp loader,  $\gamma_3\delta_1\delta'_1$ , shows that the five subunits are arranged as a circular heteropentamer (Jeruzalmi et al., 2001). In order for this clamp loader to bind two molecules of DNA polymerase III core, two of the  $\gamma$  subunits are replaced by two  $\tau$  subunits.  $\tau$  and  $\gamma$  are encoded by the same gene (*dnaX*);  $\tau$  is the full-length product and  $\gamma$  is truncated by a translational frameshift (Blinkowa and Walker, 1990; Flower and McHenry, 1990; Tsuchihashi and Kornberg, 1990). The N-terminal 47 kDa of  $\tau$  contains the sequence of the  $\gamma$  subunit, thus explaining how  $\gamma$  and  $\tau$  can replace one another in clamp loading action with  $\delta$  and  $\delta'$  (Onrust and O'Donnell, 1993). The extra 24 kDa of C-terminal sequence unique to  $\tau$  is responsible for binding

DNA polymerase III core (McHenry, 1982; Studwell-Vaughan and O'Donnell, 1991); these sequences also bind the DnaB helicase (Kim et al., 1996a; Yuzhakov et al., 1996). Thus, a single clamp loader cross links two DNA polymerases and holds the hexameric helicase into the replisome (reviewed in (O'Donnell et al., 2001)).

The  $\gamma$  ( $\tau$ ) subunits of the  $\gamma$  complex constitute the motor of the clamp loading machine, as they are the only subunits that hydrolyze ATP; neither  $\delta$  nor  $\delta'$  bind or hydrolyze ATP (Kelman and O'Donnell, 1995). The  $\delta$  subunit forms the main attachment to the  $\beta$  clamp and it can open the  $\beta$  ring single-handedly (Leu et al., 2000; Stewart et al., 2001; Turner et al., 1999).  $\delta$  is referred to as the wrench, or crowbar, of the clamp loader since it can open  $\beta$  on its own (Jeruzalmi et al., 2001b). The  $\delta'$  subunit modulates the ability of  $\delta$  to bind  $\beta$  (Leu et al., 2000; Stewart et al., 2001; Turner et al., 1999). In the absence of ATP,  $\delta'$  obscures the  $\delta$  subunit within  $\gamma$  complex from binding to  $\beta$  (Jeruzalmi et al., 2001; Turner et al., 1999). However, with ATP bound to the  $\gamma$  subunits,  $\delta$  is pulled away from  $\delta'$ , allowing  $\delta$  to bind and open the  $\beta$  ring (Jeruzalmi et al., 2001; Naktinis et al., 1995). In this state, with  $\beta$  and ATP bound to  $\gamma$  complex, a tight affinity for DNA is established (Bertram et al., 1998; Hingorani and O'Donnell, 1998). Upon recognizing a primed site, the ATP is hydrolyzed resulting in the dissociation of  $\gamma$  complex from  $\beta$ , leaving  $\beta$  to close around the DNA whereupon it may associate with the polymerase component of the holoenzyme.

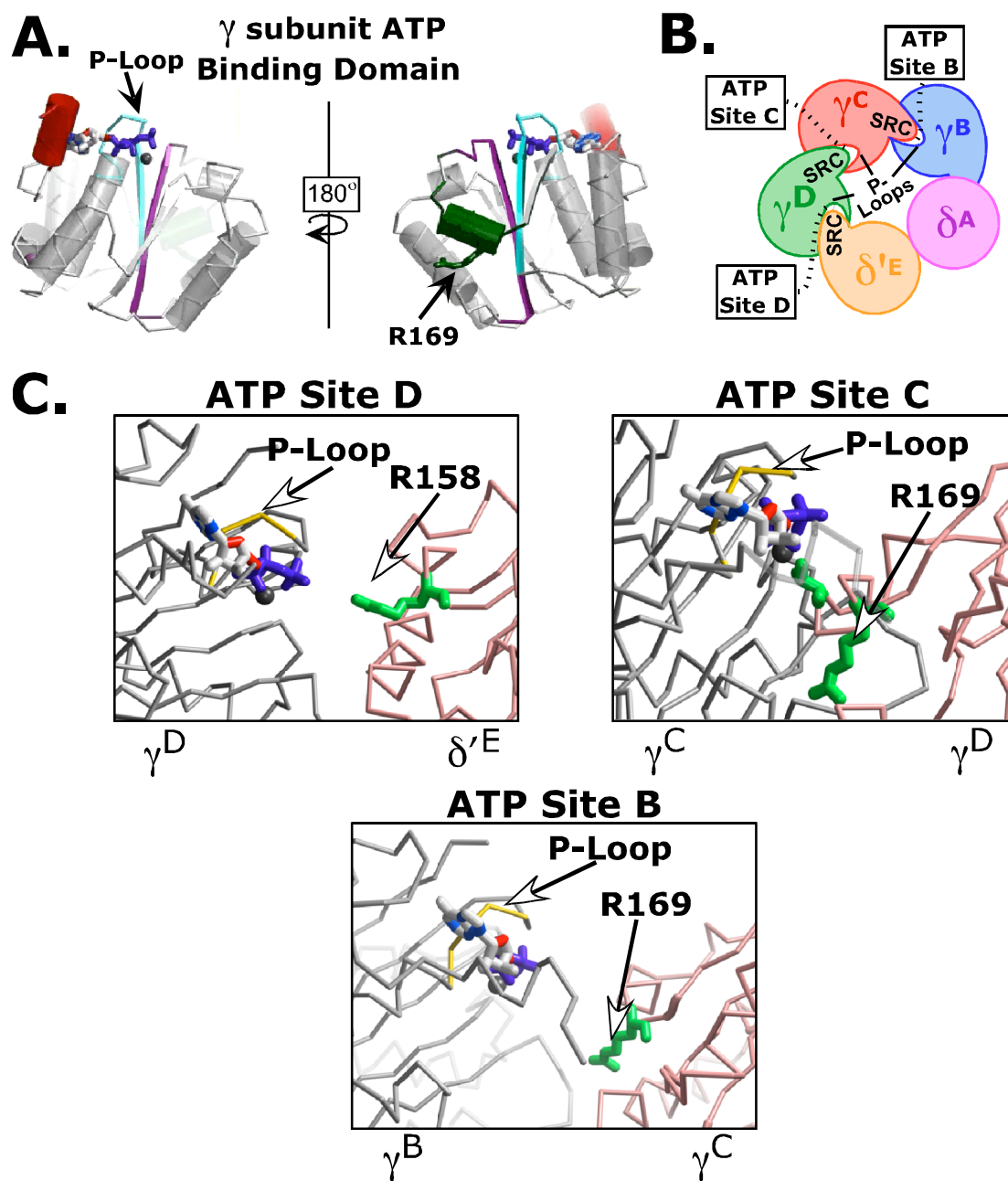
$\beta$  also interacts with several other proteins besides DNA polymerase III core. These include ligase, MutS, UvrB, DNA polymerases I, II, IV and V, and possibly many other proteins involved in DNA repair (Bonner et al., 1992;

Hughes et al., 1991; Lopez de Saro and O'Donnell, 2001; Tang et al., 1999). These additional roles of  $\beta$  in other processes besides replication may account for the presence of  $\gamma$  complex in *E. coli* that lacks  $\tau$  altogether, presumably freeing it for action at sites distinct from the replication fork.

The structure of  $\gamma_3\delta\delta'$  reveals that the ATP sites of the  $\gamma$  subunits are located at subunit interfaces (see Figure 2.1)(Jeruzalmi et al., 2001). To facilitate comparison to other clamp loaders, the five positions in the pentameric ring of  $\gamma_3\delta\delta'$  have been assigned letters that correspond to the alphabetical nomenclature adopted for RFC (Bowman et al., 2004). In this naming system  $\delta$  is in the A position, the adjacent  $\gamma$  is in the B position and the order continues in that “counterclockwise” direction, when looking from the C-terminus (Figure 2.1B). The ATP site nomenclature has been modified to fit this alphabetical nomenclature. Site A in RFC is at the RFC-A/B interface.  $\gamma$  complex lacks this ATP site, but the three remaining ATP sites are referred to henceforth as sites B-D, to correspond with the analogous RFC sites. ATP site B is at the  $\gamma^B/\gamma^C$  interface, site C is at the  $\gamma^C/\gamma^D$  interface, and site D is at the  $\gamma^D/\delta'^E$  interface (Figure 2.1B). The major pentameric contacts occur via the C-terminal domains of the five subunits. The ATP binding sites of  $\gamma$  are located in the N-terminal domains. The N-terminal domain of  $\delta$  is also where the  $\beta$  interactive element is located (Jeruzalmi et al., 2001b), although proximity of  $\delta'$  to  $\delta$  blocks access of  $\delta$  to  $\beta$  (Jeruzalmi et al., 2001). Combining several biochemical findings with the structure of  $\gamma_3\delta\delta'$ , suggests that as the ATP sites fill, conformational changes in  $\gamma$  are propagated around the pentamer to pull the  $\delta$  wrench away from the  $\delta'$  stator so that  $\delta$  can bind to  $\beta$  for clamp opening. The apparent rigidity of  $\delta'$ , compared

**Figure 2.1 Architecture of  $\gamma$  complex ATP sites.** *Panel A* Orthogonal views of the  $\gamma$  ATP binding domain. R169 is located far away from the phosphate binding loop (P-loop) on the same polypeptide chain, preventing formation of a functional intramolecular ATP site. *Panel B* Cartoon of the circular pentamer of  $\gamma$  complex looking down the center of the ring from the C-terminus. Three ATP sites are created at subunit interfaces. Each site is highlighted by the P-loop of a  $\gamma$  subunit and the Sensor 1 Arg in the SRC motif from the adjacent  $\delta'$  (ATP site D) or  $\gamma$  (sites B and C) subunit. *Panel C* ATP site D: A magnified view of the  $\delta'/\gamma^D$  N-terminal domain interface with an ATP molecule modeled against the P-loop of  $\gamma^D$  based on the NSF D2 crystal structure (Yu et al., 1998). The  $\delta'$  Sensor 1 R158 (green residue) is in close proximity to the  $\gamma$ -phosphate of ATP, suggesting a catalytic function. ATP sites B and C: the  $\gamma^B/\gamma^C$  and  $\gamma^C/\gamma^D$  interfaces, respectively, have a similar architecture to ATP site D. The  $\gamma$  Sensor 1 R169 is positioned proximal to the ATP molecule modeled into the neighboring  $\gamma$ . It should be noted that ATP modeled into site C clashes with some residues of  $\gamma^D$ , and thus a conformation change is needed to make this site accessible to ATP.

Figure 2.1 Architecture of  $\gamma$  complex ATP sites.



to  $\gamma$  and  $\delta$  which have a flexible joint for motion in clamp loading, has earned  $\delta'$  the term “stator”. ATP hydrolysis presumably reverses the conformational changes in  $\gamma$  and  $\delta$  induced by ATP binding to  $\gamma$ , thus bringing the N-terminal domain of  $\delta$  back into proximity to the  $\delta'$  stator. This effectively pushes  $\beta$  off of  $\delta$ , allowing the  $\beta$  ring to close around DNA.

The subunits of  $\gamma_3\delta\delta'$  are members of the large AAA+ family (Neuwald et al., 1999). As their name implies (ATPases Associated with a variety of cellular Activities) these proteins are generally ATPases and they function in a wide diversity of cellular processes. The structures of homohexameric AAA+ proteins NSF and p97 (membrane fusion), RuvB (branch migration) and HslU (proteasome), reveal an Arg residue that reaches over the interface to the ATP site of the neighboring subunit (Bochtler et al., 2000; Putnam et al., 2001; Yu et al., 1998; Zhang et al., 2000a). This Arg is thought to be analogous to the “arginine finger” of GTPase-activating protein (GAP), which plays a catalytic role in hydrolysis of GTP bound to Ras by stabilizing the accumulating negative charge in the transition state (Ahmadian et al., 1997). It is proposed that the use of this Arg residue in catalysis provides a means of intersubunit communication that coordinates nucleotide hydrolysis around the ring.

The  $\gamma$  complex has many similarities to the homohexameric AAA+ proteins, but also has several important differences. The largest differences are its heterooligomeric composition, use of five subunits instead of six, and presence of two subunits which do not bind ATP. Like the homohexamers, the  $\gamma$  complex subunits are arranged in a ring and the  $\gamma$  subunit ATP sites are located at interfaces where the Arg of one subunit is in proximity to ATP modeled into

the subunit adjacent to it (Figure 2.1). This Arg residue is embedded in an SRC motif that is conserved in clamp loading subunits of T4 phage, eubacteria, archaea, and eukaryotes. The  $\delta'$  subunit also contains an SRC motif and the Arg residue is proximal to ATP site D. In each structure, the Arg needs to move at least a few angstroms to be near enough to exert an influence on the bound ATP.

The contribution of these potential arginine fingers to ATP binding, hydrolysis and clamp loading is one subject of this report. The results demonstrate that these SRC motif arginine residues in both  $\gamma$  and  $\delta'$  are not required for ATP binding, however they are important to catalysis. The finding that  $\delta'$  contributes a catalytic arginine residue to an ATP site in the  $\gamma$  trimer motor demonstrates that the  $\delta'$  stator also functions as a component of the motor of the clamp loader. This conclusion has implications for analogies between  $\gamma$  complex and clamp loaders of other systems (see Discussion). The findings indicate that there is an ordered sequence to hydrolysis. In addition, further investigation has uncovered that these residues serve a pre-hydrolysis role as well. The  $\delta'$  arginine finger is involved in ATP-dependent  $\beta$  association by  $\gamma$  complex and the  $\gamma$  arginine fingers are required for high-affinity DNA binding.

## RESULTS

### Reconstitution of $\gamma$ (R169A) complex.

Arginine 169 is located in the highly conserved SRC motif of the  $\gamma$  subunit. To assess the importance of  $\gamma$  arginine 169 to clamp loading activity we mutated it to alanine and purified the  $\gamma$  (R169A) protein from an overproducing strain of *E. coli*. To study the effect of this mutation on clamp loader activity, we

reconstituted the  $\gamma$  complex using  $\gamma$ (R169A) with  $\delta$ ,  $\delta'$ ,  $\chi$  and  $\psi$ . Our previous studies have shown that fully assembled  $\gamma$  complex is stable to ion exchange chromatography on an FPLC MonoQ column, where it elutes much later than free subunits (Onrust and O'Donnell, 1993). The  $\gamma$ (R169A) mutant was mixed with an excess of  $\delta$ ,  $\delta'$ ,  $\chi$  and  $\psi$ , incubated 30 min. at 15°C, then applied to a MonoQ column followed with a gradient of NaCl. The result, in Figure 2.2A, demonstrates that the  $\gamma$ (R169A) mutant forms a " $\gamma$ (R169A) complex" in which all five subunits co-elute in fractions 41-49, while the excess subunits elute much earlier. As demonstrated later in this report (Figure 2.4), the  $\gamma$ (R169A) complex also remains intact during analysis on a gel filtration column. The subunit ratio of  $\gamma$ (R169A) complex is comparable to wild-type  $\gamma$  complex as observed in the Coomassie Blue stained SDS polyacrylamide gel of Figure 2.2B. This ability of  $\gamma$ (R169A) to assemble into a multisubunit complex with  $\delta$ ,  $\delta'$ ,  $\chi$  and  $\psi$  demonstrates that the  $\gamma$ (R169A) mutant is properly folded. It also provides reconstituted  $\gamma$ (R169A) complex for the studies to follow.

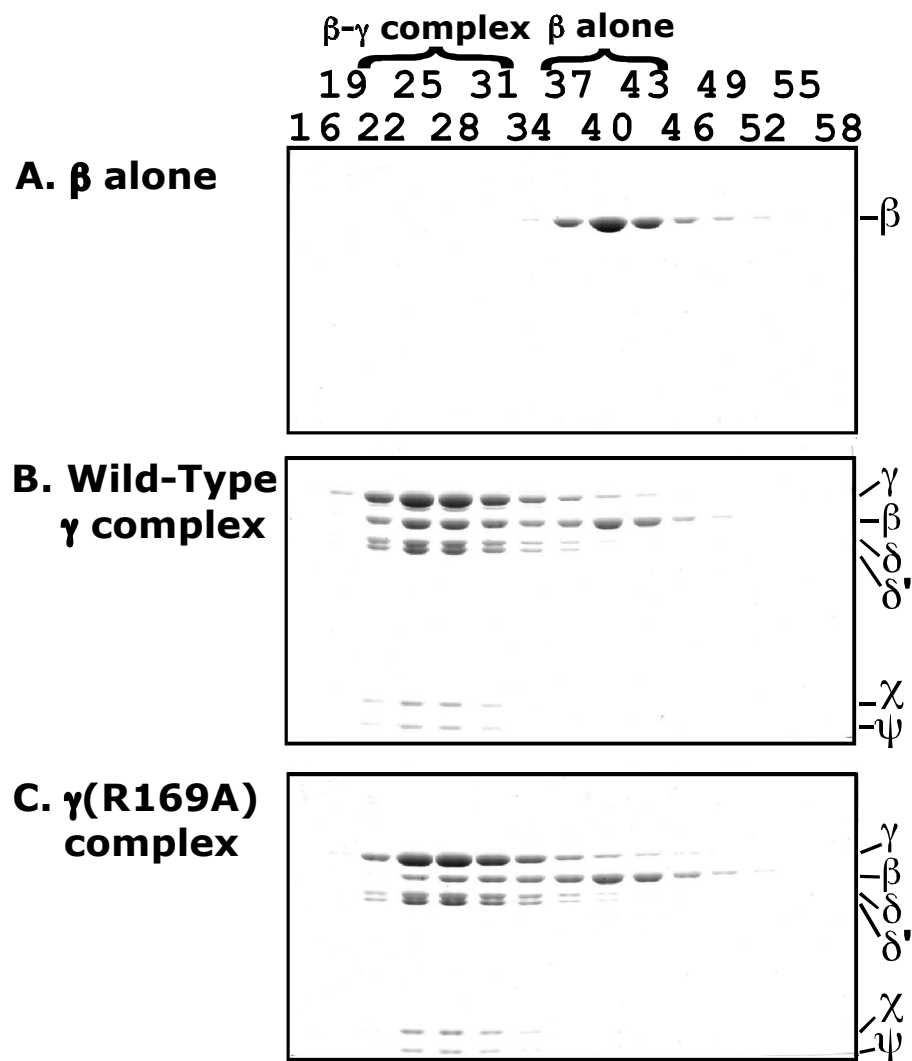
**The  $\gamma$ (R169A) complex binds three molecules of ATP with similar affinity as the wild-type.**

Next, we determined whether  $\gamma$ (R169A) complex binds ATP, and if so whether it binds ATP with similar stoichiometry and affinity compared to wild-type  $\gamma$  complex. To address these issues we used the equilibrium gel filtration technique. In this analysis a gel filtration column is equilibrated with a known concentration of  $^{32}\text{P}$ -ATP. The  $\gamma$  complex is incubated with the same concentration of  $^{32}\text{P}$ -ATP as present in the column buffer, and then is applied to



**Figure 2.4 ATP promotes  $\beta$  binding in the  $\gamma$ (R169A) complex.** The interaction between  $\beta$  and  $\gamma$  complex was analyzed by gel filtration on a Superose 12 column to separate free  $\beta$  (fractions 37-43) from  $\beta$  bound to  $\gamma$  complex (fractions 22-31). *Panel A*  $\beta$  alone in the presence of ATP. *Panel B*  $\beta$  and wild-type  $\gamma$  complex with ATP. *Panel C*  $\beta$  and the  $\gamma$ (R169A) complex with ATP.

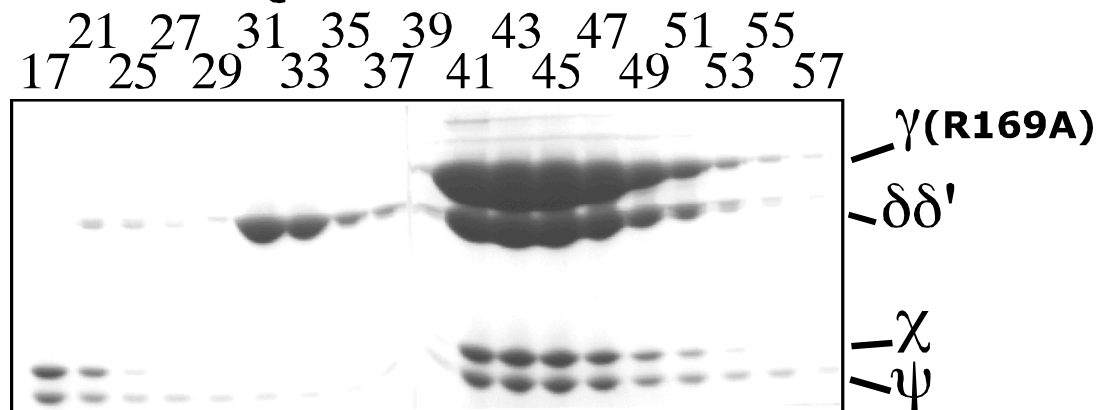
Figure 2.4 ATP promotes  $\beta$  binding in the  $\gamma$ (R169A) complex.



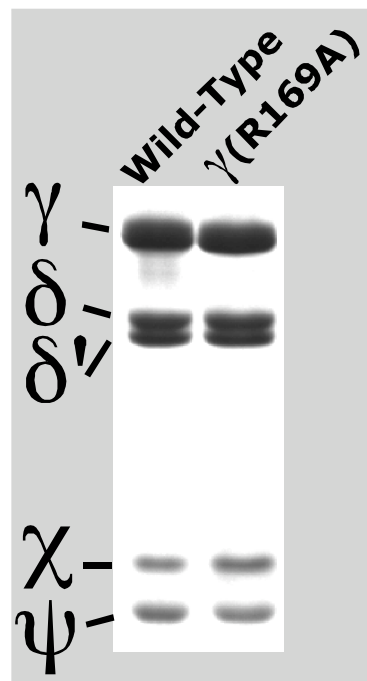
**Figure 2.2 Reconstitution of  $\gamma$ (R169A) complex.** *Panel A* Gradient elution of reconstituted  $\gamma$ (R169A) complex from a MonoQ column. Note the excess free  $\chi\psi$  (fractions 17-21), and  $\delta\delta'$  (fractions 31-33) subcomplexes that elute early in the gradient compared to the full  $\gamma$ (R169A) complex (fractions 41-49) made with limiting  $\gamma$ (R169A) subunit. *Panel B* Reconstituted wild-type  $\gamma$  complex and  $\gamma$ (R169A) complex have comparable subunit stoichiometries as analyzed in a 14% SDS polyacrylamide gel stained with Coomassie Blue.

Figure 2.2 Reconstitution of  $\gamma$ (R169A) complex.

### A. MonoQ Reconstitution



### B. SDS-PAGE



the column. Fractions are collected and the amount of protein and  $^{32}\text{P}$ -ATP in each fraction is determined. Protein bound  $^{32}\text{P}$ -ATP is carried around the beads resulting in a peak of  $^{32}\text{P}$ -ATP that elutes early, followed later by a trough which has less  $^{32}\text{P}$ -ATP than the column buffer due to its displacement from the buffer by the protein. This information can be used to calculate the  $K_d$  value for ATP binding to the complex. However, a more accurate assessment of the  $K_d$  value can be obtained by repeating the experiment at a variety of ATP concentrations (the column is equilibrated at different ATP concentrations), followed by plotting the data as a Scatchard plot. This detailed analysis also carries the advantage of providing the stoichiometry of ATP bound to the complex.

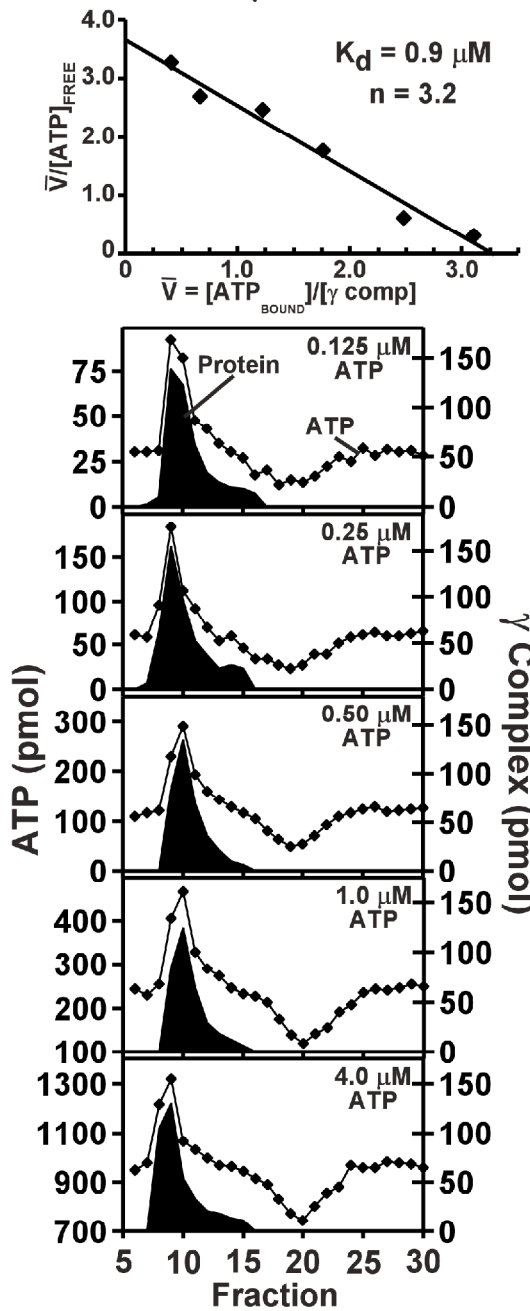
The results, in Figure 2.3, show that  $\gamma(\text{R169A})$  complex binds three molecules of ATP with similar affinity as wild-type  $\gamma$  complex ( $K_d$  1-2  $\mu\text{M}$ ). The data for both wild-type and  $\gamma(\text{R169A})$  complex fall on a relatively straight line indicating that the three sites bind ATP with similar affinity. This conclusion is also supported by a study of a monomeric  $\gamma$  subunit (missing the C-terminal oligomerization domain) which binds ATP with a  $K_d$  value of 1.36  $\mu\text{M}$ , determined by isothermal calorimetry (Podobnik et al., 2003).

Overall, these results indicate that the two ATP sites that carry alanine residues in place of arginine 169 (sites B and C) still bind ATP, and that this arginine residue contributes little, if any, to the binding affinity of ATP to  $\gamma$  complex.

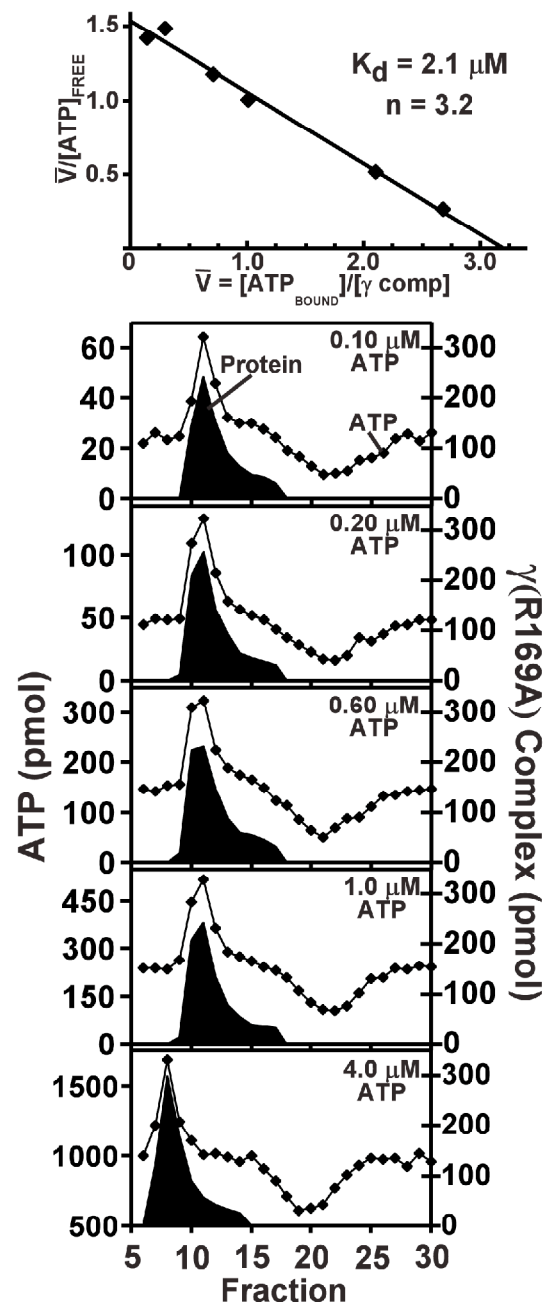
**Figure 2.3 The  $\gamma$ (R169A) complex binds three ATP molecules with similar affinity as wild-type  $\gamma$  complex.** Wild-type  $\gamma$  complex (*Panel A*) and  $\gamma$ (R169A) complex (*Panel B*) were analyzed for ATP binding affinity and stoichiometry by equilibrium gel filtration as described in “Materials and Methods.” The concentration of ATP (diamonds) and  $\gamma$  complex (black area curve) were measured in column fractions for a series of experiments performed at different concentrations of ATP. The Scatchard plot at the top is derived from the series of column analyses (each data point is one column analysis). The 10  $\mu$ M ATP analysis is included in the Scatchard plot, but is not shown in the profile below the plots. The line is the least squares fit to the data. *Panel A* Wild-type  $\gamma$  complex analysis yields a  $K_d$  of 0.9  $\mu$ M and stoichiometry of 3.2 ATP/ $\gamma$  complex. *Panel B* The  $\gamma$ (R169A) complex analysis yields a  $K_d$  value for ATP binding of 2.10  $\mu$ M with a stoichiometry of 3.2 ATP/ $\gamma$ (R169A) complex.

Figure 2.3 The  $\gamma$ (R169A) complex binds three ATP molecules with similar affinity as wild-type  $\gamma$  complex.

**A. Wild-Type  $\gamma$  Complex**



**B.  $\gamma$ (R169A) Complex**



### **$\gamma$ (R169A) complex binds $\beta$ .**

Previous studies have demonstrated that ATP binding to  $\gamma$  complex induces a conformation change that leads to the binding of the  $\beta$  subunit (Hingorani and O'Donnell, 1998). This predicts that the  $\gamma$ (R169A) complex, which binds ATP, should be capable of binding to  $\beta$ . To test this prediction, we analyzed a mixture of  $\gamma$ (R169A) complex and  $\beta$  for complex formation on a Superose 12 sizing column equilibrated with buffer containing ATP.  $\beta$  alone migrates in fractions 37-43 (Figure 2.4A). Analysis of a mixture of wild-type  $\gamma$  complex and  $\beta$  is shown in Figure 2.4B;  $\beta$  subunit co-elutes with the large  $\gamma$  complex in fractions 22-31 and resolves from unbound  $\beta$  which elutes in the later fractions. A similar analysis using  $\gamma$ (R169A) complex, shown in Figure 2.4C, demonstrates that the mutant  $\gamma$  complex is also capable of associating with  $\beta$ . The amount of  $\beta$  that comigrates with the  $\gamma$ (R169A) complex is nearly the same compared to wild-type  $\gamma$  complex indicating that it is capable of binding  $\beta$ , although its affinity for  $\beta$  may be somewhat decreased by the mutation. ATP binding, not hydrolysis, powers all the steps of clamp loading except the final stage of dissociating from the  $\beta$ •DNA complex, allowing  $\beta$  to close around the DNA (Hingorani and O'Donnell, 1998). This last step requires ATP hydrolysis. Next, the mutant  $\gamma$  complex was studied for ability to hydrolyze ATP.

### **The $\gamma$ R169 is essential to catalysis.**

We next analyzed  $\gamma$ (R169A) complex in three different assays, each of which require ATP hydrolysis. The first activity to be tested was DNA-



dependent ATPase activity of  $\gamma$  complex, followed by clamp loading and finally  $\beta$ -dependent DNA synthesis by core DNA polymerase.

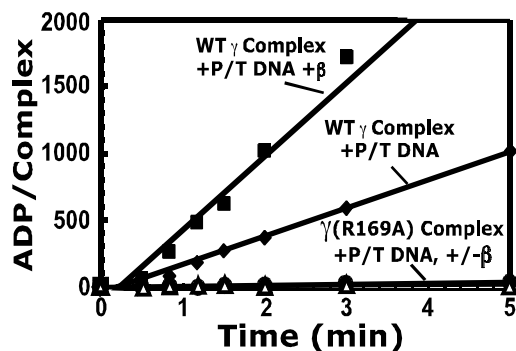
The  $\gamma$  complex requires the presence of DNA for significant ATPase activity (Onrust et al., 1991). The  $\beta$  subunit stimulates  $\gamma$  complex ATPase activity provided a primed DNA, not ssDNA, is present (Onrust et al., 1991). In the experiments of Figure 2.5A we examined the  $\gamma$  complex ATPase activity using primed DNA with and without  $\beta$ . Whereas wild-type  $\gamma$  complex hydrolyzes approximately 217 molecules of ATP per min in the presence of the primed template, the  $\gamma$ (R169A) complex shows no detectable ATPase activity (i.e. detection limit  $\sim 5$  ATP hydrolyzed/min./ $\gamma$  complex). The  $\beta$  subunit stimulates the ATPase activity of wild-type  $\gamma$  complex in the presence of primed DNA as illustrated in Figure 2.5A. However,  $\beta$  does not provide detectable ATPase activity by  $\gamma$ (R169A) complex in the presence of the primed template (Figure 2.5A), even when a very large excess of  $\beta$  is present (2  $\mu$ M, data not shown).

The absence of catalytic activity predicts that the mutant  $\gamma$  complex will be incapable of loading a  $\beta$  clamp onto primed DNA. To assay  $\beta$  clamp loading activity directly we radiolabeled  $\beta$  with  $^{32}\text{P}$ -phosphate using a derivative of  $\beta$  that carries an N-terminal tag with a kinase site. The  $^{32}\text{P}$ - $\beta$  was incubated with  $\gamma$  complex and primed M13mp18 ssDNA (SSB coated), then the reaction was analyzed for assembly of  $^{32}\text{P}$ - $\beta$  onto the primed DNA by gel filtration on a Bio-Gel A-15m column. This resin has large pores which include proteins, but exclude the larger M13mp18 primed ssDNA (see agarose gel analysis). As a result,  $^{32}\text{P}$ - $\beta$  bound to DNA elutes early and resolves from free  $^{32}\text{P}$ - $\beta$  that is not bound to DNA. A control reaction using wild-type  $\gamma$  complex is shown in Figure

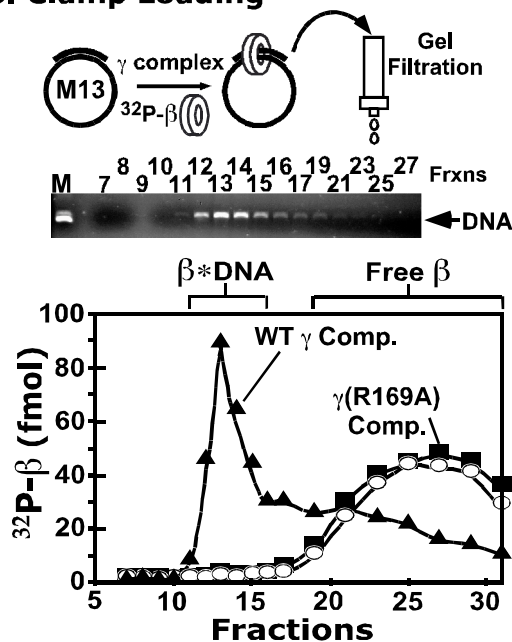
**Figure 2.5 Analysis of catalytic activity by  $\gamma$ (R169A) complex.** Wild-type and  $\gamma$ (R169A) complex clamp loaders were tested in three assays that each require hydrolysis of ATP. *Panel A* Steady-state ATPase assays in the presence of synthetic primed template + and -  $\beta$ . Squares and diamonds are wild-type  $\gamma$  complex plus and minus  $\beta$ , respectively. Circles and open triangles are  $\gamma$ (R169A) complex plus and minus  $\beta$ , respectively. *Panel B* In the clamp loading assays,  $^{32}\text{P}$ - $\beta$  was incubated with wild-type  $\gamma$  complex (triangles) or  $\gamma$ (R169A) complex (squares) in the presence of SSB coated singly primed M13mp18 ssDNA, then DNA-associated  $^{32}\text{P}$ - $\beta$  was separated (fractions 11-16) from free  $^{32}\text{P}$ - $\beta$  (fractions 17-33) on a Bio-Gel A-15m column. The open circles are a control in which only the DNA substrate was omitted from the reaction. Above the plot is an ethidium bromide-stained neutral agarose gel analysis which demonstrates that the large DNA substrate alone elutes in fractions 11-16. *Panel C* Wild-type  $\gamma$  complex (triangles) or  $\gamma$ (R169A) complex (squares) was titrated into replication reactions containing  $\beta$ , core, SSB-coated singly primed M13mp18 ssDNA, and  $^{32}\text{P}$ -dNTPs. Incorporation of  $^{32}\text{P}$ -dTTP indicates that the  $\beta$  clamps are loaded onto DNA for processive replication by core polymerase.

Figure 2.5 Analysis of catalytic activity by  $\gamma$ (R169A) complex.

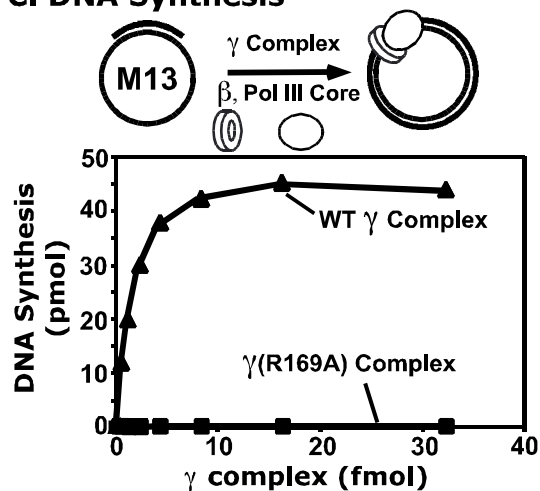
### A. ATPase Activity



### B. Clamp Loading



### C. DNA Synthesis



2.5B. Most of the  $^{32}\text{P}$ -  $\beta$  elutes with the DNA in fractions 11 - 16. An agarose gel analysis, shown in Figure 2.5B, confirms that the large SSB-coated DNA substrate alone elutes in these same fractions (11-16). However, repeating this analysis using mutant  $\gamma$  complex did not result in assembly of  $^{32}\text{P}$ - $\beta$  on the DNA, and instead the  $^{32}\text{P}$ -  $\beta$  eluted later, in fractions 17-33. A control reaction lacking the DNA template confirmed that free  $^{32}\text{P}$ - $\beta$  elutes in fractions 17-33 (Figure 2.5B). Analysis of  $\gamma$  complex migration shows that it elutes in the included fractions in the same position as free  $\beta$ , consistent with the large pore size of the A15m resin (data not shown). Hence, the  $\gamma(\text{R169A})$  complex is inactive for clamp loading action, consistent with its lack of ATPase activity.

Finally,  $\gamma(\text{R169A})$  complex and  $\beta$  were tested for ability to support DNA synthesis by core polymerase. The core Pol III is incapable of extending a primer around an SSB coated primed M13mp18 ssDNA template unless it is coupled to a  $\beta$  clamp, and thus provides another measure of clamp loading activity. Use of the wild-type  $\gamma$  complex yielded a strong signal in this assay, but as expected, when mutant  $\gamma$  complex was used in this assay no activity was detected, consistent with the inactivity of mutant  $\gamma$  complex in ATPase and clamp loading assays (Figure 2.5C). These results support and extend those of an earlier analysis in which several conserved residues of  $\gamma$  were mutated (Walker et al., 2000). Mutation of  $\gamma$  R169 resulted in loss of replication and ATPase activity, and failed to complement a conditional lethal *dnaX* gene *in vivo*, although that study did not demonstrate that the mutant  $\gamma$  was properly folded, or that it retained ability to form a stable complex with  $\delta$  and  $\delta'$ , bind ATP and associate with  $\beta$ .

### **The $\delta'$ SRC motif**

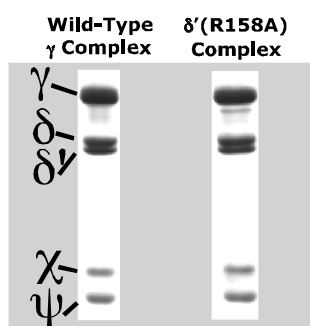
The above results strongly support an essential role of the  $\gamma$  arginine 169 in catalysis. However, the results imply something further. Mutation of  $\gamma$  arginine 169 only eliminates this catalytic residue at ATP sites B and C. The putative catalytic arginine of ATP site D is supplied by  $\delta'$ , not  $\gamma$ , and therefore this site should remain competent for ATP hydrolysis, even in the  $\gamma$ (R169A) complex. Even though  $\gamma$ (R169A) complex retains one intact ATP site, the results show that it has lost essentially all of its ATPase activity. This implies that site B and/or C must function before ATP in site D is hydrolyzed. Alternatively, site D binds ATP but is simply not catalytic. A non-catalytic site D would explain why the  $\gamma$ (R169A) complex has no ATPase, but would not explain why the SRC motif is broadly conserved in prokaryotic  $\delta'$  subunits. To test whether arginine 158 in the SRC motif of  $\delta'$  is important to catalysis, this arginine was mutated to alanine, and the  $\delta'$ (R158A) mutant was purified and reconstituted into  $\gamma$  complex for analysis. If ATP site D is not hydrolytic, the  $\delta'$ (R158A)  $\gamma$  complex should have wild-type levels of ATPase and clamp loading activity.

The  $\delta'$  (R158A) mutant was mixed with  $\gamma$ ,  $\delta$ ,  $\chi$  and  $\psi$  to reconstitute the " $\delta'$ (R158A)  $\gamma$  complex". The complex was stable to ion exchange chromatography yielding purified reconstituted  $\delta'$ (R158A)  $\gamma$  complex with similar subunit stoichiometry as wild-type  $\gamma$  complex (Figure 2.6A). ATP binding analysis by equilibrium gel filtration demonstrated that the  $\delta'$  (R158A)  $\gamma$  complex retained ability to bind three ATP molecules with similar affinity as wild-type  $\gamma$  complex (Figure 2.6B;  $K_d \sim 0.8 \mu\text{M}$ ). In contrast to the  $\gamma$ (R169A) complex, the  $\delta'$ (R158A)  $\gamma$  complex retained some activity in the catalytic assays requiring that

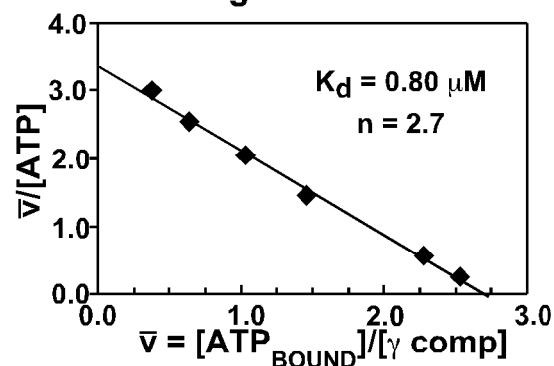
**Figure 2.6 The  $\delta'$ (R158A)  $\gamma$  complex is impaired in clamp loading and ATPase activity.** Reconstituted  $\delta'$ (R158A)  $\gamma$  complex was analyzed for ATP binding,  $\beta$  interaction, ATPase activity and clamp loading. *Panel A* The reconstituted  $\delta'$ (R158A)  $\gamma$  complex has a similar ratio of subunits as wild-type  $\gamma$  complex. *Panel B* Equilibrium gel filtration demonstrates that the  $\delta'$ (R158A)  $\gamma$  complex binds 2.7 ATP molecules with a  $K_d$  value of 0.8  $\mu$ M. *Panel C* Steady-state ATPase assays show that the  $\delta'$  (R158A)  $\gamma$  complex (diamonds,  $-\beta$ ; open squares,  $+\beta$ ) has reduced ATPase activity compared to wild-type  $\gamma$  complex  $\sim 35\%$   $-\beta$ , and  $\sim 15\%$   $+\beta$ ). The activity of wild-type  $\gamma$  complex is shown for comparison. (triangles,  $-\beta$ ; closed circles,  $+\beta$ ). *Panel D* DNA replication assays reflect the level of clamp loading activity by the  $\delta'$  (R158A)  $\gamma$  complex (diamonds) compared to wild-type  $\gamma$  complex (triangles).

Figure 2.6 The  $\delta'(R158A)$   $\gamma$  complex is impaired in clamp loading and ATPase activity.

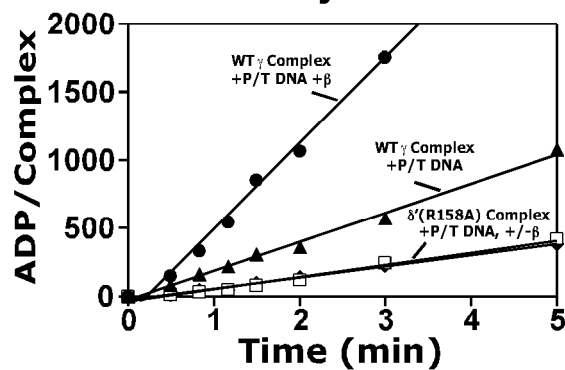
### A. Reconstitution



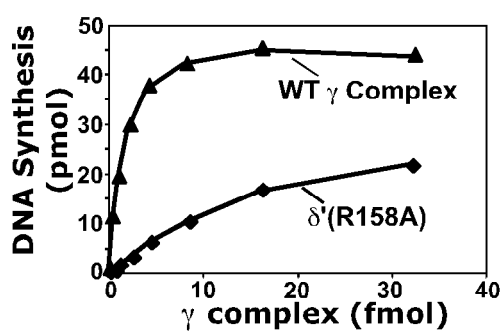
### B. ATP Binding



### C. ATPase Activity



### D. DNA Synthesis



ATP be hydrolyzed. The  $\delta'$ (R158A)  $\gamma$  complex retained approximately 30% of the DNA dependent ATPase activity of wild-type  $\gamma$  complex, although  $\beta$  no longer stimulated the ATPase as it did wild-type  $\gamma$  complex (Figure 2.6C). Also, the DNA synthetic activity of  $\delta'$ (R158A)  $\gamma$  complex was about 20-30% active compared to wild-type  $\gamma$  complex (Figure 2.6D). Hence, the  $\delta'$ (R158A)  $\gamma$  complex retains some clamp loading activity, but is nevertheless significantly compromised compared to wild-type  $\gamma$  complex.

The above results demonstrate that ATP site D is catalytic, since  $\delta'$ (R158A)  $\gamma$  complex is significantly less active than wild-type  $\gamma$  complex. If site D were only used for ATP binding, the  $\delta'$ (R158A)  $\gamma$  complex would have been expected to be fully active in the catalytic assays. Hence arginine 158 of  $\delta'$  is important to catalytic activity of  $\gamma$  complex, consistent with conservation of the SRC motif among prokaryotic  $\delta'$  subunits.

## DISCUSSION

### **The $\delta'$ stator contributes a catalytic arginine to the clamp loader motor:**

The  $\gamma$  complex clamp loader has been proposed to consist of three main components (Jeruzalmi et al., 2001b; O'Donnell et al., 2001): the  $\delta$  wrench (opens  $\beta$ ), the  $\gamma$  trimer motor (hydrolyzes ATP), and the  $\delta'$  stator (modulates  $\beta$  interaction with  $\delta$ ) (reviewed in (Jeruzalmi et al., 2002)). The  $\gamma$  and  $\delta$  subunits appear to have a flexible joint between the C-terminal domain (domain III), and the N-terminal domains (domains I/II). In contrast, the three domains of the  $\delta'$  stator appear to be held in a rigid conformation, thus the term stator, the stationary part of a machine upon which the other parts move (Jeruzalmi et al.,



2001; Podobnik et al., 2003). The C-terminal domains of all five subunits form a tight closed circular connection, holding the subunits together. However, the N-terminal domains have an interruption between  $\delta$  and  $\delta'$ . The size of this gap modulates the ability of  $\beta$  to interact with the  $\delta$  wrench. The rigid  $\delta'$  stator is proposed to function as an anvil, and when ATP is hydrolyzed, the  $\gamma$  subunits move  $\delta$  close to  $\delta'$ , forcing the  $\beta$  ring off the  $\delta$  wrench, allowing the  $\beta$  ring to close.

This report demonstrates that  $\delta'$ , besides its role as stator, also plays an instrumental role in the motor function of  $\gamma$  complex by supplying a catalytic arginine into ATP site D of  $\gamma$  complex. A catalytic role for this arginine was suggested by its proximity to ATP modeled into ATP site D of the  $\gamma$  complex structure (Jeruzalmi et al., 2001). Further, this arginine is embedded in an "SRC" motif that is highly conserved among clamp loading subunits of prokaryotes, eukaryotes and archaeobacteria. The *E. coli*  $\delta'$  subunit is a member of the AAA+ family and has the same chain fold as  $\gamma$ , yet  $\delta'$  does not bind ATP (Guenther et al., 1997). The P-loop of  $\delta'$  has been modified through evolution and the N-terminus blocks the nucleotide binding site. However, there are examples of prokaryotic  $\delta'$  subunits that contain a consensus P-loop (i.e. *Aquifex aeolicus* (Bruck et al., 2002)). Whether these  $\delta'$  subunits bind ATP is not known. However, even if these  $\delta'$  subunits bind ATP they may not hydrolyze it for lack of a catalytic arginine in the neighboring  $\delta$  subunit. Perhaps noncatalytic ATP provides rigidity to these  $\delta'$  subunits without needing the extra connections between Domains III and Domains I/II observed in the *E. coli*  $\delta'$  stator.

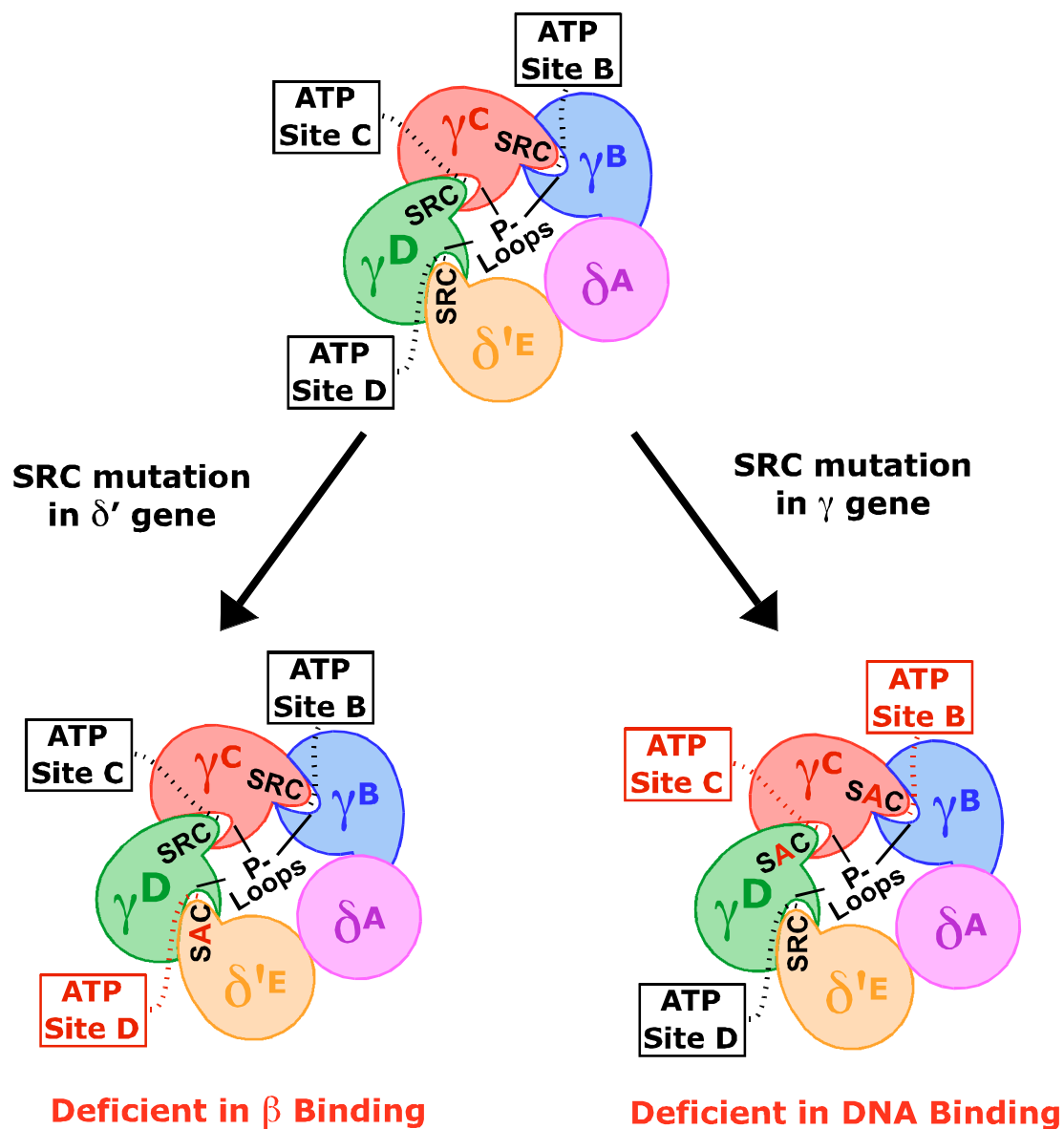
The catalytic role played by  $\delta'$  in clamp loading ATPase action may explain why the  $\delta'$  sequence is highly conserved in prokaryotes compared to the sequence of  $\delta$ . The  $\delta$  subunit is the main subunit responsible for opening the  $\beta$  clamp, but it has no catalytic role (Jeruzalmi et al., 2001b; Turner et al., 1999). Simple maintenance of protein-protein contacts, with no catalytic role to preserve, has apparently allowed the  $\delta$  sequence to drift considerably. The catalytic role played by  $\delta'$  may be responsible for the much greater conservation of the  $\delta'$  sequence.

**Pre-hydrolysis roles for arginine fingers of  $\gamma$  complex:**

Further collaborative studies have lead us to a deeper level of detail regarding the biophysical effects of the  $\delta'$  and  $\gamma$  arginine finger mutations (summary in Figure 2.7) (Snyder et al., 2004). These investigations were performed using sensitive fluorescence techniques for quantitatively measuring the affinity of  $\gamma$  complex for  $\beta$  and DNA. It was found that the  $\delta'$ (R158A) mutant is significantly deficient in ATP-dependent binding to fluorescently-labeled  $\beta$  (Snyder et al., 2004), which may partially explain why  $\beta$  does not stimulate DNA-associated ATP hydrolysis of this mutant (Figure 2.6C). However, the  $\delta'$ (R158A)  $\gamma$  complex can still load  $\beta$  for use with Pol III (Figure 2.6D), therefore, this reduction in affinity is not severe enough to abolish clamp loading activity. The  $\gamma$ (R169A) mutant complex was still active for  $\beta$  binding, but instead showed a severe deficiency in fluorescent-DNA binding, compared to wild-type (Snyder et al., 2004).

**Figure 2.7 Summary of fluorescent studies of  $\beta$  and DNA binding with  $\gamma$  complex arginine finger mutants.** In collaboration with Linda Bloom's group, investigation of the  $\delta'$ (R158A) and  $\gamma$ (R169A)  $\gamma$  complex mutants was continued (Snyder et al., 2004). A summary of the results is represented schematically. The  $\delta'$ (R158A)  $\gamma$  complex was deficient in associating with fluorescently-labeled  $\beta$  (*left*). The  $\gamma$ (R169A) complex was deficient in fluorescent DNA association (*right*).

Figure 2.7 Summary of fluorescent studies of  $\beta$  and DNA binding with  $\gamma$  complex arginine finger mutants.



### **An ordered hydrolysis model for clamp loading:**

This report demonstrates that mutation of arginine 169 in  $\gamma$ , which removes the arginine of the SRC motif near ATP sites B and C, abolishes ATPase activity even though three ATP molecules still bind the mutant  $\gamma$  complex and ATP site D remains intact. This mutant is compromised in DNA binding activity, which may partially explain this phenotype. However, this result may also suggest that site D cannot hydrolyze ATP until after ATP in sites B and/or C is hydrolyzed. Conversely, if ATP must be hydrolyzed in sites B and/or C before ATP is hydrolyzed in site D, then an ATP site D mutant may not block hydrolysis of ATP in sites B and C. Indeed, this expectation is largely upheld in this study. The  $\delta'$ (R158A)  $\gamma$  complex retains significant ATPase activity indicating that ATP in sites B and/or C can be hydrolyzed, even when site D is missing the catalytic arginine. The  $\delta'$ (R158A)  $\gamma$  complex also retains some clamp loading activity allowing it to support processive DNA synthesis. Thus, hydrolysis of ATP in sites B and C would appear to be sufficient, although not optimal, for clamp loading.

### **Generalization of these results to other clamp loaders.**

The studies of this report on the *E. coli* clamp loader have implications for subunit function in clamp loaders of other organisms. The eukaryotic RFC heteropentamer is composed of five different subunits, three of which contain both the SRC motif and consensus P-loops, suggesting they may act similarly to the  $\gamma$  trimer motor (yeast RFC B•C•D and human p36•37•40) (Cullmann et al., 1995). Indeed, these complexes contain DNA-dependent ATPase activity (Cai et

al., 1997; Ellison and Stillman, 1998). One RFC subunit (yRFC-E and human p38) contains the SRC motif, but lacks a consensus P-loop, and thus is analogous to the  $\delta'$  stator, and may contribute a catalytic arginine for ATP hydrolysis in the RFC-B/C/D motor. The yRFC1 subunit (human p128) forms a strong attachment to the PCNA clamp (Fotedar et al., 1996) and thus may be analogous to the  $\delta$  wrench. Like *E. coli*  $\delta$ , yRFC-A lacks an SRC motif, but unlike  $\delta$  it contains a consensus P-loop suggesting it binds ATP. Mutation of this P-loop is without significant effect on yRFC activity, indicating that this fourth ATP site in the eukaryotic clamp loader may be coupled to some other process (Schmidt et al., 2001).

Studies of P-loop mutants of yRFC-B, C, and D show that mutation of either RFC-D or C greatly reduce ATPase activity and clamp loading function of RFC, while mutation of RFC-B has much less of an effect on these activities (Cullmann et al., 1995). These results are similar to the current study of  $\gamma$  complex. Namely,  $\delta'$ (R158A)  $\gamma$  complex is partially active, while  $\gamma$ (R169A) complex is completely inactive. Thus, it seems quite possible that the RFC clamp loader may also hydrolyze ATP in an ordered sequence around the circular pentamer, as proposed here for  $\gamma$  complex, where some sites must first hydrolyze ATP before ATP in other sites can be hydrolyzed. These similarities will be discussed in more detail in Chapters 4 and 5.

The five-subunit clamp loader of bacteriophage T4 has two different subunits, four copies of gp44 and one gp62 subunit. Biochemical studies demonstrate that ATP is hydrolyzed to load the T4 gp45 clamp onto DNA; stoichiometry measurements range from 1-4 ATP per gp45 clamp loading event

(Berdis and Benkovic, 1996; Pietroni et al., 2001; Sexton et al., 1998; Young et al., 1996). The gp44 tetramer is an ATPase, contains both the SRC motif and P-loop, and is homologous to *E. coli*  $\gamma$  and  $\delta'$ . The gp62 is similar to  $\delta$  in that it has neither the SRC motif nor P-loop, and its sequence has diverged from  $\gamma/\delta'$ . At first glance it would seem that the T4 clamp loader has done away with the stator, and indeed it may have. However, keeping in mind that gp62 has no SRC motif, it seems likely that one gp44 subunit (i.e. adjacent to gp62) will be incapable of hydrolyzing ATP. Thus, one gp44 subunit may serve a similar role as  $\delta'$  in modulating contact between the gp44/62 clamp loader and the gp45 clamp, at the same time as providing a catalytic arginine residue for ATP hydrolysis in the neighboring gp44 subunit.

The clamp loaders of archaeobacteria have been studied, but less intensively than the clamp loaders of *E. coli*, T4, yeast and humans. Generally, archaeobacterial clamp loaders (called RFC) consist of two subunits, RFC large and RFC small (Cann et al., 1999). The stoichiometry of these subunits is not certain, with reports ranging from 1:4, like T4, to 3:2 and even 4:2 (Cann et al., 2001; Pisani et al., 2000; Seybert et al., 2002). The crystal structure of *Pyrococcus furiosus* RFC small subunit shows that its basic unit is a trimer, presumably the equivalent to the  $\gamma$  trimer motor, in which each subunit contains both a nucleotide binding site and a closely juxtaposed arginine from the neighboring subunit (Oyama et al., 2001). The *Pyrococcus* RFC large subunit contains a consensus P-loop motif and thus may bind nucleotide, but lacks the SRC motif. The lack of a SRC motif in the RFC large subunit suggests that one ATP site will

be unable to hydrolyze ATP and thus may function in an analogous fashion as the  $\delta'$  stator.

#### **Possible role of the SRC arginines in clamp loading fidelity.**

The  $\gamma$  complex is a very poor ATPase without a DNA effector. The crystal structure indicates that  $\gamma$  R169 and  $\delta'$  R158 are not quite close enough to function with ATP, they must move an extra one or two angstroms to have an effect on ATP hydrolysis (Jeruzalmi et al., 2001). Hence, it is tempting to speculate that misalignment of these arginines may underlie the very weak ATPase of  $\gamma$  complex, and their proper positioning may be used as a regulatory mechanism.

DNA stimulates the  $\gamma$  complex ATPase activity, and thus may bring the SRC motif Arg/ATP site pairs into a more favorable alignment for hydrolysis. Curiously, the  $\beta$  subunit only stimulates the  $\gamma$  complex ATPase when a primed template is used as an activator; ssDNA and duplex DNA stimulate  $\gamma$  complex in the absence of  $\beta$ , but do not give more activity when  $\beta$  is added (Onrust and O'Donnell, 1993). Consistent with this observation,  $\gamma$  complex does not load  $\beta$  onto ssDNA, even though ssDNA stimulates ATPase activity (Stukenberg et al., 1991). Furthermore, the ATPase cycle is tightly coupled to clamp loading, as only 2-3 ATP are hydrolyzed for each  $\beta$  clamp that is loaded onto a primed template (Hingorani and O'Donnell, 1998; Turner et al., 1999). Finally, the head-to-tail architecture of the  $\beta$  dimer generates two distinct “front” and “back” faces, only one of which functions with the DNA polymerase and thus it must be oriented correctly on DNA to interface with the polymerase (Kong et al., 1992; Naktinis et al., 1996). What system of checks and balances does  $\gamma$  complex have



to ensure that these criteria have been met? It seems possible that the catalytic arginines, and their juxtaposition to the ATP sites, may act as a fidelity mechanism to ensure that  $\beta$  is only loaded when primed DNA is threaded through  $\beta$ , and only when  $\beta$  is oriented correctly for function with polymerase. Perhaps when these different criteria are met, the catalytic SRC motif arginine residues are brought into register for ATP hydrolysis to propel loading of the  $\beta$  clamp onto DNA.

## Using $\gamma$ Complex Subunit Fusions to Further Dissect ATP Site Function

### ABSTRACT

The *E. coli*  $\gamma$  complex clamp loader is formed from one copy each of the  $\delta$ ,  $\delta'$ ,  $\chi$  and  $\psi$  subunits and three identical copies of the  $\gamma$  ATPase subunit. Thus, it is not possible to make a mutation in one specific  $\gamma$  subunit within the complex. In this study we examine *E. coli* clamp loader complexes formed with a mixture of mutant and wild-type ATPase subunits using two different approaches: (a) *in vitro* mixture of free subunits and (b) generation of a clamp loader reconstituted using a three-subunit fusion. We find that these strategies are successful for creating mixed wild-type/mutant clamp loader complexes. Furthermore, our studies suggest that all three ATP sites must contain an arginine finger residue for efficient  $\beta$  loading. A fusion-containing  $\gamma$  complex with an arginine finger mutation positioned in ATP site C is more deficient in DNA-stimulated ATP hydrolysis than a site B mutant. This result suggests that the  $\gamma$ R169A complex studied in Chapter 2 is deficient in DNA association due mainly to the arginine finger mutation in ATP site C, rather than site B.

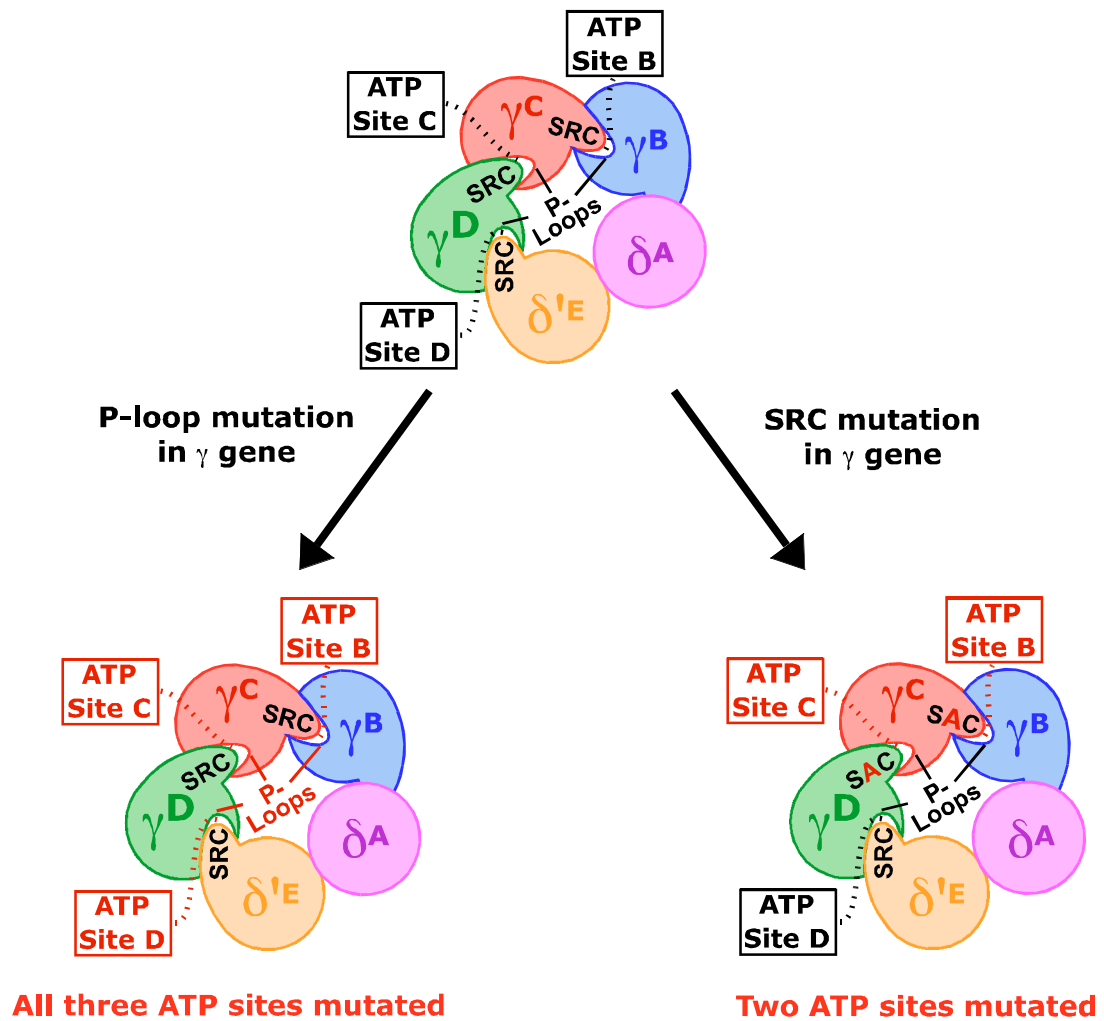
## INTRODUCTION

The studies in Chapter 2 of arginine finger mutants of *E. coli*  $\gamma$  complex indicate that certain ATP sites have specific functions during clamp loading. ATP site D acts to coordinate  $\beta$  association through an ATP sensor activity of the  $\delta'$  (E subunit) arginine finger (Snyder et al., 2004). ATP sites B and/or C are involved primarily in promoting DNA association when ATP is bound (Snyder et al., 2004). In addition, ATP sites B and C remain active and can support clamp loading when site D is mutated, but the entire complex is inactive when sites B and C are mutant (Johnson and O'Donnell, 2003). The conclusions of these previous studies were limited in detail by the fact that mutation of the arginine finger in the  $\gamma$  subunit disrupts both ATP sites B and C of the clamp loader, due to the presence of multiple  $\gamma$  subunits in the complex (Figure 3.1). We therefore wished to determine the contribution of each of these two ATP sites to  $\gamma$  complex activity. Towards this goal, we investigated ways of creating  $\gamma$  complexes with only one mutant  $\gamma$  subunit.

Several strategies have been employed previously to create mixed wild-type/mutant complexes of homooligomeric ATPase rings (Farr et al., 2000; Hishida et al., 2004; Hishida et al., 2003; Horwich et al., 1998; Martin et al., 2005). A simple strategy for introducing a mutant subunit into an otherwise wild-type oligomeric ring is to simply mix the two species and allow the subunits to exchange. It is not possible to position a mutant subunit in a specific place in an oligomer based on this approach. However, this strategy is useful for determining whether a single mutant subunit, when introduced into the

**Figure 3.1. Mutation of the  $\gamma$  subunit introduces either two or three mutations into the ATP sites of  $\gamma$  complex.** The *E. coli* clamp loader contains three copies of the  $\gamma$  subunit. Introduction of a mutation into any one of the  $\gamma$  subunit residues that contribute to the ATP binding pocket (P-loop mutation, for example) creates a  $\gamma$  complex with all three ATP sites mutated (*left*). Introduction of a mutation in the  $\gamma$  subunit residues that interact secondarily with ATP bound in the adjacent pocket (such as a mutation in the Ser-Arg-Cys (SRC) motif) generates mutations in ATP sites B and C.

Figure 3.1 Mutation of the  $\gamma$  subunit introduces either two or three mutations into the ATP sites of  $\gamma$  complex.



complex, has a severe effect on activity, or whether one wild-type subunit can remain active with all other subunits mutated. RuvB branch migration activity was analyzed in this manner to demonstrate that ATP hydrolysis can be uncoupled from branch migration (Hishida et al., 2003) and to confirm the importance of the arginine finger of RuvB (Hishida et al., 2004).

A more precise method to create a wild-type/mutant complex involves using peptide linkers that attach monomers together via C- to N-terminal linkage. Detailed structural information is useful in constructing subunit fusions to constrain the complex to adopt only one particular arrangement. This fusion strategy can allow the formation of a single-chain homooligomer in which each monomer is generated from a unique DNA sequence. Thus, each monomer has a specific identity in the oligomer, relative to the other monomers in the fusion. The fusion construct gene can then be altered to create mutations in a specific monomer. The GroEL chaperonin was previously engineered as a single-chain heptamer of covalently-linked GroEL monomers to generate point mutations at specific monomer positions in the ring (Farr et al., 2000; Horwich et al., 1998). A similar strategy has recently been employed to study the ATP-coupled activity of the AAA+ homohexamer protease ClpX (Martin et al., 2005).

$\gamma$  complex differs from the homooligomeric rings mentioned above because the pentameric ring is comprised of three different polypeptides: three identical copies of the  $\gamma$  ATPase subunit, and one each of  $\delta$  and  $\delta'$  (Jeruzalmi et al., 2001; Pritchard et al., 2000). The presence of the  $\delta$  and  $\delta'$  subunits in  $\gamma$  complex create a structural asymmetry in the  $\gamma_3\delta\delta'$  pentamer, whereas the homooligomeric rings are symmetrical. Each  $\gamma$  subunit of  $\gamma$  complex has a unique

position: either adjacent to  $\delta$ , adjacent to two  $\gamma$  subunits, or adjacent to  $\delta'$ . Even though the  $\gamma$  monomers are each in a unique environment in the complex, they are identical subunits and thus one cannot reconstitute the complex with a single mutant  $\gamma$  at a specific position in the pentamer.

In this study we examine *E. coli* clamp loader complexes formed with a mixture of mutant and wild-type ATPase subunits using two different approaches: (a) *in vitro* mixture of free subunits and (b) generation of a clamp loader reconstituted using a three-subunit fusion. We find that these strategies are successful for creating mixed wild-type/mutant clamp loader complexes. Furthermore, investigation of the fusion-construct complexes suggests that all three ATP sites must be fully functional for wild-type level clamp loading activity. A minimal but significant amount of clamp loading activity was observed in all single-site arginine finger mutants generated as fusion complexes, implying that inactivation of any one ATP site does not abolish clamp loading completely.

## RESULTS

### **$\gamma_2\tau(\text{SAC})_1$ mutant complexes are no more than 30% active in clamp loading**

A simple approach to introducing a mutant ATPase subunit into the *E. coli* clamp loader was used to test whether introducing an arginine finger mutation in ATP site C or B randomly would abolish clamp loading activity. We took advantage of the fact that the  $\tau$  subunit, which contains the entire  $\gamma$  polypeptide sequence along with a unique C-terminus of ~24 kDa, binds tightly to a MonoS column while the  $\gamma$ - $\gamma$ - $\delta'$  construct does not. Pre-mixing of  $\gamma$  and  $\tau$  and subsequent

formation of clamp loader complexes yields a mixture of complexes with DnaX ATPase subunit stoichiometries of  $\gamma_3$ ,  $\gamma_2\tau_1$ ,  $\gamma_1\tau_2$ , and  $\tau_3$  (Pritchard et al., 2000).  $\tau$  can take the place of  $\gamma$  in the clamp loader complex and all  $\tau$ -containing species are equally active in clamp loading (data not shown).

We used a step-wise reconstitution scheme whereby excess wild-type  $\gamma$  subunit was mixed with  $\tau$ (SAC) to form hetero-oligomers, then the  $\delta$ ,  $\delta'$ ,  $\chi$ , and  $\psi$  subunits were added (Figure 3.2A). Finally, the mixture was separated on a MonoS column at an ionic strength that only allowed  $\tau$ -containing complexes to bind the column (complexes containing three copies of  $\gamma$ , no copies of  $\tau$ , pass through the resin when concentrations of NaCl are above ~100 mM). In this way the unique C-terminus of the  $\tau$  subunit, not present in the shorter  $\gamma$  subunit, acts as an affinity tag for (SAC) mutant-containing complexes.

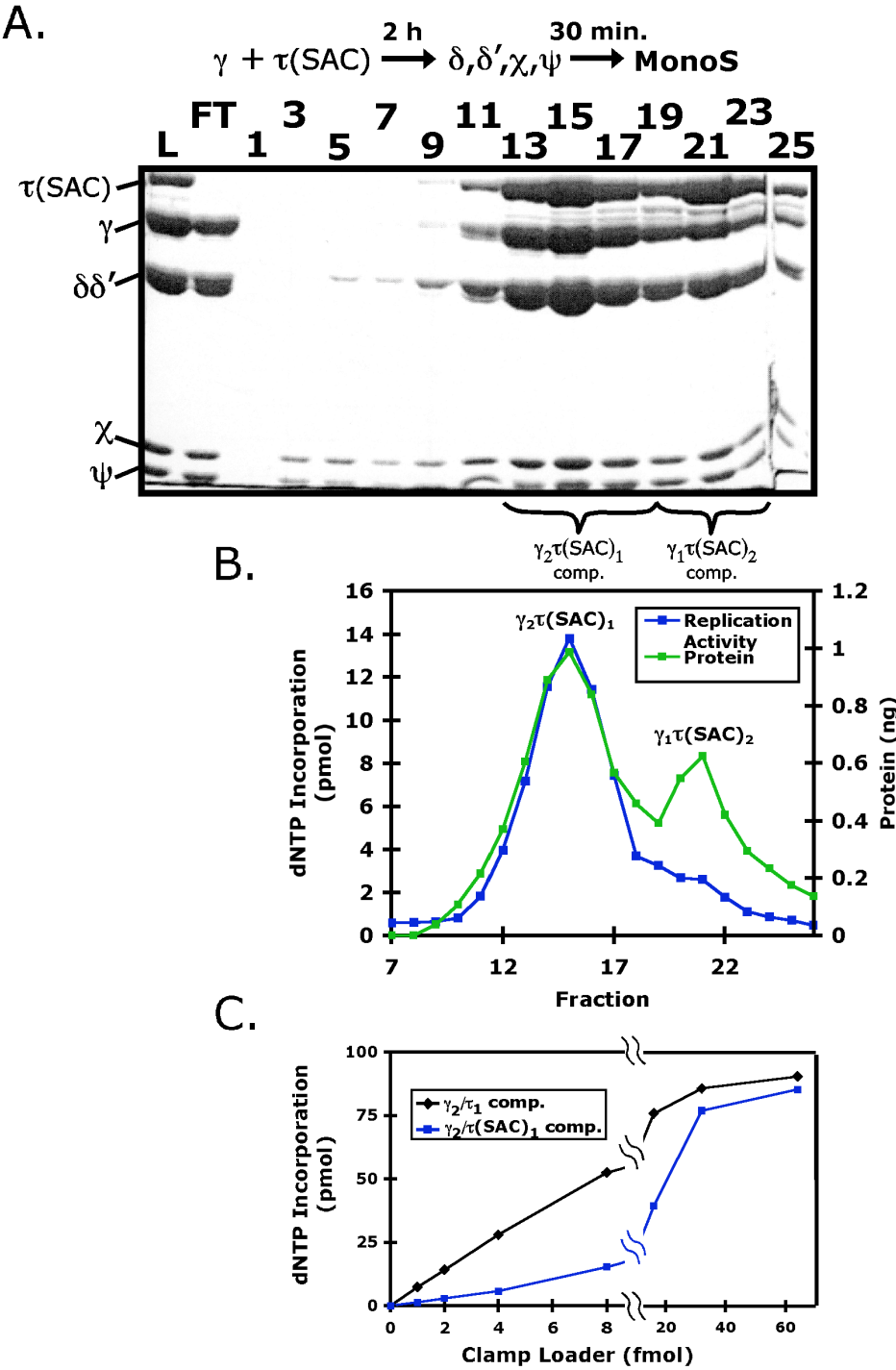
Reconstitution and purification of  $\tau$ (SAC)-containing complexes yielded a majority of  $\gamma_2\tau$ (SAC)<sub>1</sub> species, in addition to  $\gamma_1\tau$ (SAC)<sub>2</sub> and  $\tau$ (SAC)<sub>3</sub> species (Figure 3.2A). Gel filtration analysis of mixed  $\gamma/\tau$  complexes indicated that they are stable clamp loader complexes and laser densitometer scanning of SDS-PAGE-separated samples demonstrated that each complex has the expected stoichiometry (A. Johnson, P. McNerney, and M. O'Donnell, unpublished observations).

Fractions from the MonoS purification were collected and analyzed for protein concentration as well as the ability to promote processive replication of a primed DNA template by the *E. coli* core polymerase in the presence of  $\beta$  and ATP. This replication activity is indicative of  $\beta$  loading at the primer site. A peak of replication activity coincided with the  $\gamma_2\tau$ (SAC)<sub>1</sub> clamp loader complex



**Figure 3.2. Reconstitution and activity of  $\gamma/\tau$ (SAC) complexes.** *Panel A* Clamp loader complexes containing a mixture of  $\gamma$  and  $\tau$ (SAC) subunits were reconstituted in two steps. The  $\gamma$  and  $\tau$ (SAC) subunits were incubated together and then the remaining clamp loader subunits were added. The mixture of  $\gamma_3$ ,  $\gamma_2\tau$ (SAC)<sub>1</sub>, and  $\gamma_1\tau$ (SAC)<sub>2</sub> clamp loader complexes was separated by MonoS chromatography. The  $\gamma_3$  clamp loader species passes through the column in the flowthrough, while the  $\gamma_2\tau$ (SAC)<sub>1</sub> and  $\gamma_1\tau$ (SAC)<sub>2</sub> species are eluted with an NaCl gradient. *Panel B* Protein-containing fractions that eluted during the gradient were analyzed for the ability to stimulate  $\beta$ -dependent processive replication of a singly-primed M13mp18 template by the Pol III core polymerase. *Panel C* The  $\gamma_2\tau$ (SAC)<sub>1</sub> fraction from the MonoS reconstitution was titrated into a similar replication assay to that in *Panel A* and compared to the  $\gamma_2\tau_1$  complex containing wild-type  $\tau$ .

Figure 3.2 Reconstitution and activity of  $\gamma/\tau(\text{SAC})$  complexes.



protein peak (Figure 3.2B). A second replication activity peak was not observed in the later fractions containing the  $\gamma_1\tau(\text{SAC})_2$  complex, however a significant amount of residual activity, presumably from the  $\gamma_2\tau(\text{SAC})_1$  species, was present in these fractions.

Three populations of clamp loader are likely present in the  $\gamma_2\tau(\text{SAC})_1$  pool:  $\tau(\text{SAC})$ , if randomly distributed, can occupy either the B, C, or D position of the clamp loader pentamer. The arginine fingers of the C and D subunits point into ATP site B and C, respectively. The arginine finger in the “B” subunit of  $\gamma$  complex does not point into an ATP site. Instead, this residue points into the  $\delta^A$  subunit, which does not have an ATP binding pocket. The interaction of this arginine from  $\gamma^B$  with  $\delta^A$  is not predicted to be essential or serve a role in clamp loading. We therefore, assumed that a complex with this residue mutated would retain 100% wild-type activity. Assuming full randomization in the position that  $\tau$  occupies in the  $\gamma_2\tau(\text{SAC})_1$  species, the mix should represent one-third complex with three active ATP sites, one-third with two good ATP sites at B and D, and one-third with good ATP sites at C and D.

To assess the activity of the  $\gamma_2\tau(\text{SAC})_1$  complex relative to wild-type we titrated this clamp loader into a replication assay. In this experiment the “wild-type” clamp loader used was a  $\gamma_2\tau_1$  clamp loader complex generated in a similar manner to the  $\gamma_2\tau(\text{SAC})_1$  species, except that wild-type  $\tau$  subunit was used instead of  $\tau(\text{SAC})$ . The results of the titration indicate that the  $\tau(\text{SAC})$ -containing complex retains 20-30% activity compared to the “wild-type” complex (Figure 3.2C).

One may assume that a  $\gamma_2\tau(\text{SAC})_1$  complex in which  $\tau(\text{SAC})$  occupies the “B” position will be fully active. In fact the  $\gamma_2\tau(\text{SAC})_1$  mixture does not produce more than 30% activity. We conclude from this result that introducing the (SAC) mutation into either ATP site B or site C inactivates the clamp loader. Thus each ATP site is equally important for placing  $\beta$  onto DNA for use with Pol III. Alternatively, the mixing scheme we employed does not generate an equal mixture of species with one-third of the mixture containing  $\tau(\text{SAC})$  at each of the three possible positions. To address these two possibilities we pursued a strategy to generate defined clamp loader complexes with a mutation in only one ATP site.

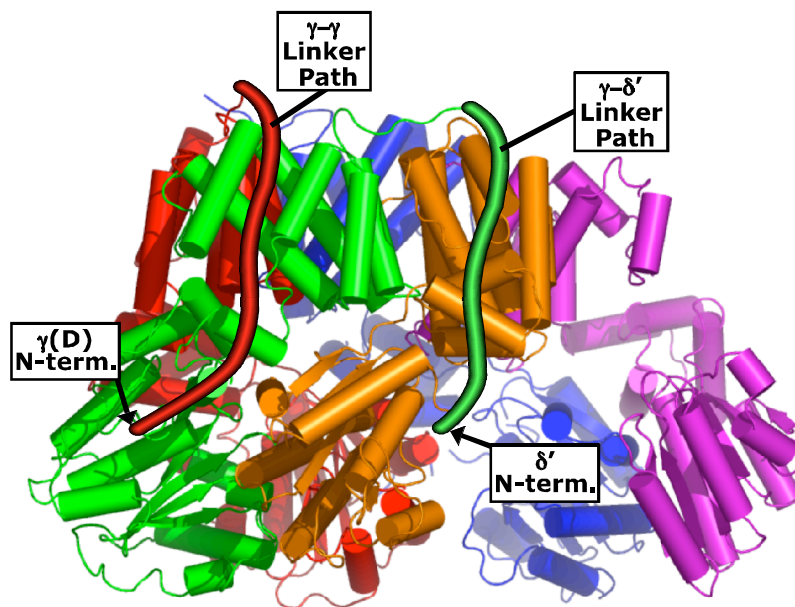
### **Generation of fused $\gamma$ complex subunits**

To introduce a mutation in a specific ATP site of  $\gamma$  complex, we developed a strategy to produce a  $\gamma$  subunit that would only be able to adopt one position in the clamp loader. This is best accomplished by tethering a  $\gamma$  subunit to one of the single-copy subunits in the  $\gamma$  complex pentameric ring, either  $\delta$  or  $\delta'$ . Based on the crystal structure of  $\gamma_3\delta\delta'$ , the distances between the N- and C-termini of adjacent subunits were determined (Figure 3.3A). The N-terminus of  $\delta'$  and the C-terminus of the adjacent  $\gamma$  subunit were chosen to be physically linked together, based on their proximity in the structure. We therefore created a fusion construct gene with this linkage, which we term  $\gamma$ - $\delta'$ . The native C-terminal sequence of  $\gamma$  was identified as unstructured and flexible by limited proteolysis (David Jeruzalmi and John Kuriyan, unpublished), therefore, the C-terminus of  $\gamma$  was fused directly to the N-terminus of  $\delta'$ , using no novel linker sequence.  $\delta'$  and

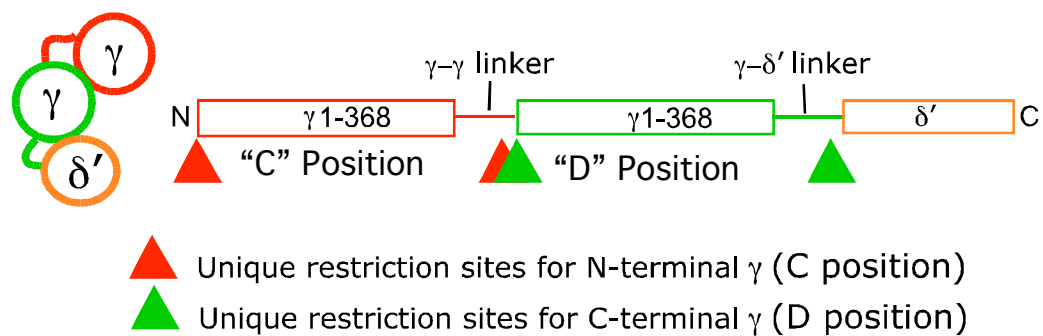
**Figure 3.3. Design of the  $\gamma$ - $\gamma$ - $\delta'$  fusion construct.** *Panel A* Crystal structure of the  $\gamma\delta\delta'$  complex depicting a hypothetical path that the two linkers in the  $\gamma$ - $\gamma$ - $\delta'$  fusion construct may follow to connect the three subunits. *Panel B* Schematic of the  $\gamma$ - $\gamma$ - $\delta'$  fusion construct polypeptide sequence. The rectangular regions denoted “ $\gamma$ 1-368” represent the three structured domains of  $\gamma$  complex present in the crystal structure. The “ $\gamma$  flexible linker” regions denote residues 369-417 that comprise the predicted unstructured C-terminal polypeptide sequence of each  $\gamma$  subunit. The colored triangles indicate the positions in the corresponding DNA sequence for each  $\gamma$  monomer that can be uniquely cut with restriction enzymes to introduce mutated  $\gamma$  sequence.

Figure 3.3 Design of the  $\gamma$ - $\gamma$ - $\delta'$  fusion construct.

A.



B.



one  $\gamma$  subunit associate in the clamp loader complex through domain III of each subunit. By fusing a  $\gamma$  subunit to  $\delta'$ , this interaction will likely be promoted, due to the intramolecular nature of the association. This fusion strategy should, in theory, provide a way to position a  $\gamma$  subunit in the “D” position of the pentamer, next to  $\delta'$  (in the E position).

The  $\gamma$ - $\delta'$  construct would presumably reconstitute with two free  $\gamma$  subunits,  $\delta$ ,  $\chi$ , and  $\psi$  to form the equivalent of the full  $\gamma$  complex. However, a  $\gamma$ - $\delta'$  construct is not sufficient to produce a clamp loader complex in which each  $\gamma$  is uniquely positioned, because free subunits would occupy the remaining two positions. Therefore, a three-subunit fusion construct was created where the C-terminus of a second  $\gamma$  subunit was fused directly to the N-terminus of the  $\gamma$ - $\delta'$  construct, generating the  $\gamma$ - $\gamma$ - $\delta'$  construct (Figure 3.3B). This construct should reconstitute with only one free  $\gamma$  subunit,  $\delta$ ,  $\chi$ , and  $\psi$  to form  $\gamma$  complex. Each  $\gamma$  subunit in this complex originates from a unique DNA sequence allowing mutation of individual  $\gamma$  subunits.

The linkers between the  $\gamma$  subunits in the  $\gamma$ - $\gamma$ - $\delta'$  construct are 49 native residues long, starting from the last ordered residue of the  $\gamma$  subunits from the  $\gamma\delta\delta'$  crystal structure (Jeruzalmi et al., 2001) (Figure 3.3) to the end of the  $\gamma$  sequence. It is possible that this linker length may not constrain the fusion construct to one specific arrangement. However the physical linkage is likely to promote subunits that are adjacent in primary structure to associate similarly in a ternary complex.

The  $\gamma$ - $\gamma$ - $\delta'$  construct was generated in the pET system and transformed into *E. coli* cells. The 132 kDa polypeptide overexpressed at high levels and a

significant fraction was soluble after cell lysis. The construct was subjected to ammonium sulfate precipitation and MonoQ chromatography to ~80% homogeneity. A number of smaller polypeptides remained in the prep, which could not be purified away from the fusion construct. These remaining impurities were removed during the reconstitution step described below (see Figure 3.4).

### **The $\gamma$ - $\gamma$ - $\delta'$ construct assembles into active $\gamma$ complex**

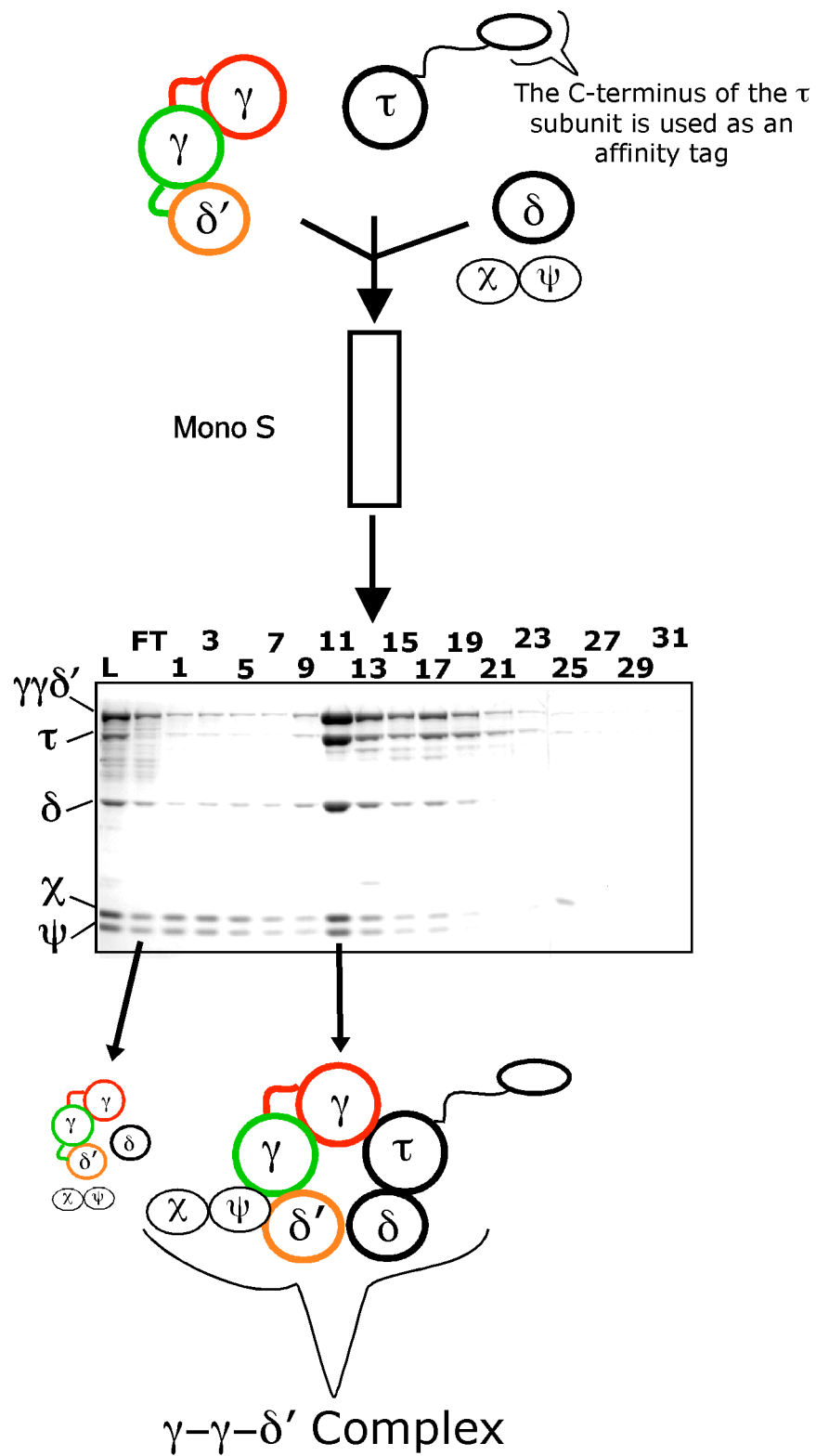
We next studied the  $\gamma$ - $\gamma$ - $\delta'$  fusion construct for the ability to associate with the remaining  $\gamma$  complex subunits into a functional clamp loader. The  $\gamma$ - $\gamma$ - $\delta'$  construct behaves similarly to free  $\gamma$  subunit during chromatographic separation. Thus, the  $\tau$  subunit was used as a type of affinity-tagged  $\gamma$  subunit, as demonstrated in Figure 3.2A, to isolate a full clamp loader complex with the fusion construct incorporated into it. An excess of  $\gamma$ - $\gamma$ - $\delta'$  construct was mixed with free  $\tau$  subunit and excess  $\delta$ ,  $\chi$ , and  $\psi$  subunits and the resulting  $\tau$ -containing complexes were purified by MonoS chromatography (Figure 3.4). A profile of this purification in Figure 3.4 shows that the free  $\gamma$ - $\gamma$ - $\delta'$ ,  $\delta$ ,  $\chi$ , and  $\psi$  pass through the resin in the flow-through, while a  $\gamma$ - $\gamma$ - $\delta'$ / $\tau$ / $\delta$ / $\chi$ / $\psi$  complex in an approximately equimolar stoichiometry is eluted during the NaCl gradient. This complex is herein referred to as the “ $\gamma$ - $\gamma$ - $\delta'$ ” clamp loader complex.

In Figure 3.5, the  $\gamma$ - $\gamma$ - $\delta'$  clamp loader complex ( $\gamma$ - $\gamma$ - $\delta'$ / $\tau$ / $\delta$ / $\chi$ / $\psi$ ) was compared to the  $\gamma_2\tau_1$  clamp loader complex ( $\gamma/\gamma/\delta'/\tau/\delta/\chi/\psi$ ) in clamp loading, replication, and ATP hydrolysis assays. First, the ability of the  $\gamma$ - $\gamma$ - $\delta'$  complex to assemble  $\beta$  onto a singly-primed circular M13mp18 single-stranded phage



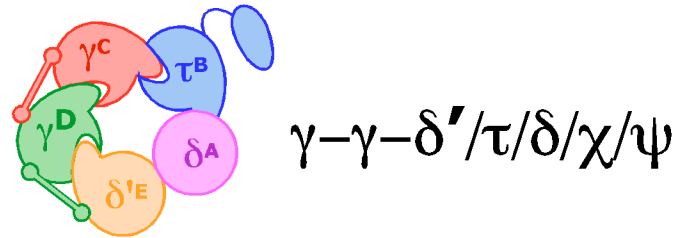
**Figure 3.4. Reconstitution of the  $\gamma\text{-}\gamma\text{-}\delta'$  complex.** Excess  $\gamma\text{-}\gamma\text{-}\delta'$ ,  $\delta$ ,  $\chi$ , and  $\psi$  were incubated with a limiting amount of  $\tau$  subunit and the mixture was separated by MonoS chromatography. A complex containing an equimolar stoichiometry of  $\gamma\text{-}\gamma\text{-}\delta'/\tau/\delta/\chi/\psi$  eluted during the NaCl gradient due to the presence of the unique C-terminal region of the  $\tau$  subunit which confers affinity for the resin.

Figure 3.4 Reconstitution of the  $\gamma$ - $\gamma$ - $\delta'$  complex.

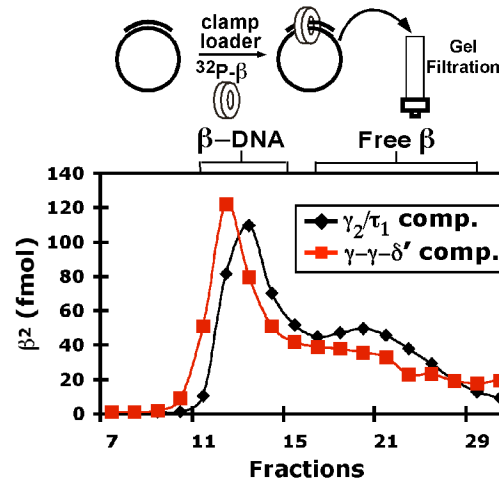


**Figure 3.5. The  $\gamma\text{-}\gamma\text{-}\delta'$  and  $\gamma_2\tau_1$  clamp loader complexes are equally active.** The  $\gamma\text{-}\delta'$  complex was examined in three assays for *E. coli* clamp loader activity and compared to the equivalent  $\gamma_2\tau_1$  clamp loader formed with all free subunits ( $\gamma/\gamma/\delta'/\tau/\delta/\chi/\psi$ ). *Panel A* The ability of the  $\gamma\text{-}\gamma\text{-}\delta'$  complex to assemble  $\beta$  onto a singly-primed circular DNA was tested. Either  $\gamma_2\tau_1$  clamp loader or  $\gamma\text{-}\gamma\text{-}\delta'$  complex was incubated with  $^{32}\text{P}$ -labeled  $\beta$ , SSB-coated singly-primed M13mp18 DNA, and ATP and the reaction was subsequently separated on a Bio-Gel A-15m column.  $\beta$  co-elutes with the large DNA in early fractions (fractions 12-16) while free  $\beta$  elutes later (fractions 17-30). *Panel B* The  $\gamma\text{-}\gamma\text{-}\delta'$  complex was tested in an assay for  $\beta$ -dependent DNA replication of the M13mp18 template by Pol III core polymerase.  $\beta$  must be loaded at the primer-template junction for the polymerase to produce a signal in this assay. *Panel C* The DNA-associated ATP hydrolysis activity in the absence (*striped bars*) and presence (*solid bars*) of  $\beta$  was analyzed by direct measurement of  $^{32}\text{P}$ -labeled ADP production from ATP.

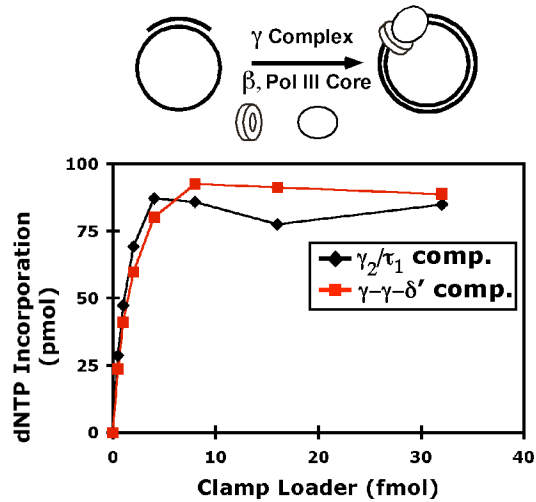
Figure 3.5 The  $\gamma$ - $\gamma$ - $\delta'$  and  $\gamma_2\tau_1$  clamp loader complexes are equally active .



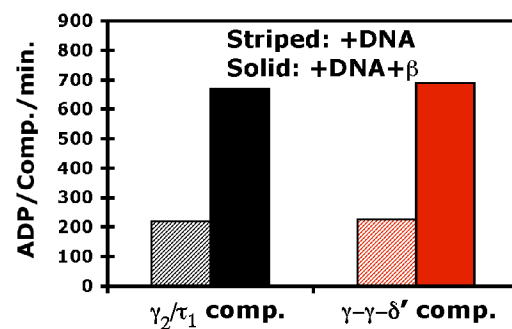
**A.**



**B.**



**C.**



genome was assayed directly. A  $\beta$  construct with a C-terminal kinase tag was used in order to radioactively label  $\beta$  with  $^{32}\text{P}$ -phosphate. The  $^{32}\text{P}$ -labeled  $\beta$  was incubated with  $\gamma_2\tau_1$  complex or the  $\gamma$ - $\gamma$ - $\delta'$  complex in the presence of ATP and SSB-coated primed-M13mp18 circular ssDNA (Figure 3.5A). The reaction was subsequently applied to a large-pore Bio-gel column. DNA-associated  $\beta$  elutes in the early fractions from this column, while free  $\beta$  elutes later. The  $\gamma$ - $\gamma$ - $\delta'$  complex performed similarly to the  $\gamma_2\tau_1$  complex in this assay, producing an early-migrating peak of DNA-associated  $\beta$  (Figure 3.5A).

Next, we tested the ability of the  $\gamma$ - $\gamma$ - $\delta'$  complex to promote  $\beta$ -dependent processive replication by *E. coli* Pol III core polymerase,  $\alpha\epsilon\theta$ . Pol III core cannot replicate singly-primed ssM13mp18 template without  $\beta$  loaded at the primer template. A titration of  $\gamma_2\tau_1$  complex a mixture of SSB-coated M13 template, Pol III core, and  $\beta$ , stimulated dNTP incorporation of Pol III core to fully replicate the M13 template (Figure 3.5B). Titration of  $\gamma$ - $\gamma$ - $\delta'$  complex into the replication assay yielded a similar stimulation of replication as the  $\gamma_2\tau_1$  complex.

The ATP hydrolysis activity of the  $\gamma$ - $\gamma$ - $\delta'$  complex was tested for its ability to be stimulated by DNA and  $\beta$ .  $\gamma$ - $\gamma$ - $\delta'$  complex displayed a DNA-associated steady-state ATPase rate comparable to  $\gamma_2\tau_1$  complex and this activity was stimulated three-fold by the presence of  $\beta$  (Figure 3.5C), presumably indicating repeated rounds of clamp loading.

The  $\gamma$ - $\gamma$ - $\delta'$  complex behaved similarly to the equivalent clamp loader formed from all free subunits in each of the above assays for  $\beta$  loading activity and ATP hydrolysis. These results indicate that the polypeptide linkers used to generate the  $\gamma$ - $\gamma$ - $\delta'$  fusion do not have negative effects on clamp loader activity.

Next we examined the effect of mutations made in the  $\gamma$ - $\gamma$ - $\delta'$  construct when the  $\gamma$ - $\gamma$ - $\delta'$  clamp loader complex was formed.

**The two single arginine finger mutant  $\gamma$ - $\gamma$ - $\delta'$  complexes are both nearly inactive for  $\beta$  loading**

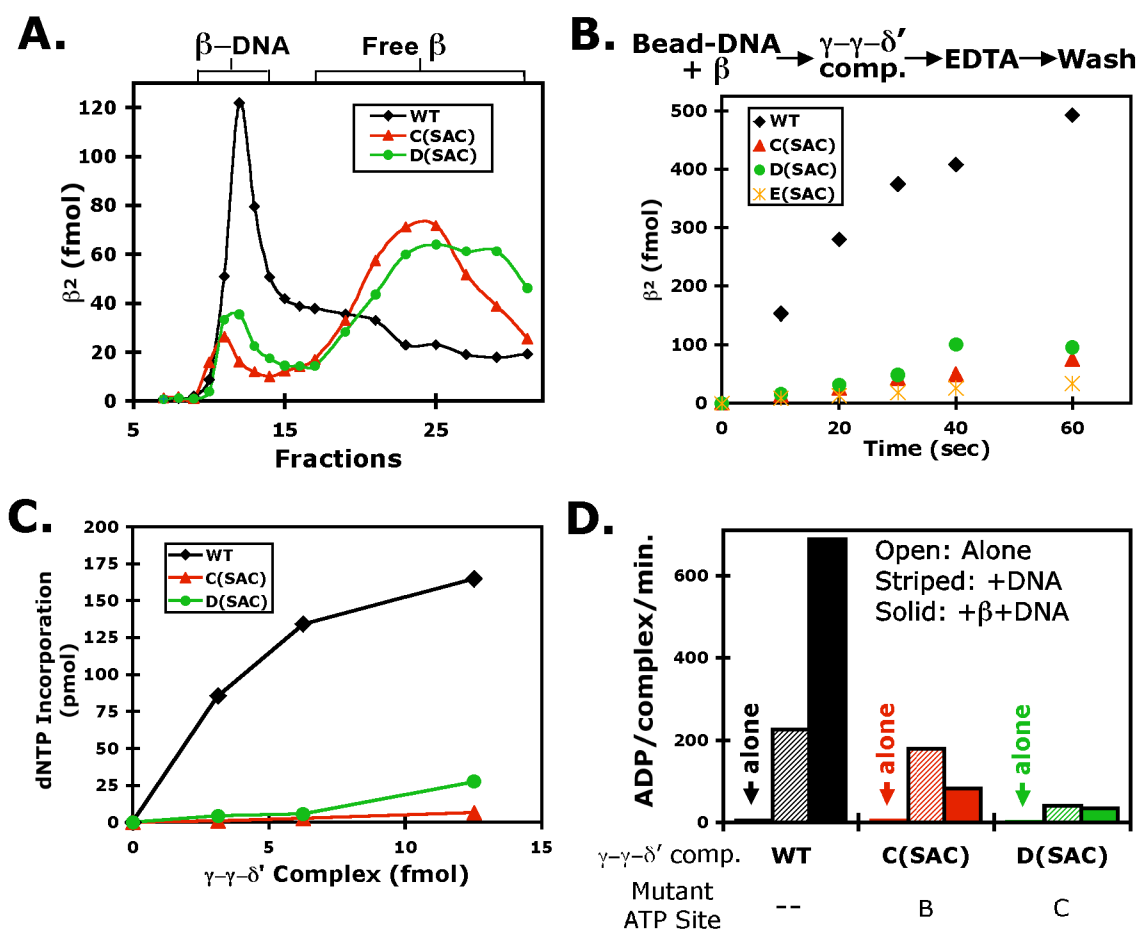
The  $\gamma$ - $\gamma$ - $\delta'$  fusion gene was altered to create an arginine finger to alanine mutant  $\gamma$  at the C position in the  $\gamma$  complex pentamer (the N-terminal  $\gamma$  of the fusion construct) or the D position (the C-terminal  $\gamma$ ). These two mutant fusion constructs are referred to as  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) or  $\gamma$ - $\gamma$ - $\delta'$  D(SAC). Both of these single point mutant fusion constructs were purified similarly to the “wild-type” fusion construct that did not contain an arginine finger point mutation. Both mutant fusion constructs were reconstituted with  $\tau$ ,  $\delta$ ,  $\chi$ , and  $\psi$  and the resulting clamp loader complexes that were purified by MonoS chromatography are referred to as  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) complex or  $\gamma$ - $\gamma$ - $\delta'$  D(SAC) complex.

We first tested these mutant  $\gamma$ - $\gamma$ - $\delta'$  complexes for the ability to load  $\beta$  onto singly-primed M13 template, in a similar experiment to that in Figure 3.5A. The  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) and the  $\gamma$ - $\gamma$ - $\delta'$  D(SAC) mutant complexes were both significantly deficient in this assay, compared to wild-type  $\gamma$ - $\gamma$ - $\delta'$  complex, displaying 20-25% wild-type activity (Figure 3.6A).

The two mutant  $\gamma$ - $\gamma$ - $\delta'$  complexes were also tested in a kinetic assay for  $\beta$  loading using the magnetic bead-conjugated DNA template. This assay is a more stringent test of the mutants because many reactions can be quenched and separated in a short period of time and quantitated to produce a timecourse. A magnetic bead-conjugated synthetic primed-template coated with SSB was used

**Figure 3.6.  $\gamma\text{-}\gamma\text{-}\delta'$  complexes with a single arginine finger mutation in ATP site B or C display similar  $\beta$  loading activity.**  $\gamma\text{-}\gamma\text{-}\delta'$  clamp loader complexes containing an arginine finger mutation in either the C or D position were analyzed for clamp loader activity, compared to the  $\gamma\text{-}\gamma\text{-}\delta'$  complex without an arginine finger mutation (referred to as “wild-type”). *Panel A* Wild-type and mutant  $\gamma\text{-}\gamma\text{-}\delta'$  complexes were assayed for the ability to load  $\beta$  on a singly-primed template as in Figure 3.6A. *Panel B* Wild-type and mutant  $\gamma\text{-}\gamma\text{-}\delta'$  complexes were assayed in a kinetic assay for  $\beta$  loading (*scheme at top*). For comparison, the activity of the  $\delta'$ (R158A)  $\gamma$  complex in the same experiment is shown (*orange stars*). *Panel C* Wild-type and mutant  $\gamma\text{-}\gamma\text{-}\delta'$  complexes were titrated into the replication assay described in Figure 3.5B. *Panel D* Wild-type and mutant  $\gamma\text{-}\gamma\text{-}\delta'$  complex ATP hydrolysis was measured alone (*open bar*), in the presence of primed DNA (*striped bars*), or in the presence of  $\beta$  and primed DNA (*solid bars*). Due to the comparatively low activity, arrows indicate the lane containing the rate of the complex alone.

Figure 3.6  $\gamma$ - $\gamma$ - $\delta'$  complexes with a single arginine finger mutation in ATP site B or C display similar  $\beta$  loading activity.





as a  $\beta$  loading template. The  $\gamma$ - $\gamma$ - $\delta'$  clamp loader complexes were incubated with the bead-conjugated template,  $^{32}\text{P}$ -labeled  $\beta$ , and ATP for a short time before the reaction was quenched with EDTA. The DNA was separated using a magnetic concentrator and the amount of  $\beta$  remaining was quantitated. A 60-second timecourse of  $\beta$  loading was measured. In this experiment, both mutants displayed almost identical clamp loading rates, both of which were  $\sim 10\%$  wild-type. The clamp loading rates observed for the  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) and D(SAC) mutants were also comparable to the  $\beta$  loading rate of the  $\delta'$ (R158A)  $\gamma$  complex (labeled "E(SAC)" complex in Figure 3.6B) in this assay. These results suggest that disrupting any one ATP site in  $\gamma$  complex with an arginine finger mutation has an equal effect on the ability to load  $\beta$  onto DNA, yet a minimal clamp loading activity remains.

#### **The $\gamma$ - $\gamma$ - $\delta'$ D(SAC) complex is less active than the C(SAC) complex in DNA-stimulated ATPase activity**

Next, we examined the effect of the single (SAC) mutations in the  $\gamma$ - $\gamma$ - $\delta'$  complex on the ability of the clamp loader to hydrolyze ATP at steady-state. A synthetic, linear DNA template was used in this assay, in order to allow for  $\beta$  to slide off the template and thus be recycled for continued use. Interestingly, the two  $\gamma$ - $\gamma$ - $\delta'$  (SAC) mutant clamp loaders displayed different activities in the presence of this DNA template alone (without  $\beta$  present). The  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) complex retained near wild-type steady-state ATP hydrolysis activity under these conditions ( $\sim 178$  ATP hydrolyzed/min.), but the  $\gamma$ - $\gamma$ - $\delta'$  D(SAC) mutant ATPase activity was five-fold less than the C(SAC) mutant (Figure 3.6D). In the

presence of DNA and  $\beta$ , both mutants displayed similar activities, only ~5-10% that of wild-type  $\gamma$ - $\gamma$ - $\delta'$  complex. The  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) activity was depressed two-fold from the DNA alone activity, while the D(SAC) mutant activity remained the same as with DNA alone. The relative activities of the two  $\gamma$ - $\gamma$ - $\delta'$  (SAC) mutant complexes in the presence of DNA and  $\beta$  together, compared to wild-type, resembled the activities of these two mutants in the  $\beta$  loading assays described in Figures 3.6A-C.

Although it is clear that an (SAC) mutation in either one of the  $\gamma$  genes in the  $\gamma$ - $\gamma$ - $\delta'$  fusion causes a comparable deficiency in clamp loading activity, a significant difference in DNA-stimulated ATP hydrolysis activity was observed. Attempts to address the DNA binding activities of these mutant complexes were not successful, due to the significant DNA binding activity present in the C-terminus of the  $\tau$  subunit that is not present in  $\gamma$ . Further investigation of these ATP sites will require a new strategy for reconstitution that does not rely on the  $\tau$  subunit to purify the complex.

## DISCUSSION

The composition of the  $\gamma$  complex pentamer, containing three identical  $\gamma$  ATPase subunits (Jeruzalmi et al., 2001; Pritchard et al., 2000), makes site-directed mutagenesis to study the ATP-dependent steps of the mechanism difficult. In order to dissect the contributions of each  $\gamma$  subunit to these steps, we constructed a three-subunit fusion,  $\gamma$ - $\gamma$ - $\delta'$  (Figure 3.3B), which allowed for site-directed mutagenesis of  $\gamma$  subunits in two specific positions in the clamp loader (position D for the  $\delta'$ -adjacent  $\gamma$  and position C for the other  $\gamma$ ). This fusion

construct was reconstituted with the  $\tau$  subunit, which is comprised of the  $\gamma$  polypeptide sequence plus a 24 kDa C-terminal extension, and  $\delta$ ,  $\chi$ , and  $\psi$  to form an active clamp loader complex. The  $\tau$  subunit occupies the last  $\gamma$  position in the clamp loader pentamer ring (the B position), which allowed purification of the reconstituted clamp loader on MonoS.

The fusion strategy used in this study was successful in generating a soluble three-subunit construct that was capable of reconstituting into a fully-active clamp loader complex. The goal of this study is to introduce a mutation into one specific  $\gamma$  subunit position while leaving the other two subunits unaltered. The length of linker that we used herein may not, in theory, constrain the fusion construct monomers to one specific arrangement. Modeling from the structure indicates that a 20-amino acid linker is long enough to connect  $\gamma^D$  to  $\delta'$  or  $\gamma^C$  to  $\gamma^D$ , but not  $\gamma^C$  to  $\delta'$ .

Both the  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) and D(SAC) mutants were inactive in  $\beta$  loading (Figure 3.6A-C). This result was actually predicted from the experiment in Figure 3.2 in which the  $\gamma_2\tau$ (SAC)<sub>1</sub> complex mixture displayed no more than 30% activity. In the presence of  $\beta$  and DNA, both  $\gamma$ - $\gamma$ - $\delta'$  (SAC) mutants had less than 15% steady-state ATP hydrolysis activity (Figure 3.6D). Interestingly, the D(SAC) mutant was more deficient in DNA-stimulated ATP hydrolysis, while the C(SAC) mutant displayed ~80% wild-type activity. The fact that the two mutants have distinctly different activities in this assay suggests that the mutant  $\gamma$  is in a different position in each complex. In addition, the clamp loading assays with the two mutant  $\gamma$ - $\gamma$ - $\delta'$  complexes (Figure 3.6A-C) suggest that an arginine finger mutation in either ATP site 2 or 3 will cause a severe deficiency in clamp

loading. This conclusion arises independently of the actual arrangement of the  $\gamma$ - $\delta'$  complex.

In the arginine finger mutant study using free  $\gamma$  complex subunits (see Chapter 2), the  $\gamma$ (R169A) mutant complex contained arginine finger mutation in both ATP sites B and C (Johnson and O'Donnell, 2003). This mutation causes a loss of DNA binding that results in abolished DNA-stimulated ATP hydrolysis (Johnson and O'Donnell, 2003; Snyder et al., 2004). We presume that the  $\gamma$ - $\gamma$ - $\delta'$  D(SAC) mutant complex introduces an arginine finger mutation into ATP site C only. The fact that the site C  $\gamma$ - $\gamma$ - $\delta'$  complex mutant was deficient in DNA-stimulated ATP hydrolysis while the site B mutant (the  $\gamma$ - $\gamma$ - $\delta'$  C(SAC) complex) was considerably less affected in this activity suggests that the mutation in ATP site C alone may result in loss of DNA binding ability, or at least a deficiency in responding to DNA association with increased ATP hydrolysis. Unfortunately, the design of the  $\gamma$ - $\gamma$ - $\delta'$  clamp loader complex precludes examining the ATP-dependent DNA binding ability of the clamp loader due to the ATP-independent DNA binding activity of the  $\tau$  C-terminus which was used in this study to affinity purify the  $\gamma$ - $\gamma$ - $\delta'$  complex. An alternate strategy for reconstitution of this complex using a His-tagged version of the free  $\gamma$  subunit would presumably allow for purification of a  $\gamma$ - $\gamma$ - $\delta'$ /His- $\gamma$ / $\delta$ / $\chi$ / $\psi$  complex. This may prove to be a more powerful strategy for future studies of the biophysical activities of the  $\gamma$ - $\gamma$ - $\delta'$  clamp loader complexes.

In conclusion, we present a successful strategy for fusing the subunits of the  $\gamma$  complex heteropentamer to generate a mutation in one of three  $\gamma$  (or  $\tau$ ) subunits. The generation of a soluble fusion construct was surprisingly

straightforward, which is encouraging for further studies using similar constructs. Although we have not identified an ATP site in  $\gamma$  complex that is expendable for  $\beta$  loading, our results do suggest that each  $\gamma$  subunit may play a particular role during the clamp loading mechanism.

### **The RFC Clamp Loader Requires Arginine Finger Sensors to Drive DNA Binding and PCNA**

#### **ABSTRACT**

Replication factor C (RFC) is an AAA+ heteropentamer that couples the energy of ATP hydrolysis to the loading of the DNA polymerase processivity clamp, proliferating cell nuclear antigen (PCNA), onto DNA. The RFC complex contains four ATP sites, each located at the interface of two subunits. In each ATP site, an arginine residue from one subunit is located near the  $\gamma$ -phosphate of ATP bound in the adjacent subunit, but this arginine does not contribute to ATP binding. These arginines act as “arginine fingers” that can potentially perform two functions: sensing that ATP is bound and catalyzing ATP hydrolysis. In this study, the arginine fingers in RFC were mutated to examine the steps in the PCNA loading mechanism that occur after RFC binds ATP. This report finds that the ATP sites of RFC function in distinct steps during loading of PCNA onto DNA. ATP binding to RFC powers recruitment and opening of PCNA and activates a  $\gamma$ -phosphate sensor in ATP site C that promotes DNA association. DNA binding to the RFC-PCNA complex then triggers the ATP hydrolysis cycle which starts in site C and ends with ATP site D. ATP hydrolysis in site D is uniquely stimulated by PCNA and we propose that this site is coupled to closure of PCNA around DNA. PCNA closure severs contact to RFC subunits D and E (RFC2 and RFC5), and the  $\gamma$ -phosphate sensor of ATP site C is switched off, resulting in a low-affinity of RFC for DNA, leading to ejection of RFC from the site of PCNA loading.

## INTRODUCTION

All cellular organisms utilize a multi-protein, ATP-driven DNA replicase which functions with other proteins to duplicate chromosomal DNA prior to cell division (Kornberg and Baker, 1992). DNA replicases are comprised of a DNA polymerase, a ring-shaped processivity clamp, and a clamp loader ATPase (reviewed in (Johnson and O'Donnell, 2005)). The DNA polymerase binds to the processivity clamp protein, which completely encircles DNA. The clamp slides on DNA and is pulled along by the polymerase during chain extension, continuously holding polymerase to DNA and constraining it to act in a highly processive manner.

The eukaryotic ring-shaped processivity factor, called proliferating cell nuclear antigen (PCNA), is a trimer of three identical subunits arranged head-to-tail to generate a ring with a large central cavity for encircling DNA (Gulbis et al., 1996; Krishna et al., 1994). PCNA confers processivity on DNA polymerase  $\delta$  (Prelich et al., 1987a; Prelich et al., 1987b; Tan et al., 1986) and also interacts with other factors involved in DNA metabolism such as DNA polymerase  $\epsilon$ , flap endonuclease -1 (FEN-1), DNA ligase, mismatch repair proteins and many others (Maga and Hubscher, 2003; Warbrick, 2000). An interface between two PCNA monomers must be disrupted and opened in order to place DNA into the center of the ring. The ring must then be re-closed for the clamp to remain bound to the DNA and function with the polymerase. The eukaryotic clamp loader complex, replication factor C (RFC), uses the energy of ATP binding and hydrolysis to recruit the processivity clamp to DNA, break one clamp interface, and topologically link the clamp to primed-template DNA (Majka and Burgers, 2004; Tsurimoto and Stillman, 1991a). The *E. coli* clamp loader and DNA

polymerase compete for the clamp and therefore the clamp loader must eject from the clamp in order for the DNA polymerase to use it (Naktinis et al., 1996). Likewise, RFC and Pol  $\delta$  have also been shown to compete for binding to PCNA (Mossi et al., 1997; Oku et al., 1998). Therefore, after PCNA is linked to DNA, RFC must eject from the clamp to allow the polymerase access to the clamp (summarized in Figure 4.1A).

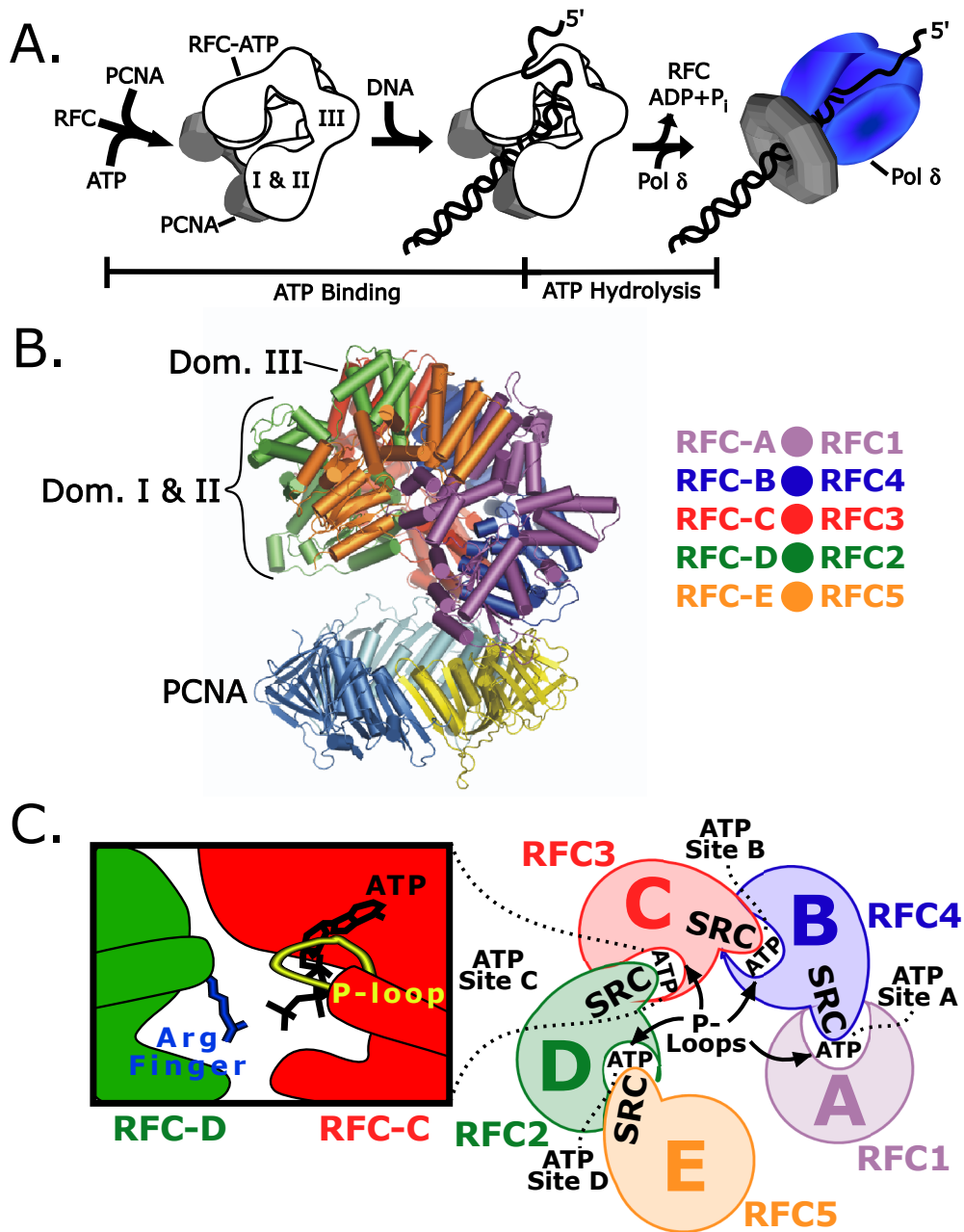
A crystal structure of *S. cerevisiae* RFC-ATP $\gamma$ S-PCNA reveals RFC to be a circular pentamer (Figure 4.1B) (Bowman et al., 2004). This subunit arrangement is similar to the *E. coli*  $\gamma$  complex clamp loader (Jeruzalmi et al., 2001), which loads the ring-shaped  $\beta$  clamp onto DNA (Stukenberg et al., 1991). An alphabetical nomenclature (e.g. subunits A-E) has been proposed to correlate subunits that occupy spatially similar positions in diverse clamp loader structures. The current study adopts the alphabetical nomenclature to refer to the five RFC subunits (see Figure 4.1C).

The structural homology between prokaryotic and eukaryotic clamps and clamp loaders suggests that the clamp loading mechanisms may be similar, and biochemical studies in both systems support this suggestion (O'Donnell et al., 2001) (Gomes and Burgers, 2001; Hingorani and O'Donnell, 1998). The clamp loader binds ATP to promote clamp binding and opening. The clamp loader-ATP-open clamp complex then associates with primed-template DNA. The association of clamp loader-ATP-clamp complex with DNA does not appear to require ATP hydrolysis and it is thought that DNA is positioned inside the central channel of the clamp during this step (Bowman et al., 2005; Bowman et al., 2004; Goedken et al., 2005; Zhuang et al., 2006). DNA binding triggers ATP



**Figure 4.1 ATP-coupled mechanism of RFC.** *Panel A* Cartoon model of the ATP binding- and hydrolysis-coupled steps of PCNA loading. After PCNA is loaded, RFC must disengage from PCNA to allow the polymerase access to the clamp. *Panel B* Crystal structure of RFC-ATP $\gamma$ S-PCNA (Bowman et al., 2004). RFC is partially-engaged with the closed PCNA ring. The C-terminal “collar” of RFC (*top*) is comprised of domain III of each RFC subunit and domains I and II form the AAA+ modules that bind ATP and PCNA. *Panel C* Schematic of the arrangement of ATP sites in the AAA+ modules of the RFC heteropentamer. Each ATP site is at a subunit interface. The neighboring subunit contains an arginine finger in a conserved SRC motif that interacts with the  $\gamma$ -phosphate of the ATP bound to the adjacent subunit. Right: Cartoon depicting ATP site C from the crystal structure of RFC-PCNA (*left*). ATP (*black*) bound to RFC-D (RFC2) interacts with the P-loop of RFC-C (RFC3) (*yellow*). The SRC arginine finger (*blue*) of RFC-D is proximal to the  $\gamma$ -phosphate of the ATP. *Panel D* List of mutations used in this study.

Figure 4.1 ATP-coupled mechanism of RFC.



**D. SRC → SAC Mutation**

- RFC-B<sub>4</sub> Arg157 → B(SAC) Site A
- RFC-C<sub>3</sub> Arg160 → C(SAC) Site B
- RFC-D<sub>2</sub> Arg183 → D(SAC) Site C
- RFC-E<sub>5</sub> Arg184 → E(SAC) Site D

hydrolysis in the clamp loader, which then closes the clamp and ejects from DNA, leaving the closed ring on DNA. This final stage of the clamp loading mechanism requires hydrolysis of ATP and is currently viewed as a single step, although multiple ATP molecules are hydrolyzed and thus this stage of the reaction is likely comprised of multiple steps.

The five RFC subunits, RFC-A through RFC-E (RFC1-5), share three domains of homology (domains I-III); only the RFC-A (RFC1) subunit has additional N- and C-terminal regions (Cullmann et al., 1995; Uhlmann et al., 1997b). The RFC pentamer complex is mainly held together by a tightly packed “collar” region consisting of domain III of each RFC subunit (see Figure 4.1A and B). The N-terminal ATPase modules, comprised of domains I and II, are arranged in a right-handed helix with a pitch that closely matches dsDNA (Bowman et al., 2004). A cavity exists in the center of this protein helix that is sufficiently large to accommodate dsDNA and conserved polar and positively charged residues line this cavity and are proposed to interact with DNA (Bowman et al., 2005; Bowman et al., 2004). There is a gap between two subunits, RFC-A and RFC-E (RFC1 and RFC5) (illustrated in Figure 4.1A and C), that is presumed to provide an entry path for DNA into the central cavity (Bowman et al., 2004). Mutation of the conserved residues that line the central cavity in  $\gamma$  complex causes a decrease in DNA binding, which supports the hypothesis that DNA binds in the central cavity of these clamp loaders (Goedken et al., 2005). A similar study in RFC shows that residues within the central cavity are required for DNA binding (Yao et al., 2006).

A recent electron micrograph study of *Pyrococcus furiosus* RFC reveals that PCNA adopts a helical conformation when held open in a complex of RFC-

ATP $\gamma$ S-PCNA-DNA (Miyata et al., 2005). Consistent with this observation, molecular dynamics simulations of yeast PCNA indicate that PCNA alone may readily adopt a right-handed helix when open (Kazmirski et al., 2005). The helical conformation of open PCNA may allow a more intimate contact between the clamp and the five subunits of RFC.

The ATPase modules of the five RFC subunits are homologous in sequence and structure to the AAA+ family of ATPases (Neuwald et al., 1999). This family includes a wide variety of factors that harness ATP to remodel protein substrates. A common feature of oligomeric AAA+ complexes is the location of ATP sites at the interface of two subunits. Interactions between AAA+ modules of RFC subunits create four interfacial ATP sites that are competent for hydrolysis (Bowman et al., 2004; Yao et al., 2003). These ATPase sites are referred to as ATP sites A-D (Figure 4.1C). RFC-E (RFC5) also binds nucleotide (Bowman et al., 2004), but the nucleotide binding pocket of RFC-E is not competent for hydrolysis, nor is it needed for RFC activity (Cai et al., 1998).

Each of the four interfacial ATP sites in RFC consist of one subunit that binds the ATP, plus side chains from the adjacent subunit (Figure 4.1C). The most prominent of the residues from the adjacent subunit is a highly conserved arginine in RFC Box VII, a region of homology that contains a Ser-Arg-Cys (SRC) motif found in all clamp loaders. The positive charge of the arginine sidechain is thought to facilitate interaction with the negatively charged  $\gamma$ -phosphate of ATP and is referred to as an “arginine finger”. In the *E. coli*  $\gamma$  complex clamp loader these arginine fingers have been shown to have two functions: they sense bound ATP and also play a catalytic role in ATP hydrolysis (Johnson and O'Donnell,

2003; Snyder et al., 2004). The sensor functions of the arginine fingers in the  $\gamma$  complex clamp loader are thought to be coupled to conformational changes that promote binding to the clamp and DNA (Snyder et al., 2004). The arginine fingers are needed for ATP hydrolysis (Johnson and O'Donnell, 2003) and are presumed to act by stabilizing the growing negative charge as the  $\gamma$ -phosphate is cleaved from ATP (Ahmadian et al., 1997; Jeruzalmi et al., 2001; Ogura et al., 2004). In both RFC and  $\gamma$  complex, ATP $\gamma$ S binding promotes ring opening and DNA binding, but the clamp does not reclose (Burgers, 1991; Hingorani and O'Donnell, 1998; Lee and Hurwitz, 1990; Tsurimoto and Stillman, 1991a; Zhuang et al., 2006); DNA binding is stabilized instead by direct contacts between the clamp loader and DNA (Ason et al., 2003; Goedken et al., 2005; Hingorani and O'Donnell, 1998; Yao et al., 2006; Zhuang et al., 2006). The arginine fingers are hypothesized to be involved in PCNA clamp opening as the RFC-ATP $\gamma$ S-PCNA structure, which utilized an RFC complex with the arginine fingers mutated, shows the PCNA ring is closed yet ATP $\gamma$ S is bound to all five subunits (Bowman et al., 2004). However, the crystal structure study utilized an RFC that also contains a deletion in the C-terminus of RFC-A, which appears to contact PCNA in EM reconstruction images, (Miyata et al., 2005; Nishida et al., 2005) and thus may underlie the closed ring in the structure.

Previous studies of prokaryotic and eukaryotic clamp loaders suggest that individual ATP sites play specific roles in clamp loading (Schmidt et al., 2001; Seybert and Wigley, 2004; Snyder et al., 2004). Mutational analysis in yeast RFC has shown that ATP binding to sites C and D of RFC is essential for DNA binding (Schmidt et al., 2001), but it is not clear how ATP binding is coupled to

DNA binding. In *E. coli*  $\gamma$  complex, the triphosphate sensor of the arginine finger in ATP site D promotes  $\beta$  clamp association, and the arginine fingers in sites B and/or C are involved in DNA binding (Snyder et al., 2004). These findings support the suggestion that there is a division of activities among ATP sites in clamp loaders. Perhaps specific ATP sites control other important clamp loader actions such as clamp closing and clamp loader ejection from the clamp and DNA.

This report examines different RFC complexes that have mutations in one or more arginine fingers. We show that none of the arginine fingers of RFC are needed for the ATP-binding-dependent steps of PCNA interaction and ring opening. However, our results demonstrate that certain ATP sites of RFC play distinct roles downstream of PCNA opening. The arginine finger in ATP site C is needed for RFC to bind DNA. DNA then triggers ATP hydrolysis in site C which leads to a loss of affinity to DNA. Continued interaction with PCNA holds RFC to DNA. However, ATP hydrolysis in site D is specifically triggered by PCNA and we propose that hydrolysis in this site leads to closure of PCNA around DNA. The crystal structure of RFC-PCNA shows that closed PCNA no longer interacts with RFC-D and RFC-E, and thus ring closure is presumed to lead to loss of RFC affinity for PCNA and dissociation of RFC from the PCNA-DNA complex.

## RESULTS

### Arginine finger mutants of RFC bind ATP similarly to wild-type

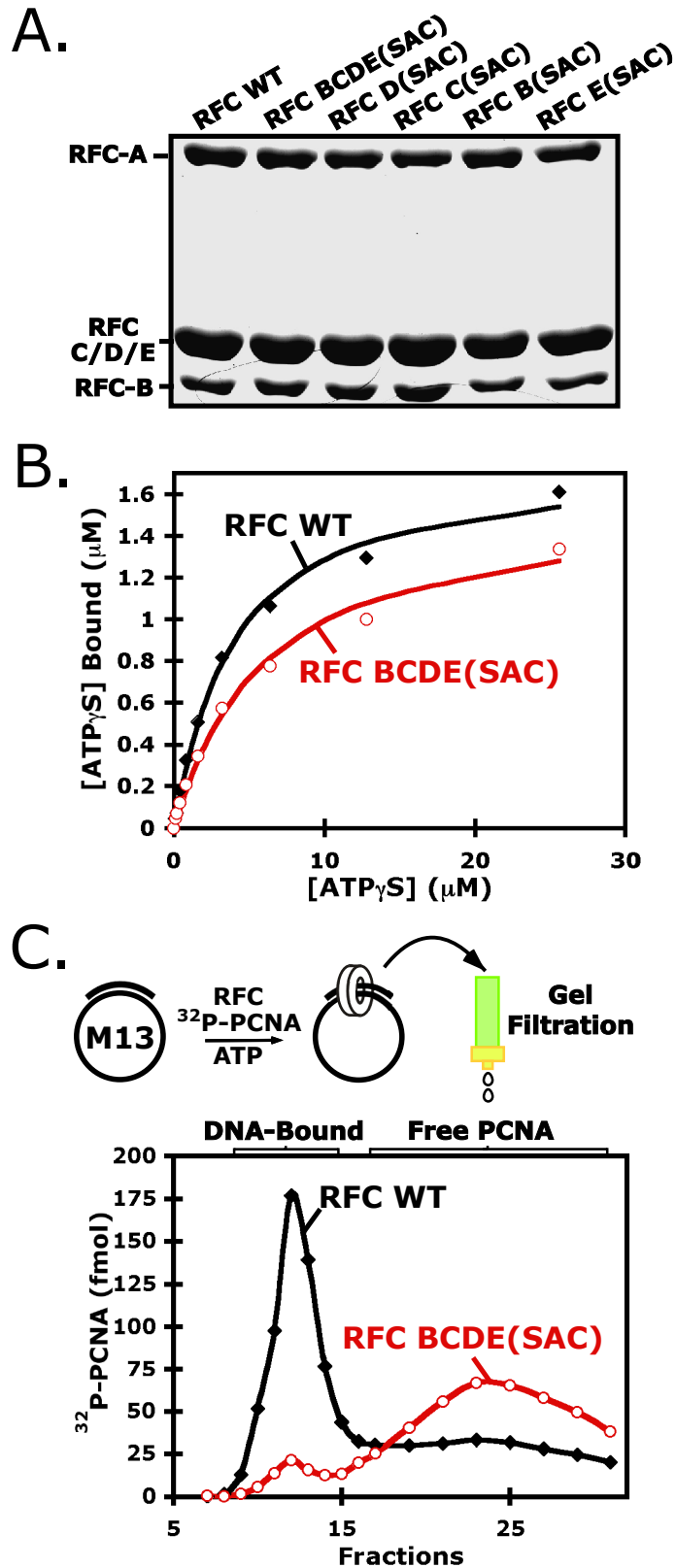
Arginine to alanine mutations were constructed in each of the SRC motifs of *S. cerevisiae* RFC (Figure 4.1D). Five-subunit RFC complexes containing the mutation in either RFC-B, RFC-C, RFC-D, or RFC-E were expressed in *E. coli* using a two-plasmid system and purified as described previously for wild-type RFC (Finkelstein et al., 2003). These single mutant five-subunit complexes are referred to as: RFC B(SAC), RFC C(SAC), RFC D(SAC), and RFC E(SAC). RFC-A does not contain an SRC motif. Therefore, no point mutants of RFC-A were used in this study. However, all RFC complexes used in this study carry a deletion of the N-terminal 283 residues of RFC-A; this region is not required for cell viability, displays non-specific DNA binding activity, and deletion of this region results in a complex with higher specific activity than RFC containing full-length RFC-A (Gomes et al., 2000; Uhlmann et al., 1997a). The RFC complex containing the truncated RFC-A is referred to herein as “wild-type” RFC.

We also constructed a quadruple arginine finger mutant, RFC BCDE(SAC). RFC BCDE(SAC) complex lacks arginine fingers entirely. Residual activities inherent to RFC BCDE(SAC) may be presumed to be independent of the arginine fingers. All of the single RFC (SAC) mutants should therefore be able to progress through the PCNA loading mechanism at least as far as the RFC BCDE(SAC) mutant. The RFC BCDE(SAC) complex and the single RFC (SAC) complexes were expressed and purified similarly to wild-type RFC and have comparable appearance in an SDS-polyacrylamide gel (Figure 4.2A), suggesting that the RFC (SAC) mutants fold properly.

**Figure 4.2 The RFC arginine fingers are required for PCNA loading, but not for ATP binding.** *Panel A* Analysis of 6  $\mu$ g each of wild-type and mutant RFC complexes in a 10 % SDS-PAGE stained with Coomassie Brilliant Blue (G250). Migration positions of the five RFC subunits are indicated to the left. *Panel B* Wild-type RFC (*black diamonds*) and RFC BCDE(SAC) (*red circles*) were incubated with the indicated amounts of  $^{35}\text{S}$ -ATP $\gamma$ S followed by analysis of bound nucleotide on nitrocellulose filters as described in “Materials and Methods.” *Panel C* Wild-type RFC (*black diamonds*) and RFC BCDE(SAC) (*red circles*) were tested for ability to load  $^{32}\text{P}$ -PCNA onto singly-primed circular M13mp18 ssDNA coated with *E. coli* SSB (see scheme at top). DNA-bound  $^{32}\text{P}$ -PCNA migrates early in the elution profile (fractions 9-15) while unbound  $^{32}\text{P}$ -PCNA migrates later (fractions 18-31).



Figure 4.2 The RFC arginine fingers are required for PCNA loading, but not for ATP binding.



Previous studies of the arginine fingers in the *E. coli*  $\gamma$  complex clamp loader concluded that these residues do not contribute to ATP binding (Johnson and O'Donnell, 2003). To test whether this is true for the RFC arginine fingers, we examined the ability of the RFC BCDE(SAC) mutant to bind nucleoside triphosphate. Using a nitrocellulose filter binding assay we determined the  $K_d$  value of wild-type RFC for ATP $\gamma$ S to be  $\sim 3.3 \mu\text{M}$ . The RFC BCDE(SAC) quadruple mutant displayed a  $K_d$  for ATP $\gamma$ S of  $5.5 \mu\text{M}$ , comparable to wild-type RFC (Figure 4.2B). This result indicates that the arginine fingers of RFC do not significantly contribute to ATP binding and this is consistent with the position of these residues across the subunit interface from the ATP binding pocket (Bowman et al., 2004).

#### **The RFC BCDE(SAC) complex cannot load PCNA**

To test whether ATP binding to RFC can promote PCNA loading in the absence of the arginine fingers, we measured the ability of the RFC BCDE(SAC) mutant to recruit PCNA to a primer-template DNA. A circular single-stranded M13mp18 phage DNA, uniquely primed with a DNA oligonucleotide and coated with *E. coli* SSB, was used as a substrate for PCNA loading. To follow clamp loading on DNA we used a PCNA construct containing a six-residue N-terminal kinase recognition sequence that enables it to be labeled with  $\gamma$ - $^{32}\text{P}$ -phosphate. The large DNA template, and  $^{32}\text{P}$ -PCNA attached to it, elutes in the early fractions (fractions 9-15) of a BioGel A15m gel filtration column, while the comparatively small  $^{32}\text{P}$ -PCNA that is not attached to DNA elutes in later fractions (fractions 18-31) (Figure 4.2C). Incubation of  $^{32}\text{P}$ -PCNA with the

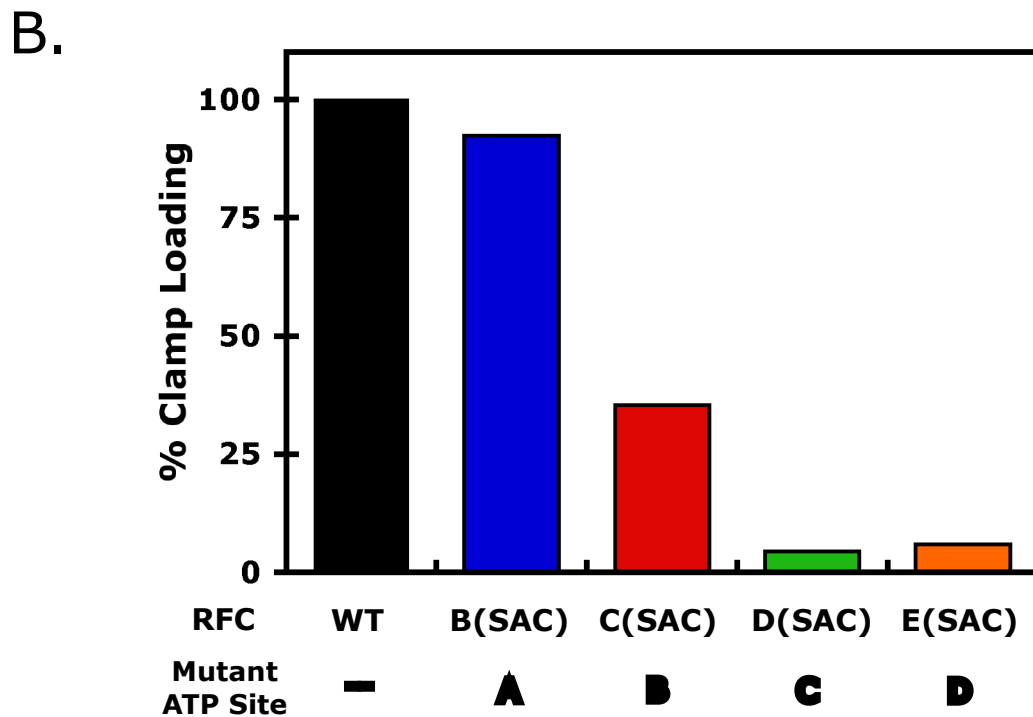
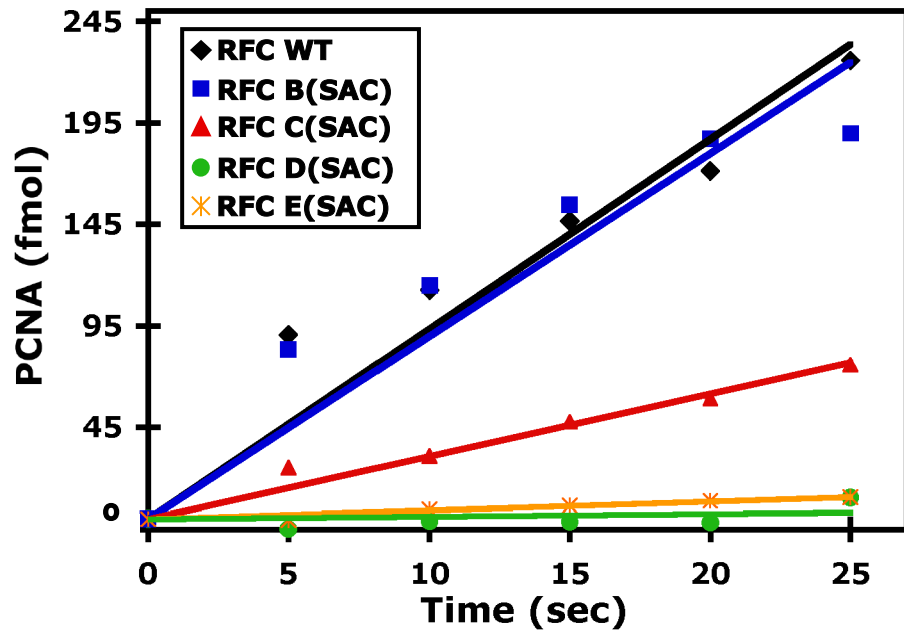
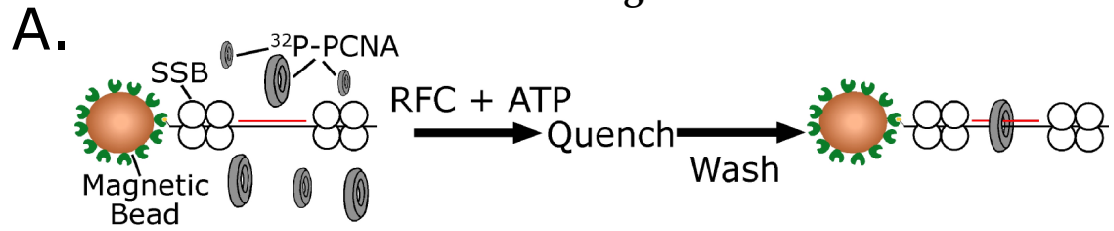
primed-DNA, ATP, and wild-type RFC generates a peak of  $^{32}\text{P}$ -PCNA that co-elutes with DNA in the early fractions (Figure 4.2C). However, the RFC BCDE(SAC) mutant does not load PCNA onto DNA. Therefore, one or more arginine fingers are needed to load PCNA onto DNA.

### **The arginine fingers of ATP sites C and D are required for efficient PCNA loading**

We next addressed which arginine fingers are required for RFC to load PCNA by testing single RFC (SAC) mutants for PCNA loading activity. Since wild-type RFC can load PCNA onto DNA in a very short time frame we designed a PCNA loading assay that allows rate measurements over a 25-second time course (see scheme in Figure 4.3). In this assay, a synthetic primed-template is first conjugated to a magnetic bead. To prevent PCNA sliding off the free end of the linear DNA, the template was designed with sufficient single-stranded DNA on both sides of the primer to facilitate the binding of *E. coli* SSB, which blocks PCNA from sliding off.  $^{32}\text{P}$ -labeled PCNA was added to the magnetic bead-coupled primed DNA and clamp loading was initiated upon addition of RFC. Reactions were rapidly quenched at timed intervals upon adding EDTA and then placed at 4°C. PCNA remains stably associated with the SSB-coated DNA with a half-life of ~ 50 min. at 4 °C (data not shown). The magnetic bead-conjugated DNA template enabled rapid separation of PCNA-DNA complexes from free PCNA within one minute. Western analysis showed that RFC does not remain bound to the PCNA-DNA complex (see Appendix Figure A1).

**Figure 4.3 RFC D(SAC) and RFC E(SAC) are severely deficient in PCNA loading.** *Panel A* The scheme at the top illustrates the magnetic bead-conjugated primed DNA assay to measure the kinetics of  $^{32}\text{P}$ -PCNA loading. *E. coli* SSB blocks the end of the DNA to prevent  $^{32}\text{P}$ -PCNA sliding off the linear DNA. The timecourse of  $^{32}\text{P}$ -PCNA loading by the indicated RFC complexes was performed as described in “Experimental Procedures.” *Panel B* Bar plot comparing the PCNA loading rates of mutant RFCs relative to wild-type RFC. Data is from Panel A.

**Figure 4.3 RFC D(SAC) and RFC E(SAC) are severely deficient in PCNA loading.**



The single (SAC) mutants displayed a spectrum of PCNA loading rates (Figure 4.3A and 4.3B). The RFC B(SAC) mutant was comparable to wild-type RFC. The RFC C(SAC) mutant was moderately deficient, displaying ~35 % the rate of clamp loading by wild-type RFC. In contrast, the RFC D(SAC) and RFC E(SAC) mutants were less than 5 % the rate of wild-type RFC, suggesting that ATP sites C (location of the RFC-D(SAC) mutation) and ATP site D (location of the RFC-E(SAC) mutation), play important roles in driving PCNA loading.

### **The arginine finger mutants of RFC can bind and open PCNA**

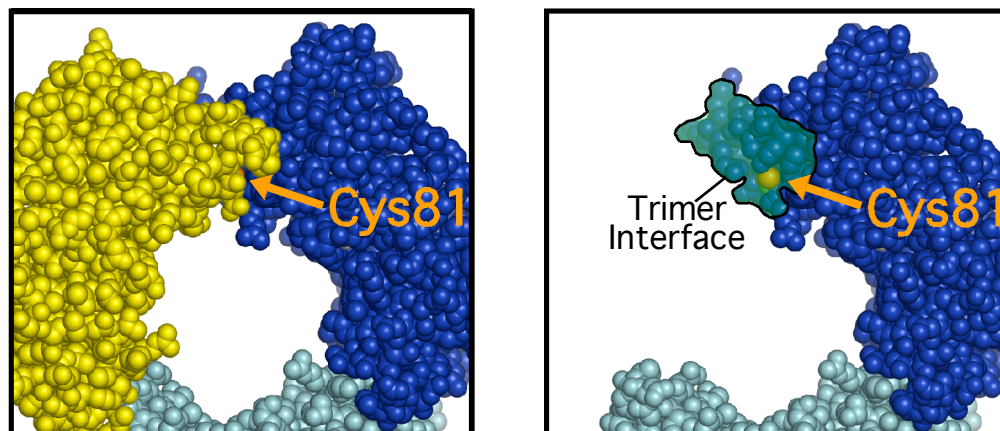
Which steps in clamp loading are the arginine finger mutants of RFC deficient in? Do any of the steps occur prior to ATP hydrolysis? Next we used the non-hydrolyzable ATP analogue, ATP $\gamma$ S, to test the single RFC (SAC) mutants for a role prior to ATP hydrolysis. PCNA ring opening and DNA binding do not require ATP hydrolysis by RFC, and therefore we developed assays to examine these two steps.

First we developed a PCNA opening assay based on a similar assay for ring opening that we used previously for the *E. coli* system (Hingorani and O'Donnell, 1998; Turner et al., 1999). We prepared a construct of PCNA in which the three surface-exposed cysteine residues were mutated to serine, leaving only one cysteine, Cys81, that is buried at each trimer interface (Figure 4.4A). This "PCNA (Cys81-Only)" clamp is resistant to labeling by the thiol-reactive dye, eosin-5-maleimide (Figure 4.4B). RFC and ATP $\gamma$ S stimulate labeling of Cys81 in PCNA (Cys81-Only) which is likely due to PCNA ring opening, thus increasing exposure of the buried Cys81 for reaction with the fluorescent maleimide. As a control we tested an RFC complex that lacks domains I and II of RFC-E (RFC5),

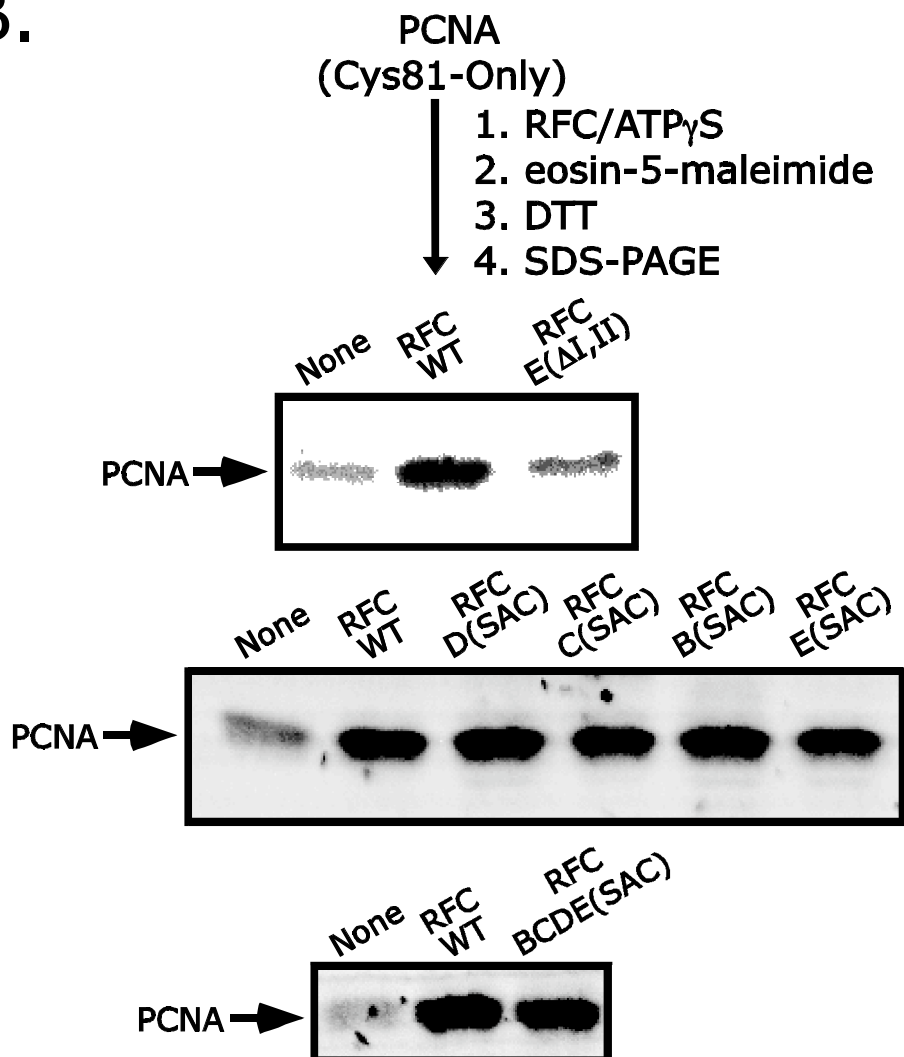
**Figure 4.4 PCNA ring opening assays.** *Panel A* Space-filling representation of the PCNA clamp structure. The three PCNA monomers are colored blue, yellow, and cyan. Cysteine 81 (*sulphur atom in orange*) of PCNA is buried at each trimer interface of the ring (left diagram). In the diagram to the right, one monomer is removed to expose the Cys81 at the trimer interface. *Panel B* Scheme of eosin-5-maleimide labeling assays using PCNA (Cys81 only). The top panel is a control reaction comparing eosin-labeling reactions containing no RFC, wild-type RFC, or RFC lacking domains I and II of RFC-E (RFC E( $\Delta$ I, II)). The middle panel compares single RFC (SAC) mutants and wild-type RFC in the PCNA opening assay. The bottom panel compares PCNA ring opening reactions with no RFC, wild-type RFC, or RFC BCDE(SAC). Proteins were separated by 10 % SDS-PAGE and visualized by UV scanning at 305 nm on a Fluor-S Multi-Imager (Bio-Rad).

Figure 4.4 PCNA ring opening assays.

A.



B.





which binds but does not open or unload PCNA from DNA (Yao et al., 2006). This mutant RFC did not stimulate labeling of the PCNA (Cys81-only) in the presence of ATP $\gamma$ S (Figure 4.4B, top). We were unable to perform a control reaction in the absence of ATP $\gamma$ S because in the absence of nucleotide RFC and PCNA form an insoluble aggregate (unpublished observation, G. Bowman and J. Kuriyan).

Next, we tested various RFC (SAC) mutants for ability to open PCNA in the presence of ATP $\gamma$ S. The single RFC (SAC) mutants were all active for PCNA opening (Figure 4.4B, middle). In fact, even the RFC BCDE(SAC) mutant was capable of opening PCNA (Figure 4.4B, bottom). Therefore the clamp loading deficiency of the RFC D(SAC) and RFC E(SAC) mutants (e.g. see Figure 4.3) must be blocked at a later step in the PCNA loading mechanism after PCNA opening.

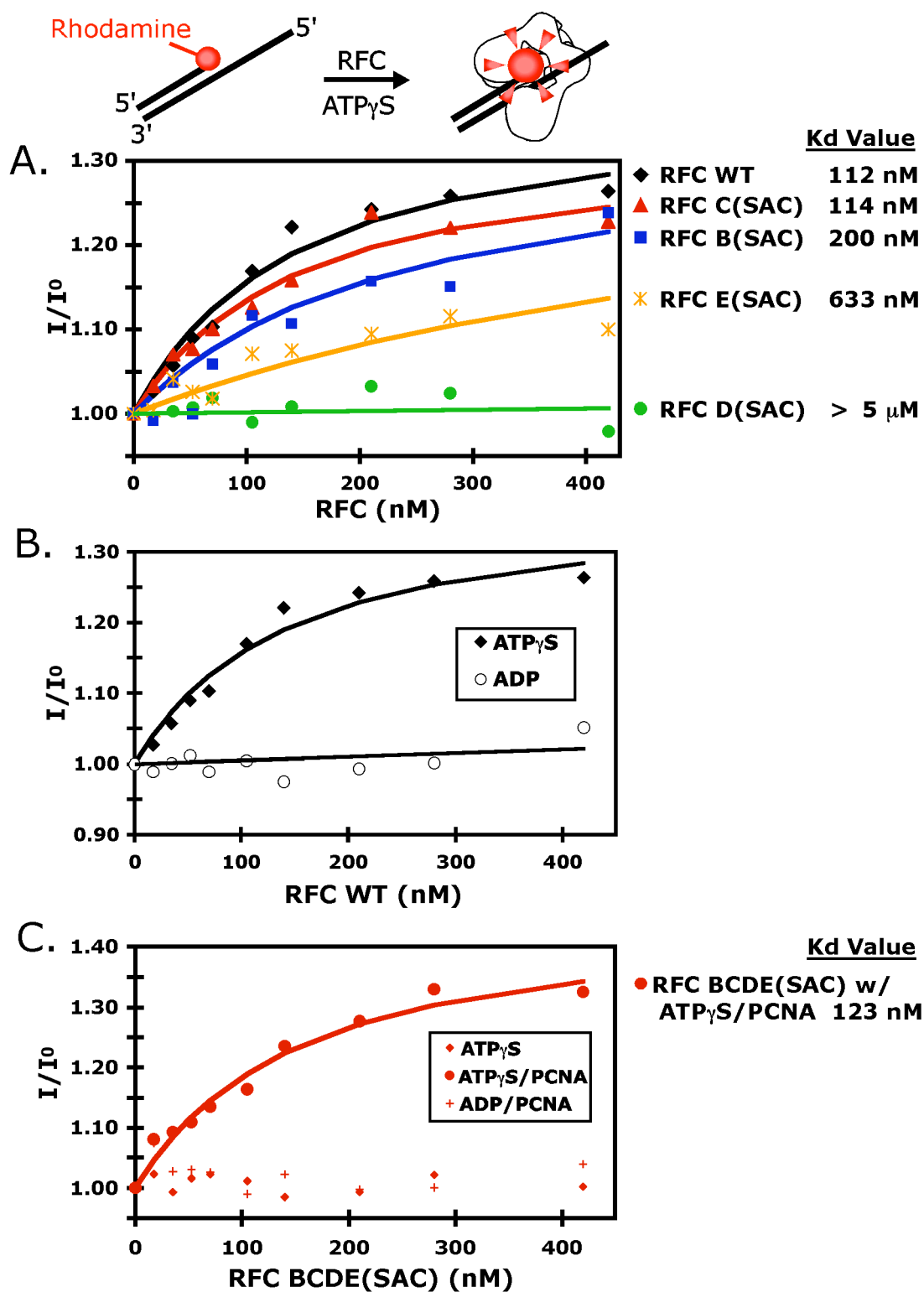
### **A $\gamma$ -phosphate sensor in ATP site C of RFC regulates DNA binding**

We next examined the RFC mutants for ability to bind DNA. ATP binding is known to promote RFC association with DNA, even in the absence of PCNA (Gomes and Burgers, 2001). To analyze DNA binding by RFC, we used a synthetic DNA with a fluorescent rhodamine derivative attached to the 3' terminus of the primer strand (see scheme in Figure 4.5). We refer to this template as "Rh-P/T". A change in fluorescence intensity indicates association of RFC at the primer-template junction and we have shown previously that RFC binds this DNA substrate with an affinity comparable to that of unmodified DNA (Gomes and Burgers, 2001; Yao et al., 2006).

**Figure 4.5 The RFC-D arginine finger is required for DNA binding by RFC.**

The fluorescent primed-template contains a 3' terminal rhodamine moiety attached to the primer strand. Addition of RFC in the presence of ATP $\gamma$ S results in an increase in fluorescence intensity (see scheme at top). *Panel A* Wild-type RFC and RFC(SAC) mutants were titrated into a reaction containing the fluorescent primed template and the relative fluorescence intensity change ( $I/I_0$ ) was measured. The  $K_d$  values for RFC interaction with the primed-template are indicated to the right. *Panel B* Wild-type RFC was titrated into reactions containing the fluorescent primed template in either the presence of ATP $\gamma$ S (*black diamonds*) or ADP (*red open circles*). *Panel C* RFC BCDE(SAC) was titrated into reactions containing the fluorescent primed template and ATP $\gamma$ S in the presence (closed circles) or absence (closed diamonds) of a saturating concentration of PCNA (810 nM). RFC BCDE(SAC) was also titrated into the reaction in the presence of ADP and PCNA (crosses).

Figure 4.5 The RFC-D arginine finger is required for DNA binding by RFC.



In Figure 4.5A, we measured the affinity of wild-type and arginine finger mutant RFC complexes for DNA by titrating RFC into a fixed concentration of Rh-P/T DNA in the presence of ATP $\gamma$ S. Wild-type RFC produces an increase in fluorescence intensity in this assay. Figure 4.5B shows that ADP does not suffice to power the conformation change in wild-type RFC needed for DNA binding. The RFC C(SAC) and RFC B(SAC) mutants had affinities for the primed DNA that were equal or two-fold reduced, respectively, compared to wild-type RFC; the RFC E(SAC) mutant displayed a six-fold reduced affinity for the primed DNA. However, RFC D(SAC) lost essentially all detectable DNA binding in this assay ( $K_d > 5 \mu\text{M}$ ). Therefore, the arginine finger of RFC-D is likely needed to recognize ATP in site C to promote the conformation change in RFC required for DNA binding.

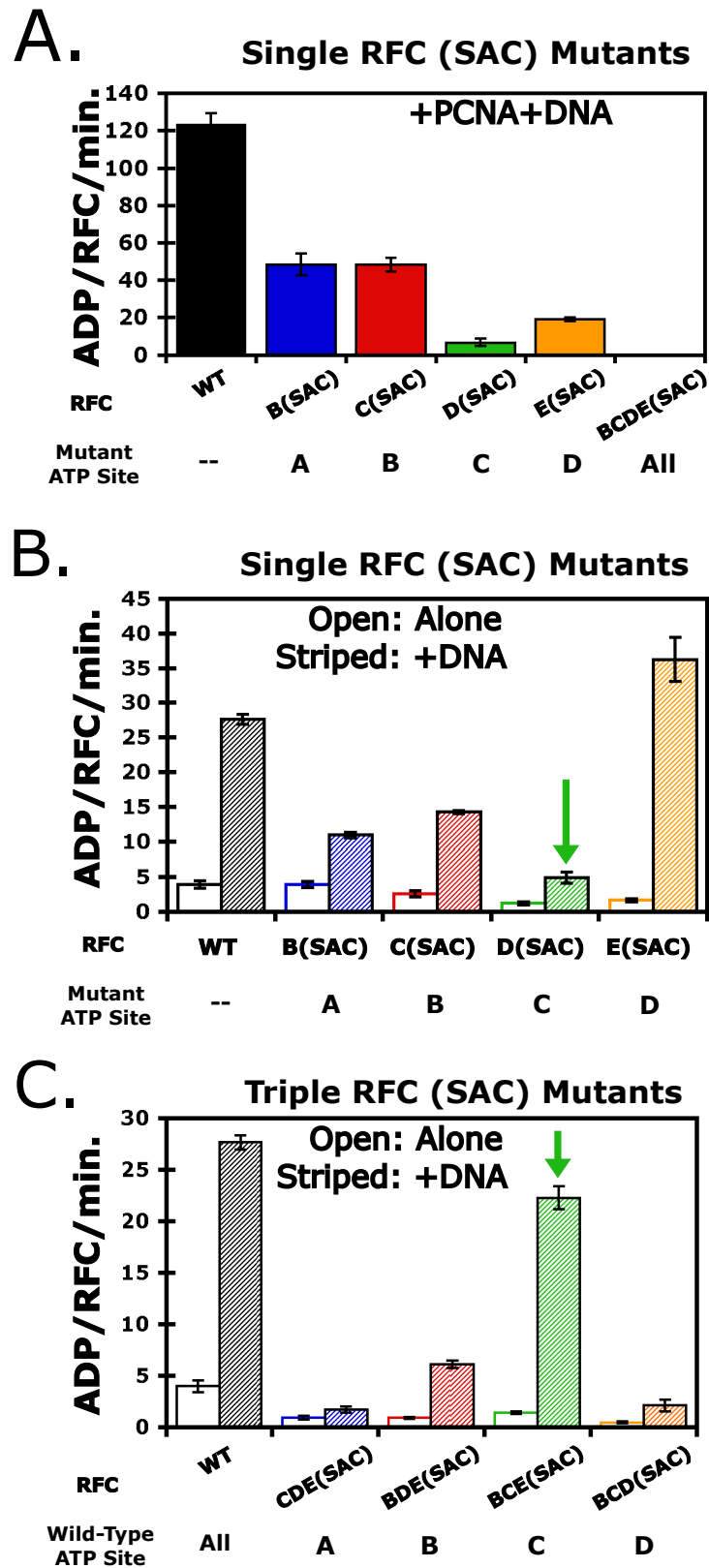
As expected, the RFC BCDE(SAC) complex did not display significant DNA binding activity, presumably due to the D(SAC) mutation (Figure 4.5C). Surprisingly, when the RFC BCDE(SAC) mutant was titrated into a reaction with the Rh-P/T DNA in the presence of saturating PCNA, a significant amount of DNA binding activity was observed (Figure 4.5C). ADP could not substitute for ATP $\gamma$ S to promote this PCNA-dependent DNA binding (Figure 4.5C), possibly because ATP binding is needed for RFC to interact with PCNA. This result demonstrates that the DNA binding deficiency of the RFC D(SAC) mutant can be partially compensated by PCNA association. In experiments below, we use the fact that PCNA enables RFC (SAC) mutants to bind DNA to investigate the ATPase activity of RFC (SAC) mutants in the presence of PCNA and DNA.

### **The arginine fingers of RFC-D and RFC-E are most important for the ATPase cycle of RFC**

The experiments described above show that the arginine fingers of RFC are not needed for ATP binding or PCNA opening, but are needed for DNA binding (i.e. the RFC-D arginine finger). The arginine fingers are also needed for ATP hydrolysis which drives the steps of PCNA loading after formation of the RFC-PCNA-DNA complex, resulting in closure of the clamp and ejection of RFC. Next we examine the effect of individual arginine finger mutations on the steady-state rate of ATP hydrolysis of RFC using a synthetic primed / template in the presence of PCNA. The short linear primed DNA allows PCNA to slide off the DNA after it is loaded, and thus RFC can perform repeated clamp loading events without running out of PCNA and DNA substrate. Wild-type RFC produced a steady-state rate of approximately 123 ATP hydrolyzed / RFC / min. (Figure 4.6A). The RFC BCDE(SAC) mutant gave no ATP hydrolysis within the time frame of the assay. Next, we examined the rate of ATP hydrolysis of the single RFC (SAC) mutants. If all four ATP sites contribute equally to the ATPase cycle of RFC, each single RFC (SAC) mutant may be expected to hydrolyze ATP at approximately 75% the rate of wild-type RFC. However, the results show that two of the RFC (SAC) mutants, RFC D(SAC) and RFC E(SAC), hydrolyze ATP at less than 20% the rate of wild-type RFC. We have shown earlier in this report that the RFC D(SAC) mutant is deficient in DNA binding. If the RFC D(SAC) mutant does not bind DNA, ATP hydrolysis will be compromised since DNA stimulates ATP hydrolysis by RFC (Figure 4.6B). However, the ATPase reaction is performed in the presence of sufficient PCNA to enable the RFC D(SAC) mutant to bind to DNA. Therefore the RFC D(SAC) and RFC E(SAC) mutants

**Figure 4.6 DNA stimulates ATP hydrolysis in RFC ATP site C.** All ATPase assays were performed under DNA concentrations that promoted maximal activity. All samples were quenched with EDTA and  $\alpha$ -<sup>32</sup>P-ADP production from  $\alpha$ -<sup>32</sup>P-ATP was analyzed by TLC and PhosphorImager analysis. Error bars indicate one standard deviation of the data set. *Panel A* Steady-state ATPase activity of single RFC (SAC) and RFC BCDE(SAC) mutants in the presence of PCNA and a synthetic primed-template. Results are the analysis of three independent experiments. *Panel B* ATPase activity of RFC (SAC) mutants was determined without PCNA but in the presence or absence of primed DNA. *Panel C* ATPase activity of RFC (SAC) triple mutants (i.e., three mutant subunits in each complex) in the absence or presence of DNA.

Figure 4.6 DNA stimulates ATP hydrolysis in RFC ATP site C.



are likely bound to PCNA and DNA, but the ATP hydrolysis cycle is blocked, preventing clamp loading. One possible explanation for this is that ATP bound at the mutant site must hydrolyze before the remaining ATP sites can fire. The RFC C(SAC) and RFC B(SAC) mutants each hydrolyze ATP at about half the rate of wild-type RFC. Therefore, inability to hydrolyze ATP in sites A or B also hinders hydrolysis in other sites, but the block is less severe than when sites C or D are mutated.

These assays were performed in the presence of PCNA and DNA. Addition of either PCNA or DNA alone to RFC also stimulates the rate of ATP hydrolysis by RFC, but to a lesser extent than when they are both present with RFC simultaneously. Do DNA and PCNA trigger hydrolysis in different ATP sites of RFC? We address this question next using the individual arginine finger mutants of RFC.

### **DNA triggers ATP hydrolysis in Site C**

At 1  $\mu$ M DNA, which is saturating in the ATPase assay, ATP hydrolysis by wild-type RFC and all four single RFC (SAC) mutants reaches a maximum. The rate of ATP hydrolysis of wild-type RFC is stimulated approximately seven-fold by primed-template DNA, from a basal rate of 4 ATP hydrolyzed in 1 min. (in the absence of PCNA and DNA) to a rate of 27 ATP hydrolyzed/min with DNA (Figure 4.6B). DNA stimulates the rate of ATP hydrolysis by the RFC E(SAC) mutant to a level slightly higher than the rate of ATP hydrolysis by wild-type RFC (Figure 4.6B). The rate of ATP hydrolysis by the RFC C(SAC) and RFC B(SAC) mutants are also stimulated by DNA, but level off at a rate about half that of wild-type RFC (Figure 4.6B). However, the rate of ATP



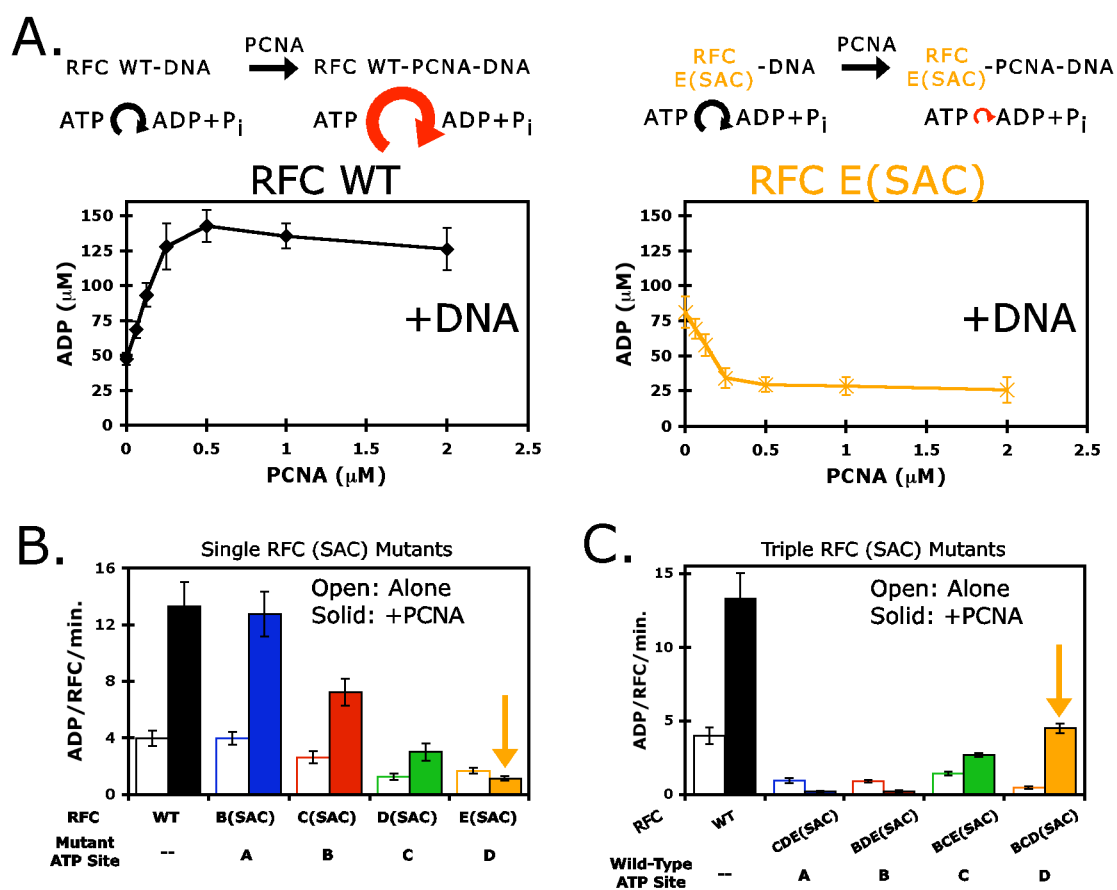
hydrolysis of the RFC D(SAC) mutant remained quite low in the presence of DNA, approximately 20% the rate of wild-type RFC (Figure 4.6B). These results indicate that the inability to hydrolyze bound ATP in site C blocks DNA-stimulated ATP hydrolysis in other ATP sites of RFC. In addition, this result suggests that DNA may trigger ATP hydrolysis in the unmutated ATP site C of wild-type RFC. To test this possibility we compared the rate of DNA-stimulated ATP hydrolysis by the triple RFC (SAC) mutants. Triple RFC (SAC) mutants contain only one ATP site that is competent for hydrolysis. The RFC BCE(SAC) mutant, which only contains a competent ATP site C, is most strongly stimulated by DNA, and in fact displays a rate of approximately 23 ATP hydrolyzed/RFC/min., about 80% the rate of wild-type RFC (Figure 4.6C). This result confirms that DNA triggers ATP hydrolysis in site C which presumably precedes DNA-stimulated ATP hydrolysis by the other sites in RFC, or blocks the DNA stimulated ATPase cycle.

#### **PCNA Triggers ATP hydrolysis in ATP site D**

Comparison of Figure 4.6A and 4.6B reveals that PCNA inhibits DNA-dependent ATP hydrolysis by RFC E(SAC). In contrast, PCNA synergistically stimulates ATP hydrolysis by wild-type RFC in the presence of DNA, presumably reflecting repeated cycles of PCNA loading. This PCNA effect is examined more closely in Figure 4.7A by titrating PCNA into reactions containing RFC and primed-template DNA. The steady-state rate of ATP hydrolysis by wild-type RFC in the presence of primer-template DNA is stimulated by PCNA, while PCNA has the opposite effect on the ATPase activity of the RFC E(SAC) mutant. PCNA inhibition of ATP hydrolysis by the RFC E(SAC)

**Figure 4.7 PCNA triggers ATP hydrolysis RFC ATP site D.** Reactions were performed as in Figure 4.6 unless indicated. Error bars indicate one standard deviation of the data set. *Panel A* PCNA was titrated into a reaction containing primed DNA and either wild-type RFC or RFC E(SAC); ADP production was analyzed after 2 min. incubation. *Panel B* ATPase activity of RFC (SAC) single site mutants was analyzed in either the presence or absence of PCNA. *Panel C* ATPase activity of RFC (SAC) triple site mutants was analyzed in either the presence or absence of PCNA.

Figure 4.7 PCNA triggers ATP hydrolysis RFC ATP site D.



mutant implies that when ATP in site D cannot be hydrolyzed, PCNA blocks the ATPase cycle.

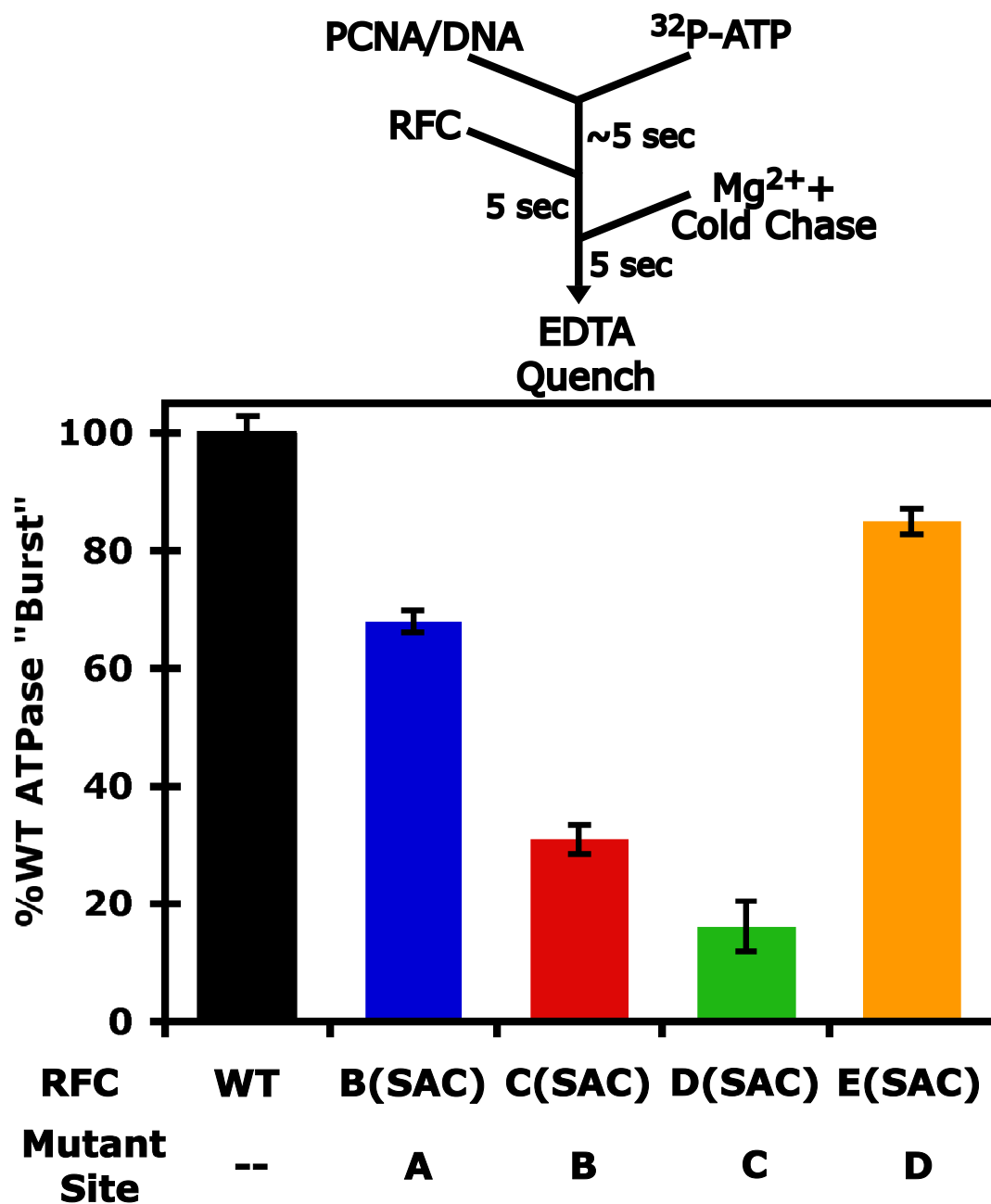
In Figure 4.7B we tested the RFC E(SAC) mutant and the other single RFC (SAC) mutants for ATPase activity in the absence and presence of PCNA, without DNA. The RFC E(SAC) mutant is not significantly stimulated by PCNA. This result suggests that association of PCNA with RFC-ATP normally stimulates the hydrolysis of ATP bound to site D. To test this proposal, we examined the triple RFC (SAC) mutants for PCNA-stimulated ATPase activity (Figure 4.7C). The results show that PCNA has the greatest stimulatory effect on ATP hydrolysis by the RFC BCD(SAC) triple mutant, which only has a competent ATP site D (Figure 4.7C). This result suggests that PCNA stimulates hydrolysis of ATP in site D of RFC. PCNA stimulates the basal rate of the triple RFC BCD(SAC) mutant 10-fold, which is significantly more than the 3-4 fold stimulation by PCNA on wild-type RFC ATP hydrolysis.

#### **RFC ATP hydrolysis occurs sequentially from ATP site C → B → A → D**

The studies described above on the rates of ATP hydrolysis by individual RFC (SAC) mutants indicate that hydrolysis in ATP site C is triggered by DNA, and ATP hydrolysis in site D is triggered by PCNA. These steady-state ATPase activities are a measure of successive turnovers, and therefore do not indicate the order in which hydrolysis in sites C and D occur during the clamp loading cycle. To investigate the order of ATP hydrolysis in RFC, we designed an assay to measure ATP hydrolysis during a single clamp loading cycle (see scheme in Figure 4.8). RFC, DNA, and PCNA are pre-incubated with <sup>32</sup>P-labeled ATP in the absence of magnesium. <sup>32</sup>P-ATP binds

**Figure 4.8 Order of ATP hydrolysis in RFC.** An assay was designed to address the amount of ATP hydrolyzed by wild-type RFC and RFC (SAC) single site mutants in a burst of ATP hydrolysis (*scheme at top*). Briefly, a mixture of PCNA and primed DNA was mixed with a  $\alpha$ - $^{32}\text{P}$ -ATP, then RFC was added in the absence of magnesium to allow ATP binding, but not hydrolysis. After 5 seconds, magnesium and a 50-fold excess of unlabeled ATP $\gamma$ S were added together and the reaction was quenched after 5 seconds with EDTA, followed by analysis of ADP produced by TLC. Reactions were performed in triplicate and error bars indicate the standard deviation of the data.

Figure 4.8 Order of ATP hydrolysis in RFC.



RFC without magnesium, thus “charging” the ATP sites, but magnesium is necessary for hydrolysis. ATP hydrolysis was initiated upon addition of a solution containing magnesium and a 50-fold molar excess of ATP $\gamma$ S. Thus the bound,  $^{32}$ P-ATP should become hydrolyzed, and the 50-fold excess of ATP $\gamma$ S should prevent further hydrolysis by filling the ATP sites after the bound ADP is released. After 5 seconds, EDTA was added to quench the reaction. This assay yields a burst of  $\sim 1.1$  ATP/RFC which may be explained by some exchange of  $^{32}$ P-ATP for ATP $\gamma$ S before magnesium binding triggers hydrolysis. Removing PCNA or DNA from the assay reduced the burst amplitude by at least 80% (data not shown), indicating that the synergistic effect of the two substrates still operates in this design.

To examine the ATP site order of hydrolysis during clamp loading we tested the single RFC (SAC) mutants in the assay described above. If a defined order of ATP hydrolysis occurs during PCNA loading, the experiment may be expected to yield a different burst amplitude for each single RFC (SAC) mutant. The results showed that each single mutant did, in fact, produce a different burst amplitude, lower than the burst of ATP hydrolyzed by wild-type RFC (Figure 4.8). From least to greatest, the burst of ATP hydrolyzed by the single RFC (SAC) mutants was: RFC D(SAC) < RFC C(SAC) < RFC B(SAC) < RFC E(SAC). These results suggest the following order of ATP hydrolysis: ATP site C  $\rightarrow$  site B  $\rightarrow$  site A  $\rightarrow$  site D.

## DISCUSSION

The circular heteropentameric RFC clamp loader from *Saccharomyces cerevisiae* has four bipartite ATP sites, each located at interfaces of adjacent

subunits. At each site one subunit contributes a catalytic arginine finger that is contained within a highly conserved SRC motif in RFC box VII of all known clamp loaders. The other subunit contains the P-loop and other elements of ATP binding sites. In this report, the conserved arginine fingers are mutated in single subunits and combinations of subunits and the resulting mutant RFC complexes are examined for ability to progress through the different steps in clamp loading. The results point to individual functions of different ATP sites during the clamp loading cycle.

### **Role of Arginine Fingers in PCNA Opening**

In the absence of ATP, neither *E. coli*  $\gamma$  complex nor eukaryotic RFC bind to their respective clamp or to DNA (Ason et al., 2003; Gerik et al., 1997; Gomes and Burgers, 2001; Naktinis et al., 1995). Previous studies of both *E. coli*  $\gamma$  complex and yeast and human RFC have shown that ATP binding powers a conformation change that enables the clamp loader to bind the clamp and DNA (Burgers, 1991; Hingorani and O'Donnell, 1998; Lee and Hurwitz, 1990; Tsurimoto and Stillman, 1991a; Zhuang et al., 2006). Use of ATP $\gamma$ S promotes ring opening and ring loading onto DNA, but the ring stays open and the clamp loader also remains with the open clamp on DNA. This was demonstrated in the *E. coli* system using a labeled maleimide and a  $\beta$  clamp containing a cysteine residue at a buried position within the clamp interface (Hingorani and O'Donnell, 1998). In the presence of ATP $\gamma$ S, clamp opening is signaled by an increase in reactivity of the buried cysteine residue at the interface. Several lines of evidence exist for RFC holding PCNA open while it is bound to DNA with



ATP $\gamma$ S. 1) An electron micrographic reconstruction of a *Pyrococcus furiosus* RFC-ATP $\gamma$ S-PCNA-DNA complex shows an open PCNA ring in a right-handed helix (Miyata et al., 2005). 2) A FRET study of yeast RFC indicates that the clamp remains open upon DNA binding (Zhuang et al., 2006), similar to the EM study of the *Pyrococcus furiosus* clamp loader. 3) Study of RFC mutants indicates that the ring is open in the RFC-ATP $\gamma$ S-PCNA-DNA complex and that RFC is the component that holds RFC-ATP $\gamma$ S-PCNA to DNA (Yao et al., 2006).

The PCNA ring is closed in the RFC-ATP $\gamma$ S-PCNA structure (Bowman et al., 2004). However, RFC used in the structure analysis contained two types of mutations, the four SRC motifs were mutated to SQC to prevent ATP $\gamma$ S hydrolysis during crystal growth, and the other mutation was in RFC-A which contained deletions in both the N- and C- terminal regions. One or more of these mutations may have prevented PCNA from opening in the RFC-ATP $\gamma$ S-PCNA structure. The N-terminal region of RFC-A binds DNA nonspecifically and can be eliminated both *in vivo* and *in vitro*, and in fact results in a substantial increase in clamp loading activity *in vitro* (Gomes et al., 2000; Uhlmann et al., 1997a). The effects of C-terminal deletions of eukaryotic RFC-A have not been studied, but the electron microscopic image reconstruction of archaeal RFC-PCNA-DNA complex suggests that the C-terminal region of the large RFC subunit (analogous to yeast RFC-A (RFC1)) connects to the PCNA ring near the open interface (Miyata et al., 2005). Thus the C-terminal region of eukaryotic RFC-A is a likely candidate for a role in PCNA clamp opening. The four arginine finger mutations used in the structure determination could also affect the ring-opening step. However, the studies of the current report show that the RFC with four

SRC→SAC mutations (i.e. RFC BCDE(SAC)) is still active in binding and opening PCNA. The RFC BCDE(SAC) mutant is also capable of assembling PCNA onto DNA with ATP $\gamma$ S, forming the RFC-ATP $\gamma$ S-PCNA-DNA complex (Figure 4.5C and unpublished results), which is consistent with the ability of RFC BCDE(SAC) to open PCNA (Figure 4.4B, *bottom*). Hence, PCNA may be closed in the structure due to the truncation mutation in the RFC-A C-terminus, possibly in combination with the arginine finger mutations.

The current study of arginine finger mutants of RFC demonstrates that the arginine finger of RFC-D (RFC2) is required for RFC to bind DNA. This ATP site corresponds to site C of *E. coli*  $\gamma$  complex, the one site that remains closed in  $\gamma$  complex when it is cocrystallized with ATP $\gamma$ S (Kazmirski et al., 2004). Furthermore, the  $\gamma$  complex structure with two ATP $\gamma$ S bound is in essentially the same conformation as inactive  $\gamma$  complex with no bound nucleotide. Therefore, binding of ATP to site C is presumed to power the conformation change in  $\gamma$  complex required to bind both  $\beta$  and DNA. The ATP site C arginine finger mutation of RFC is the only one that blocks a step powered by ATP binding, and thus one may infer that ATP site C of RFC may carry a similar importance to the ATP-dependent conformation of RFC as ATP site C in *E. coli*  $\gamma$  complex. However, structures of the fully ATP bound state of  $\gamma$  complex and of the unliganded state of RFC will be needed to confirm whether this prediction is indeed the case.

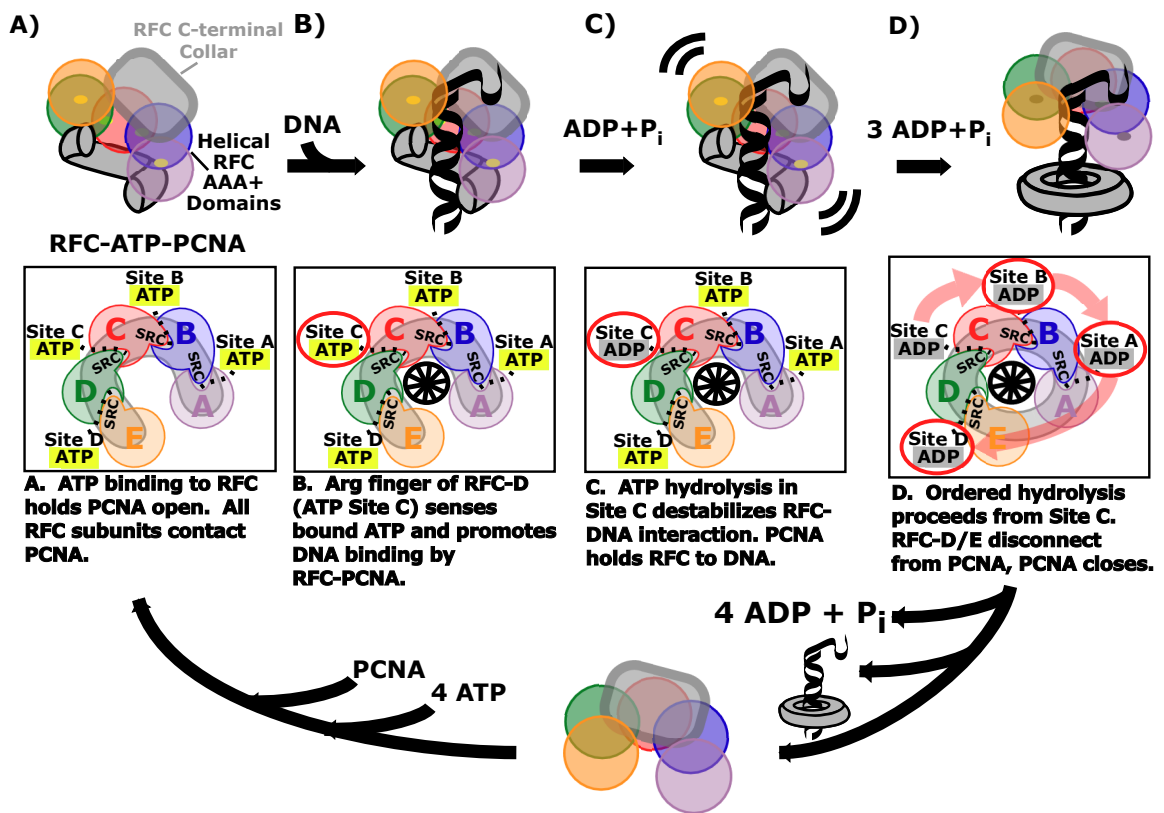
### **Role of Arginine Fingers in DNA Binding and ATP Hydrolysis**

The scheme in Figure 4.9 summarizes the steps in the ATP hydrolysis cycle of RFC as understood from this report and previous studies. ATP binding powers all the steps that lead to formation of the complex of RFC bound to an open, helical PCNA clamp (diagram A). To take the next step, binding to DNA, the arginine finger of RFC-D (RFC2) is needed to sense the  $\gamma$ -phosphate of ATP bound to site C. This ATP sensing step brings RFC-PCNA complex into the proper conformation to bind primed DNA (diagram B). This conformation change could be either subtle, for example by bringing the DNA binding residues of RFC-D (RFC2) into proper alignment for interaction with the phosphate backbone; or could be a more global change, such as altering the helical pitch of the AAA+ domains of the five subunits of RFC, thus bringing all the subunits into register for DNA interaction. Further studies will be needed to more fully understand the nature of this step and function of the arginine finger of RFC-D (RFC2).

RFC-PCNA binds to primed DNA through the slot between RFC subunits A and E (RFC1 and RFC5), and through the open gap in PCNA. Interaction with DNA triggers a round ATP hydrolysis, the cycle of which appears to start with the ATP bound to site C (diagram C). Hydrolysis of ATP in site C will be sensed by the arginine finger of RFC-D (RFC2), thus lowering the affinity of RFC for DNA. However, RFC will not dissociate at this point in the mechanism because of its connection to the PCNA clamp, which we have shown here enables RFC to bind DNA even with a missing arginine finger in ATP site C. The ATP hydrolysis cycle continues to the other ATP sites until all the sites have hydrolyzed bound ATP. Previous studies have shown earlier that RFC-E (RFC5),

**Figure 4.9 Model of RFC ATP site function during clamp loading.** Schematic models depicting the findings of this study in the context of the PCNA loading cycle. *Panel A* ATP binding powers binding and opening of PCNA by RFC. The arginine fingers of RFC are not involved in the formation of this RFC-ATP-(open)PCNA complex. *Panel B*  $\gamma$ -phosphate recognition by the ATP site C arginine finger coordinates RFC-(open)PCNA binding to DNA through the central chamber. *Panel C* When the RFC-PCNA supercomplex binds DNA, ATP hydrolysis is triggered first in ATP site C. This hydrolysis event likely decreases the affinity of RFC for DNA, but PCNA helps RFC maintain its grip on DNA. *Panel D* ATP hydrolysis continues sequentially from ATP site C  $\rightarrow$  site B  $\rightarrow$  site A  $\rightarrow$  site D, resulting in PCNA closure around DNA. RFC dissociates from the site of loading, aided by the lowered affinity for DNA due to the lack of triphosphate in site C.

Figure 4.9 Model of RFC ATP site function during clamp loading.



context of the full RFC complex this interaction likely promotes a complex where all five RFC subunits are bound to a helical, open PCNA (Kazmirski et al., 2005; Miyata et al., 2005). RFC subunits D and E (RFC2 and RFC5), together, form ATP site D. We show here that ATP hydrolysis in ATP site D is specifically stimulated by PCNA, and we propose that this closes PCNA around DNA thereby disconnecting the interaction between RFC-D/E (RFC2/5) and the closed PCNA ring (diagram D). Loss of contact between PCNA and RFC-D/E (RFC2/5) is mandatory upon closure of the ring, because the RFC subunits are helical while the closed PCNA forms a plane. The RFC-ATP $\gamma$ S-PCNA structure shows that the RFC-A, RFC-B, and RFC-C subunits bind to the closed PCNA, and RFC-D and RFC-E are lifted above the plane of the closed ring (Bowman et al., 2004). Loss of contact between RFC-D/E and PCNA minimizes interaction of RFC with the clamp, and combined with the decrease in RFC-DNA affinity, due to loss of ATP at site C, may result in the observed dissociation of RFC from the PCNA-DNA complex at the end of a clamp loading cycle. A decrease in affinity of RFC for DNA is also promoted by the absence of nucleotide in ATP sites C and D as indicated by a study of P-loop mutants of RFC that no longer bind ATP (Schmidt et al., 2001).

### **Comparison to Other Clamp Loaders**

Unlike RFC, *E. coli*  $\gamma$  complex contains only three ATP sites. These sites correspond to ATP sites B, C and D of RFC. In  $\gamma$  complex, the arginine finger of ATP site D is contained in  $\delta'$  (E subunit), and mutation of this arginine yields a clamp loader complex that has lower affinity for the  $\beta$  clamp (Snyder et al., 2004).

Arginine fingers of the other ATP sites cannot be mutated individually since the  $\gamma$  subunits are encoded by the same gene. However, mutation of the arginine fingers in both ATP sites B and C yields a complex that no longer interacts tightly with DNA (Snyder et al., 2004). Thus, there are striking parallels between arginine finger mutants of RFC and  $\gamma$  complex. The arginine finger of RFC in ATP site C is needed for DNA binding, and thus has similar behavior to the arginine finger mutation in ATP sites B and C in  $\gamma$  complex. If RFC and  $\gamma$  complex act in a similar fashion, then one may predict that the arginine finger in ATP site C of  $\gamma$  complex, and not the arginine in ATP site B, is responsible for the ATP binding-induced DNA affinity of  $\gamma$  complex. The results of arginine finger mutations in the  $\gamma$ - $\gamma$ - $\delta'$  complex (Chapter 3) support this suggestion. Mutation of the  $\gamma$  complex arginine finger in ATP site D (between the D and E positions), like the same mutation in RFC, prevents ATPase activity in response to the clamp (Johnson and O'Donnell, 2003). Interestingly, the RFC-D and RFC-E subunits of RFC are needed to open the PCNA clamp (Yao et al., 2006). However, in a significant departure between the activities of the eukaryotic RFC and  $\gamma$  complex, the  $\delta$  wrench (A subunit) of  $\gamma$  complex opens the  $\beta$  clamp, while RFC-A lacks this activity (Yao et al., 2006).

The RFC of *Archaeoglobus fulgidus* is similar to *E. coli*  $\gamma$  complex and eukaryotic RFC in the ability to load a clamp onto DNA prior to ATP hydrolysis, and in all cases the clamp loader remains on DNA with the clamp. One may presume that the clamp remains open from the work in the *E. coli* system and from the electron micrographic reconstruction of the RFC-ATP $\gamma$ S-PCNA-DNA complex from the archeon *Pyrococcus furiosus* (Miyata et al., 2005). Archaeal RFC

complexes consist of two different subunits: RFC-s (s for small) and RFC-l (l for large), in a 4:1 ratio, respectively. Studies on *A. fulgidus* RFC suggest that four ATP molecules bind to archaeal RFC and are hydrolyzed during a full clamp loading cycle (Seybert and Wigley, 2004). *A. fulgidus* RFC with arginine finger mutations in the 4 RFC-s subunits, like eukaryotic RFC, still binds ATP but is no longer functional in formation of the RFC-PCNA-DNA ternary complex (Seybert et al., 2006), suggesting that a step prior to hydrolysis is disrupted by this mutation. Identification of individual roles of different ATP sites of archaeal RFC is made difficult by the fact that four ATPase subunits (RFC-s) are encoded by the same gene, and thus site-directed mutations are produced in all four RFC-s subunits of RFC at the same time.



## Concluding Discussion

PCNA and  $\beta$  are each required components of the leading and lagging strands at the replication fork for processive nucleotide incorporation and subsequent Okazaki fragment processing (Bruck and O'Donnell, 2001; Maga and Hubscher, 2003). Previous studies have shown that  $\gamma$  complex can effectively load  $\beta$  clamps on the lagging strand to keep up with the moving fork (Stukenberg et al., 1994; Turner et al., 1999). At the eukaryotic replication fork, the lagging strand is replicated discontinuously in Okazaki fragments of approximately 150 nucleotides and PCNA must be loaded at the primer for extension of each fragment. The replication fork moves at a speed of  $\sim 50$ -100 nt/s (Raghuraman et al., 2001); therefore, RFC must load PCNA correctly every 2-3 seconds. The steady-state ATPase rate of RFC during clamp loading is approximately  $\sim 2$  ATP hydrolyzed/s, corresponding to 2 seconds per full turnover of four ATP molecules. This suggests that RFC operates on the same timescale as Okazaki fragment synthesis. This time constraint requires that RFC act with high-fidelity to harness each ATPase cycle to correctly link the PCNA ring onto DNA. Our studies in Chapter 4 reveal important ATP-mediated events that underlie the high-fidelity of the RFC clamp loader and give insight into the conservation of the clamp loading mechanism.

### **Clamp and DNA association: lessons from mutant clamp loaders**

In Chapter 4 we demonstrate that the arginine finger of RFC ATP site C acts as a triphosphate sensor to promote DNA binding. When a triphosphate adenine nucleoside occupies ATP site C, a conformational change must occur in RFC, mediated by the arginine finger in site C, that promotes DNA binding. Removing the arginine finger sensor by mutation breaks the signal and prevents the conformational change for DNA binding. Arginine fingers are thought to serve these non-catalytic functions in other AAA+ proteins such as SV40 large T-antigen (Gai et al., 2004) and NSF (Matveeva et al., 2002). Interaction with the  $\gamma$ -phosphate of ATP is hypothesized to cause the arginine to act as a lever of sorts, to propagate conformational changes back into the subunit that is contributing the arginine. In the absence of the  $\gamma$ -phosphate interaction, the arginine lever is not active and the corresponding subunit may be in a more relaxed conformational state.

Double-stranded DNA binds in the center of the spiral arrangement of ATPase modules of RFC (Bowman et al., 2004; Yao et al., 2006). ATP site C presumably plays a role in modulating this DNA binding activity. Consistent with this possibility, removing three proposed DNA-interacting residues from either RFC-B, RFC-C, or RFC-D causes a deficiency in DNA binding similar to that of the ATP site C arginine finger mutant (Appendix Figure A.2). This result suggests that disrupting the interaction between any one RFC subunit and DNA may inhibit association of the entire complex with DNA. RFC-D appears to serve this modulator function, perhaps coupling nucleotide occupancy in ATP site C to modulation of the DNA-interacting residues of RFC-D. When ATP site C is

empty, RFC-D may be out of register with the helix of ATPase modules. Another possibility is that the mutations in the RFC-D arginine finger or the RFC-B, -C, or -D DNA binding residues prevent a more global conformational change in RFC that is utilized to recognize DNA. The difference in the conformations of the  $\gamma_3\delta\delta'$  and RFC crystal structures suggest that the helix of ATPase modules is at least somewhat dynamic. Perhaps the interactions perturbed in our mutational studies disrupt the dynamics of the helix.

The fact that the DNA binding activity of the quadruple arginine finger RFC mutant is stimulated by PCNA (Figure 4.5C) suggests that either PCNA binds directly to DNA in the RFC-PCNA-DNA complex, or PCNA changes the conformation of RFC to promote DNA binding. If PCNA associates directly with DNA in the RFC-PCNA-DNA complex, then the clamp should stimulate DNA binding activity in an RFC mutant that is missing DNA-interacting residues. Contrary to this prediction, addition of PCNA is not sufficient to stimulate the DNA binding activity of a mutant that lacks the main DNA-interacting residues of RFC-B, RFC-C, and RFC-D (Appendix Figure A.3), yet this mutant can still bind and open PCNA (Yao et al., 2006). The fact that PCNA cannot rescue this mutant suggests that PCNA, when associated with RFC, does not likely bind directly to DNA in the RFC-PCNA-DNA complex. Yet, the RFC BCDE(SAC) complex, which retains the DNA-interaction residues, is stimulated by PCNA to bind DNA. Perhaps the putative right-handed helical conformation of the open PCNA ring (Kazmirski et al., 2005) helps stabilize the spiral conformations of the N-terminal domains of the five RFC subunits, promoting recognition of the dsDNA helix. A previous study of RFC/DNA association demonstrated that

PCNA binding to RFC does, indeed, aid in the specificity of RFC for primer-template versus single-stranded DNA (Hingorani and Coman, 2002).

Interestingly, the RFC/PCNA interaction is less sensitive to arginine finger mutation than the  $\gamma$  complex/ $\beta$  interaction. Previous work showed that mutation of the ATP site D arginine finger in the  $\delta'$  subunit of  $\gamma$  complex causes a significant decrease in the ATP-dependent affinity for the  $\beta$  clamp (Snyder et al., 2004). Perhaps  $\gamma$  complex must perform more work to drive the open  $\beta$  clamp into a helical conformation and arginine finger mutation in ATP site D disrupts this step. The RFC-PCNA interaction may need to be more robust, thus less sensitive to mutation, to prevent partial disassembly of the trimeric clamp during opening. The dimeric  $\beta$  clamp, when opened by  $\gamma$  complex, must maintain only one closed subunit interface to prevent disassembly, while open PCNA requires two closed interfaces to maintain a stable open trimeric ring. The RFC-B/C subcomplex stabilizes PCNA on DNA, presumably by tightening one or more trimer interfaces (Yao et al., 2006). Perhaps the tolerance of the RFC-PCNA interaction to RFC ATP site mutation is also a consequence of the requirement for RFC to stabilize the open PCNA trimer.

### **Hydrolysis-coupled closure of the clamp around DNA**

Mutation of the arginine finger of  $\delta'$ , in ATP site D of  $\gamma$  complex, disrupts the association of  $\gamma$  complex with  $\beta$  (Snyder et al., 2004). We observed that the analogous mutation in RFC ATP site D also causes a clamp-related deficiency. The RFC E(SAC) mutant binds and opens PCNA, but is deficient in clamp loading and ATPase activity because ATP site D must fire to close PCNA. At

high  $\beta$  clamp concentration, the  $\delta'$  (SAC)  $\gamma$  complex does not display wild-type activity (Snyder et al., 2004), which suggests that  $\beta$  affinity is not the only deficiency of this mutant. Perhaps ATP site D of  $\gamma$  complex acts similarly to site D of RFC, coupling hydrolysis to closure of the clamp on DNA.

Both RFC and  $\gamma$  complex regulate interactions with the clamp via ATP site D. In the crystal structure of RFC-PCNA, the RFC-D and RFC-E subunits do not interact with the closed clamp (Bowman et al., 2004). It is not clear how PCNA is opened. The ATPase modules of RFC-D and RFC-E can, together as a subcomplex, open PCNA (Yao et al., 2006). However, in the context of the full RFC complex, these two subunits would have to break the helical structure of the RFC ATPase modules to interact with the closed clamp in order to open it. Computational modeling of this movement suggests that it is not favorable (Kazmirski et al., 2005). Perhaps, when RFC binds PCNA, RFC-A, RFC-B, and RFC-C disrupt one interface of the clamp and the RFC-D/E interaction simply stabilizes the open form. This is consistent with the fact that PCNA, when open, can adopt a helical conformation to “find” RFC-D/E (Kazmirski et al., 2005). The association of PCNA with RFC-D/E is likely stabilized by ATP in site D and disrupted when ATP is hydrolyzed to ADP or when ADP dissociates; however, we have shown that the obligatory sensing of the change in nucleotide must be accomplished by residues other than the arginine finger of RFC-E (Figure 4.4).

#### **The mechanism of ordered ATP hydrolysis in RFC and $\gamma$ complex**

In Figure 4.9 we propose that RFC follows a defined order of ATP hydrolysis during PCNA loading, where each hydrolysis event is dependent on

the previous one for efficient catalysis. The following order is predicted: ATP site C  $\rightarrow$  site B  $\rightarrow$  site A  $\rightarrow$  site D. This order is consistent with the roles we have assigned to ATP sites C and D and the order in which those roles are likely to be performed. This order of hydrolysis also explains one surprising phenomenon we observed for the double and triple RFC arginine finger mutants. When a mutation of the RFC-D arginine finger, which by itself results in low ATP hydrolysis in RFC, is combined with an RFC-B mutation in the RFC pentamer, ATP hydrolysis is restored (Appendix Figure A.4). In light of the order of ATP hydrolysis, we interpret this result as evidence of de-regulation of the ATP hydrolysis order, causing site D to fire without site C firing first. This de-regulated RFC complex is not functional in PCNA loading (data not shown), which supports the idea that clamp loader ATP sites cannot work independently to perform the function of the complex. This is in contrast to molecular motor complexes, such as the ClpX unfoldase, in which a single good ATP site in the hexamer can support protein unfolding (Martin et al., 2005).

ATP sites A and D are located on opposite sides of the gap between the ATPase modules of RFC-A and RFC-E. How does ATP hydrolysis proceed across this gap from ATP site A to site D? RFC-A has a unique C-terminal domain (IV) of unknown function that is not present in the other four RFC subunits. Domain IV of RFC-A reaches across the gap in ATPase domains and makes contacts with the ATPase module of RFC-E, the subunit that contributes the catalytic arginine finger to ATP site D (Bowman et al., 2004). Domain IV of RFC-A also makes contacts near ATP site A. This unique C-terminal domain of RFC-A may transmit a signal from ATP site A that triggers hydrolysis in site D, utilizing an allosteric mechanism to promote the terminal hydrolysis event. A

similar allosteric mechanism governs ATP hydrolysis regulation in the AAA+ chaperone Hsp90 (Hattendorf and Lindquist, 2002). Alternatively, changes in PCNA or DNA during clamp loading may promote hydrolysis in site D without direct communication through RFC.

Our studies using arginine finger mutants of  $\gamma$  complex and  $\gamma$ - $\gamma$ - $\delta'$  fusion clamp loader complex in Chapters 2 and 3 suggest that  $\gamma$  complex harnesses ATP binding and hydrolysis in a very similar mechanism to RFC. In fact, the ATP hydrolysis cycle may begin in site C and end in site D, as it does in RFC.  $\gamma$  complex does not have an ATP site in position A; therefore, the hydrolysis order would be site C  $\rightarrow$  site B  $\rightarrow$  site D. If  $\gamma$  complex obeys a similar order of ATP hydrolysis as RFC, then ATP site D will be the last to fire during  $\beta$  loading, perhaps leading to  $\beta$  closure around DNA. This order of hydrolysis is consistent with the observation that disengagement of  $\gamma$  complex from  $\beta$  occurs after all ATP is hydrolyzed (S. Anderson and L. Bloom, personal communication).

How is the signal for ATP hydrolysis in  $\gamma$  complex propagated from site B to site D? The  $\delta$  subunit of  $\gamma$  complex, in position A, does not contain a Domain IV like RFC-A. Perhaps, because the  $\gamma$  complex order of hydrolysis proceeds from site B to D, the closer proximity of these sites within the complex allows a more local signal. Alternatively, the role of domain IV of RFC-A may be played by the  $\chi$  and  $\psi$  subunits of  $\gamma$  complex.  $\chi$  and  $\psi$ , together as a complex, are known to stimulate the activity of the minimal clamp loader  $\gamma_3\delta\delta'$  (Xiao et al., 1993). Recently it has been speculated that these two subunits may partially fill the gap between  $\delta$  and  $\delta'$  (Gulbis et al., 2004), perhaps bridging the distal portions of the clamp loader.

### **Conservation of mechanism among all clamp loaders**

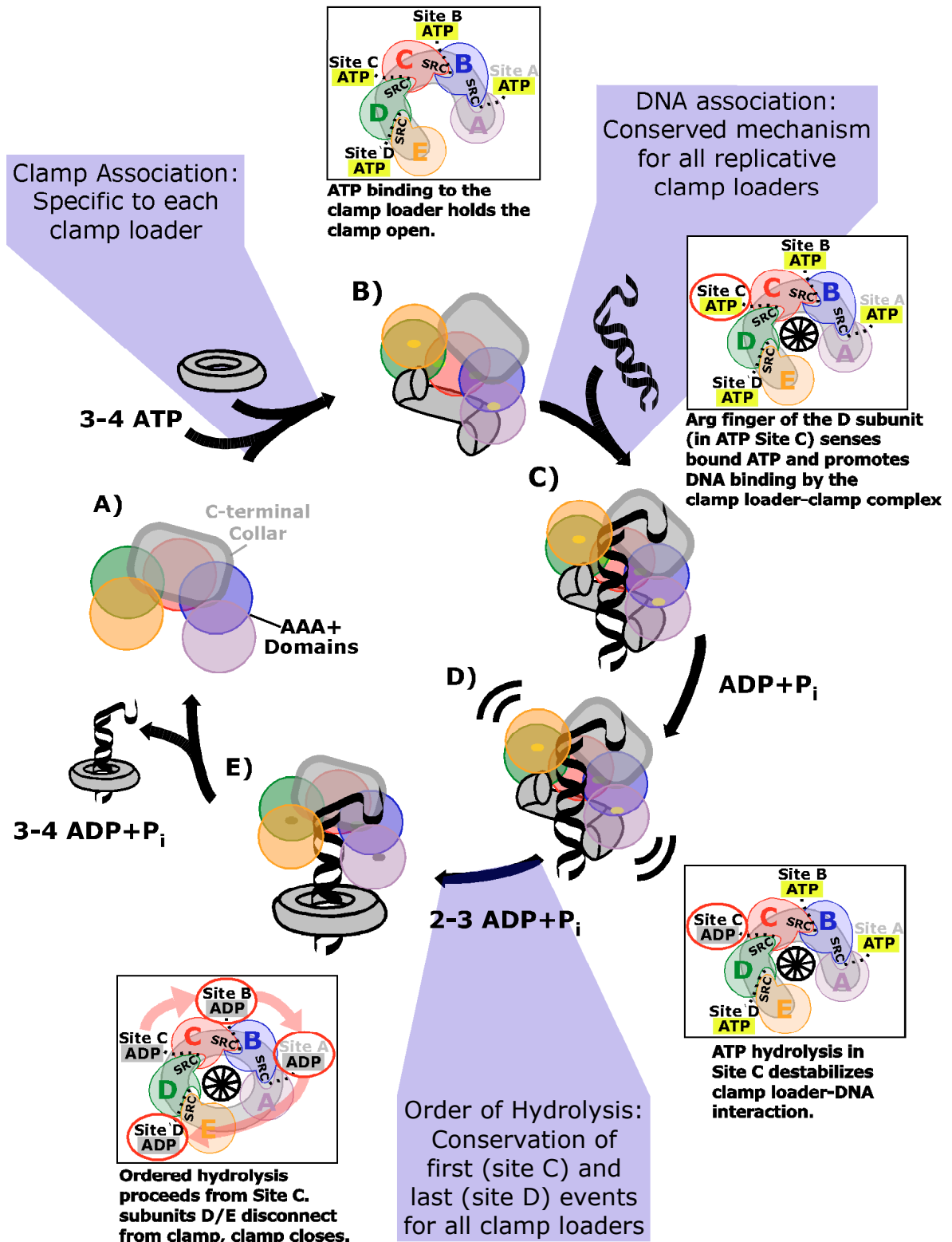
We provide evidence in this thesis for conservation of ATP site function between the *E. coli*  $\gamma$  complex and yeast RFC. RFC has one more competent ATPase site than  $\gamma$  complex, at the RFC-A/B interface, but activity in this site is not essential for clamp loading in yeast RFC (current study, (Schmidt et al., 2001)). The two ATP sites that are most important for clamp loading by RFC, ATP sites C and D, have conserved analogous positions in  $\gamma$  complex.  $\gamma$  complex may utilize these two ATP sites in a similar manner to drive DNA binding and  $\beta$  clamp loading. Clamp loaders of most DNA replicases are heterooligomers with one subunit that lacks an SRC motif (Davey et al., 2002b). This composition results in one subunit lacking a competent ATP site, yet contributing an arginine finger to the adjacent ATP binding pocket. In *E. coli*, this subunit is  $\delta'$  and in RFC, the D subunit. Thus all clamp loaders are predicted to have ATP sites D, and the adjacent site C, but some clamp loaders, such as  $\gamma$  complex, lack ATP site A. The important activities that our studies have ascribed to sites C and D may have led to their conservation in clamp loaders of all kingdoms. Based on the conservation of clamp loader architecture, we predict that all AAA+ machines that load processivity clamps onto DNA will share certain steps in their mechanism (see Figure 5.1).

Sites C and D are present in the “core” RFC-B/C/D/E subcomplex that associates with other RFC-like proteins to create “alternate” RFC complexes. These complexes act in other cellular processes such as DNA damage repair and sister chromatid cohesion (Majka and Burgers, 2004). For example, the Rad24



**Figure 5.1 The conserved mechanism of processivity clamp loading.** Proposed model detailing the steps in clamp loading that may be conserved in all clamp loaders. *Panel A* The AAA+ domains of the inactive nucleotide-free clamp loader are presumably out of alignment for clamp and DNA association. Binding of 3-4 molecules of ATP drives association of the clamp loader with the clamp. All five ATPase modules presumably dock onto the clamp during clamp opening. These intimate interactions are likely specific to each clamp loader. In most systems, the clamp is opened prior to ATP hydrolysis. *Panel B* DNA association may be promoted by ATP in site C in all clamp loaders, as in *E. coli* and *S. cerevisiae*. *Panel C* DNA bound in the central channel of the RFC-PCNA spiral triggers ATP hydrolysis in site C. Hydrolysis in site C may destabilize RFC-DNA interaction; however, open-PCNA promotes continued association of RFC with DNA. *Panel D* Site C hydrolysis may begin ordered ATP hydrolysis that ends in site D. The first hydrolysis event (in site C) and the last (in site D) may be conserved in all clamp loaders. The intermediate hydrolysis events may vary, as in  $\gamma$  complex, which does not contain ATP site A. *Panel E* Hydrolysis in site D may cause subunits D and E to disconnect from the clamp, causing the clamp to close. Once the clamp is closed, and hydrolysis is complete RFC ejects from the site of clamp loading and ADP/P<sub>i</sub> dissociate to complete the cycle.

Figure 5.1 The conserved mechanism of processivity clamp loading.



subunit substitutes for RFC-A to form the “Rad 24 alternate RFC” complex and loads a PCNA-like clamp onto DNA (Bermudez et al., 2003; Majka and Burgers, 2003). Previous studies have determined that the Rad24 alternate RFC complex binds its clamp in an ATP-dependent manner mediated by the Rad24 subunit (Majka et al., 2004). Perhaps downstream events after this association, such as DNA binding and clamp closing, are driven by ATP sites C and D in a similar manner to that of the replicative RFC complex.

### **Correlation with the heteromeric AAA+ MCM and ORC/Cdc6 complexes**

Our results mutating individual ATP sites of yeast RFC bring up interesting parallels to work that has analyzed the ATP site function of other heterooligomeric AAA+ complexes involved in DNA replication. The putative replicative helicase in eukaryotes is the MCM2-7 complex, a heterohexameric ring of AAA+ proteins (Forsburg, 2004). Previous work has identified that two MCMs are needed to form a functional ATPase site and MCMs use a conserved arginine finger to promote hydrolysis (Davey et al., 2003; Schwacha and Bell, 2001). It was shown that, in the context of the full MCM2-7 hexamer, certain single-site P-loop mutations cause a dramatic loss of ATPase activity while addition of a second P-loop mutation rescues the activity (Schwacha and Bell, 2001). This result was interpreted as a de-regulation of ordered ATP hydrolysis producing a futile ATPase cycle. Our finding that addition of an ATP site A mutation in RFC restores activity to an ATP site C-containing mutant by de-regulating site D (APPENDIX) suggests that RFC and MCM2-7 may share certain features of an ordered ATP cycle.

Recent mutational analyses of the ORC/Cdc6 complex (Bowers et al., 2004; Randell et al., 2006), which loads the MCM2-7 ring onto origin DNA, also reveal similarities with our RFC ATP site work. Multiple Orc subunits and Cdc6 are AAA+ proteins that form a DNA-dependent ATPase when in complex with each other. Inhibition of hydrolysis in the ATP site of Cdc6 was found to support an ORC/Cdc6/MCM/DNA complex, yet it prevented topological association of the MCM 2-7 ring with DNA (Randell et al., 2006). It is unclear how closely MCM loading onto DNA is related to PCNA loading. However, it is interesting to note that disruption of the ATP site of Cdc6 has a similar result to disruption of ATP site D in RFC, which prevents topological linking of PCNA to DNA.

#### **Clamp loader arrangement: why a heteropentamer?**

Clamp loaders are structurally asymmetric, which distinguishes them from other ring-shaped ATPases that have ATP sites evenly distributed around the ring. This asymmetry may be utilized by clamp loaders to achieve the necessary conformational changes needed for clamp loading that symmetric ring complexes cannot achieve. For example, when clamp loader subunits dock onto PCNA, they break the symmetry of the clamp and cause PCNA to adopt an asymmetric open structure that DNA can enter.

Many symmetric ring-shaped ATPases are considered to go through a “rotary” hydrolytic cycle of ATP binding, hydrolysis, and ADP exchange that obeys an alternating pattern around the ring. In this way, every ATP site proceeds through the same steps right before or right after the adjacent site and only a fraction of sites are bound to triphosphate nucleoside at any one time. The F1-ATPase mechanism is a canonical example of this type of cycle (Abrahams et

al., 1994), and the AAA+ protein p97 is proposed to act in a similar manner (DeLaBarre and Brunger, 2005). These complexes act as molecular motors, coupling many rounds of hydrolysis in each site to achieve their function. Clamp loaders, in contrast, are readily capable of filling all sites with ATP at the same time and one full round of hydrolysis completes the mechanism. In fact, clamp loaders are sometimes referred to as “nucleotide switches” (Hingorani and O'Donnell, 1998), similar to the DnaC *E. coli* helicase loader (Davey et al., 2002a) and the *E. coli* DnaA initiation protein (Lee and Bell, 2000), both of which are also AAA+ family members. The switch is turned “on” upon ATP binding and “off” after hydrolysis. Switching drives the most important aspects of the mechanism. We have demonstrated that clamp loaders are not strictly switches, because there is an order to ATP hydrolysis that contributes to the fidelity of threading a clamp through DNA.

The T7 gp4 hexamer is a helicase that follows a rotary hydrolysis cycle (Hingorani et al., 1997). Structural studies of gp4 have revealed that when all NTP sites of gp4 are bound to nucleotide, the six-subunit complex is forced out of the active planar ring form and into a helical conformation (Sawaya et al., 1999; Singleton et al., 2000). The fact that clamp loaders become fully-charged with triphosphate adenine nucleoside may contribute to the formation of the helix of ATPase modules, seen most clearly in the structure of RFC-ATP $\gamma$ S-PCNA (Bowman et al., 2004). Perhaps the asymmetry of all clamp loaders contributes to the ability of these complexes to adopt non-planar structures. Clamp loaders are constructed from at least two types of subunits: one type with all consensus ATPase motifs, present in multiple copies, and one type with certain motifs having diverged, usually present as a single copy. The break in continuity of

subunit composition, in addition to the gap between two subunits for DNA, may force or allow helix formation as opposed to planar ring formation.

### **Future Directions**

The past six years have seen a dramatic leap in the amount of structural information available for both prokaryotic and eukaryotic clamp loaders. The wealth of information that these elegant investigations have delivered has only begun to be tapped. Some of the key elements of this thesis involved biochemical and biophysical testing of the predictions from structural work.

Many molecular details of clamp loader function have yet to be unraveled. It is still not known how a clamp loader-open clamp supercomplex is stabilized in order to search for a primer-template junction and what molecular recognition events underlie the triggering of clamp closure at that particular DNA structure. What structural features of a 3' end of a primer distinguish it from a 5' end? Does the clamp loader read the polarity of the single-stranded DNA of the template strand? How does the replacement of RFC-A by an alternate RFC subunit confer different specificity on that clamp loader, for both alternate clamps and alternate DNA structures?

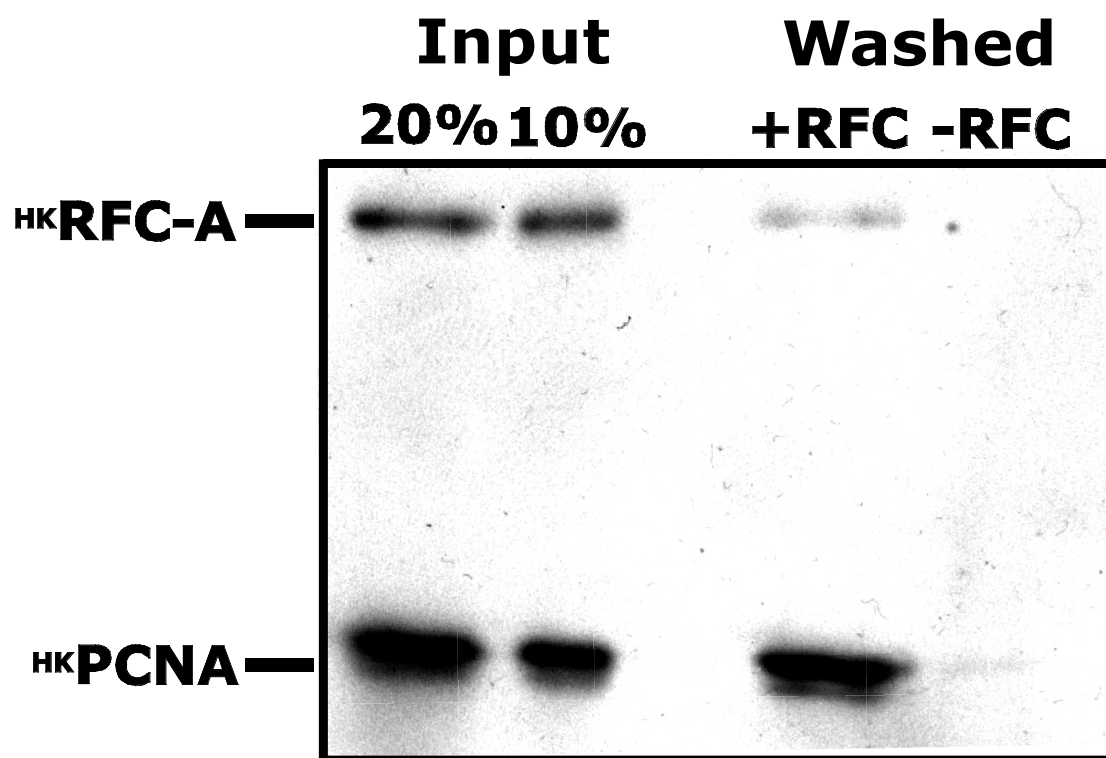
The model for ordered ATP hydrolysis in RFC put forth in Chapter 4 must be more rigorously examined. Mutations in the RFC subunit DEXX box motif, which is thought to be responsible only for coordination of  $Mg^{2+}$  for ATP hydrolysis, may aid in investigating the sequence of ATP hydrolysis. Examination of the effect of these mutations using fluorescence-based stopped-flow assays for ATP hydrolysis, similar to work on  $\gamma$  complex (Williams et al., 2004), will be key to confirming the model in Figure 4.9.

Many questions still remain as to how the clamp loader acts at the replication fork. Particularly in eukaryotes, because it is not even clear how RFC may associate with the replication fork. It is speculated that a similar connection may occur to that in *E. coli*, between clamp loader and polymerase and/or helicase (see Figure 1.7). There are many additional factors at the replication fork, compared to *E. coli*, such as Cdc45, the GINS complex, Sld2 and Sld3. It seems likely that RFC will association with these other factors at the fork into a eukaryotic replisome. Investigations of RFC dynamics at the fork will be an exciting line of work. In addition, the dynamics of the *E. coli* clamp loader at the fork, specifically the way that the C-termini of  $\tau$  may function in clamp loader action, have yet to be thoroughly investigated. The fusion strategy employed in Chapter 3 may be a powerful tool to investigate the action of the  $\tau$  C-terminus. A fusion construct could be made that would place  $\tau$  at a specific position in the clamp loader. Perhaps a clamp loader with  $\tau$  at a certain position is more active in lagging strand clamp loading than when  $\tau$  does not occupy that position.

New technologies offer a breath of fresh air to investigations of DNA replicases. Real-time multi-wavelength fluorescence analysis of protein-protein and protein-DNA dynamics is a powerful tool for studying pre-steady-state behavior of clamp loaders and polymerases. Single-molecule analysis, still in its infancy as a biological tool, will surely deliver exciting new advances in understanding the molecular machines that ensure faithful genome duplication.

# APPENDIX





**Figure A.1 RFC does not remain associated with bead-conjugated DNA after the separation step of PCNA loading reactions.** Reactions of 120  $\mu$ L containing unlabeled <sup>HK</sup>PCNA were incubated with and without RFC for 2 min., quenched with EDTA, and washed as in Figure 4.3. Protein remaining on the beads was stripped with 1% SDS, separated on a 10% SDS-polyacrylamide gel, and subjected to Western analysis to visualize His-tagged RFC-A (*top*) and PCNA (*bottom*). As a reference, 10% and 20% of the amount of RFC and PCNA input into the reaction were analyzed (*left*).

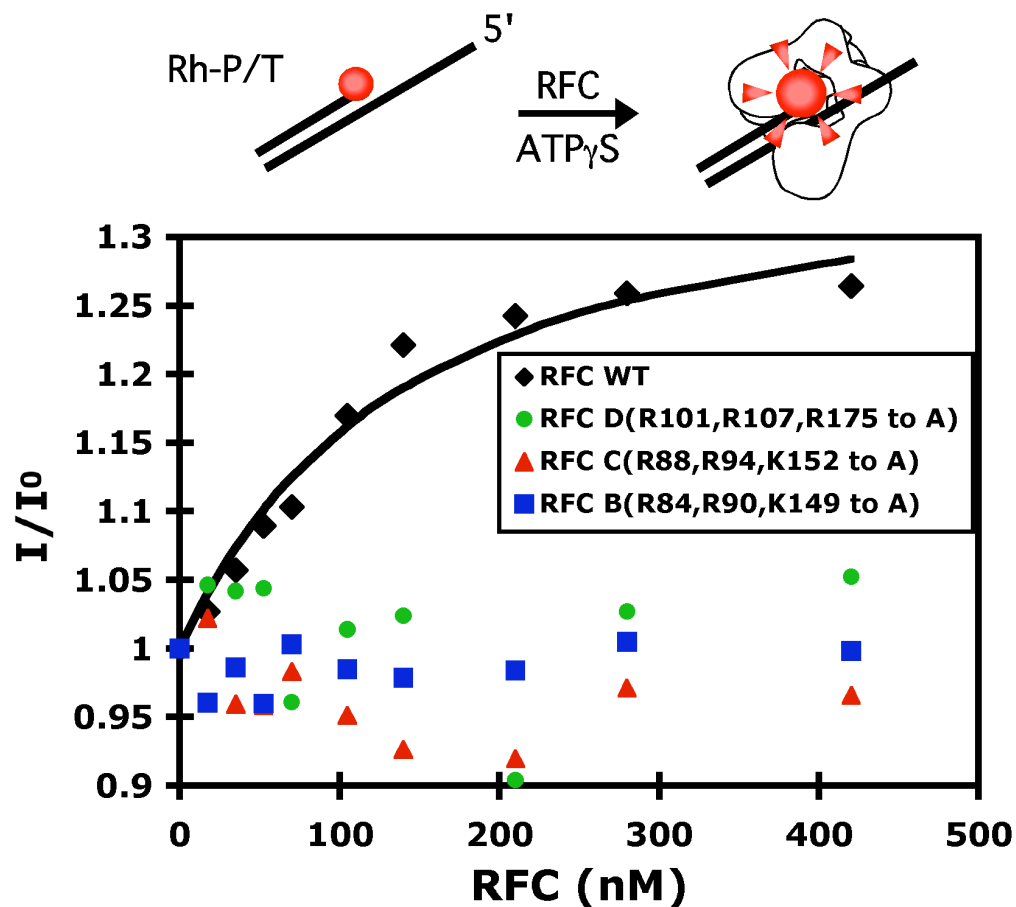


Figure A.2 Removing DNA-interacting residues from one RFC subunit prevents RFC complex association with the primed template. DNA binding assays were carried out with a primer-template DNA labeled at the 3' end of the primer (*see scheme at top*). An increase in the relative fluorescence ( $I/I_0$ ) indicates association of RFC with the DNA. RFC complexes with mutations in DNA-interacting residues of either RFC-B (R84,R90,K149 to A), RFC-C (R88,R94,K152 to A), or RFC-D (R101,R107,R175 to A) were assayed along with wild-type RFC.

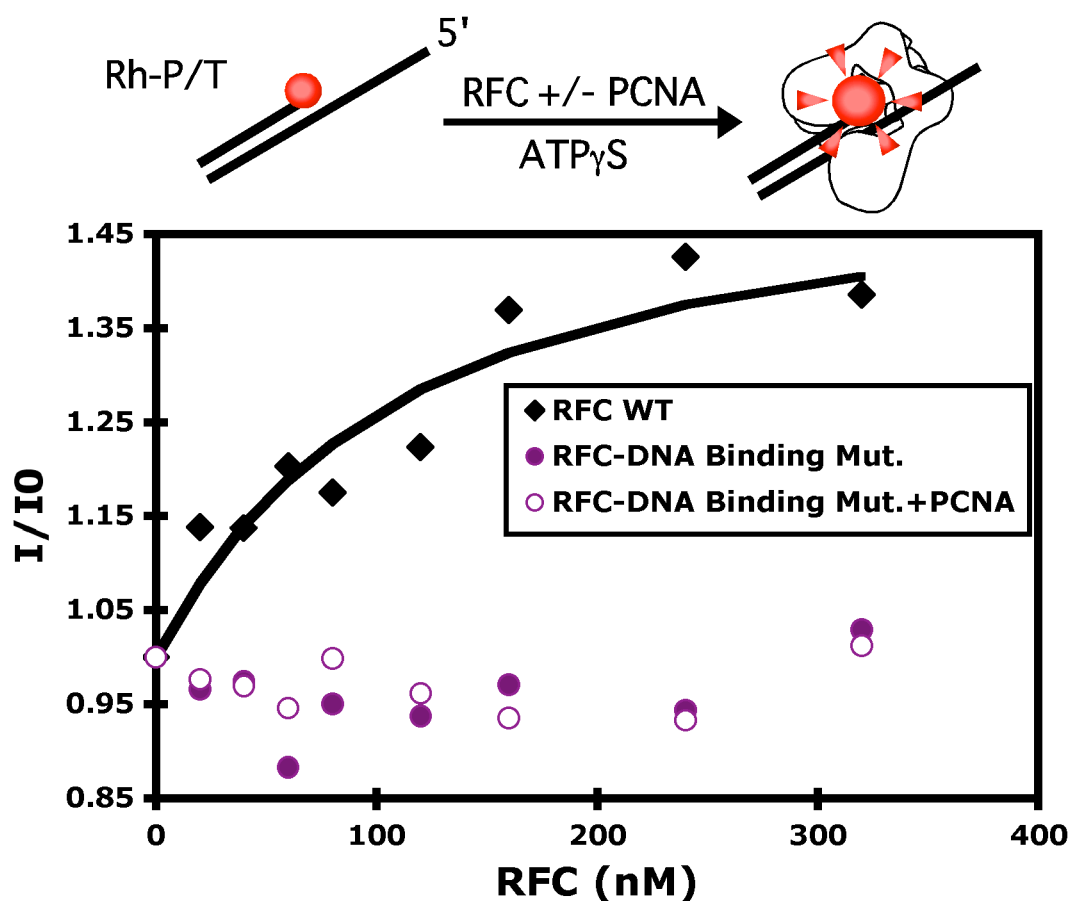


Figure A.3 PCNA does not rescue RFC that is missing DNA-interacting residues from RFC-B, -C and -D. DNA binding assays were carried out with a primer-template DNA labeled at the 3' end of the primer (*see scheme at top*). An increase in the relative fluorescence ( $I/I_0$ ) indicates association of RFC with the DNA. RFC with mutations in DNA-interacting residues of RFC-B (R84,R90,K149 to A), RFC-C (R88,R94,K152 to A), and RFC-D (R101,R107,R175 to A) was tested for primer-template association in the absence (*closed circles*) and presence (*open circles*) of PCNA. Wild-type RFC alone is shown for reference.

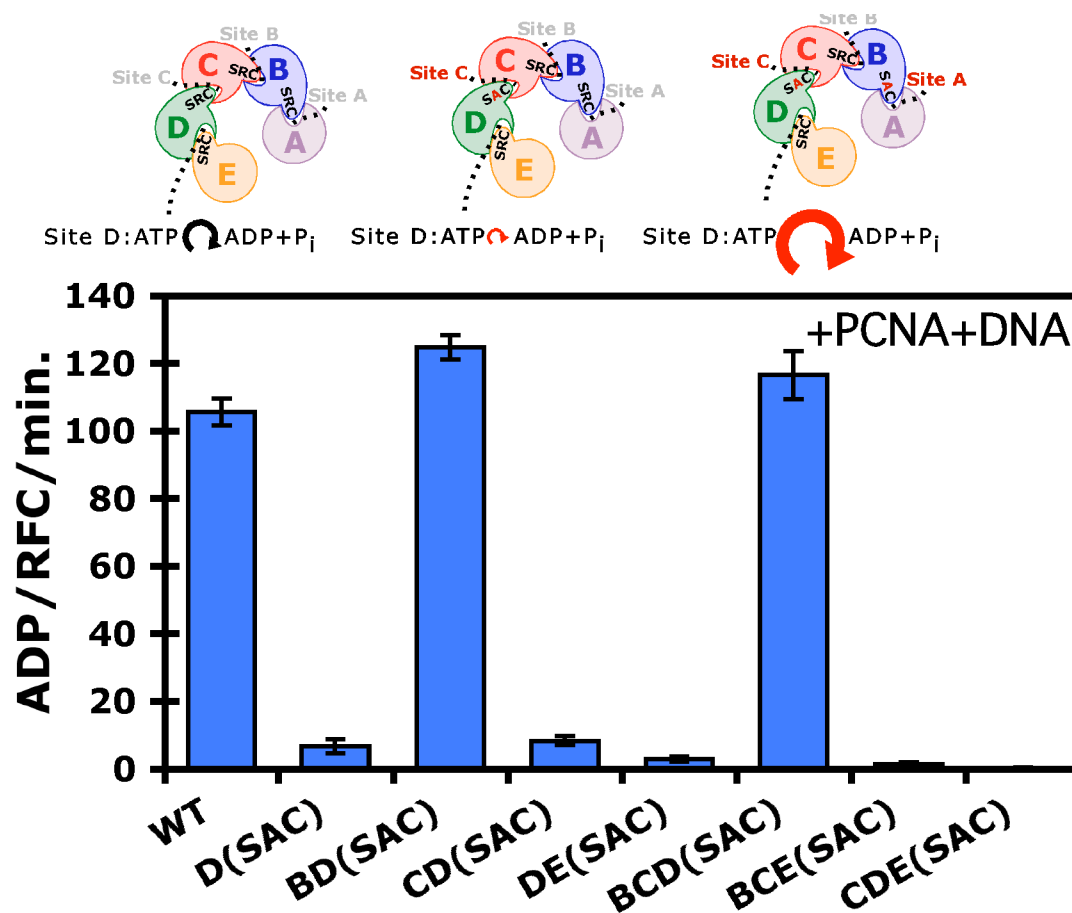


Figure A.4 ATPase activities of all permutations of (SAC) complexes containing a mutant RFC-D subunit and one or two additional (SAC) mutations. Steady-state ATP hydrolysis assays were performed for all RFC complexes with an arginine finger mutation in RFC-D. Assays were performed as in Figure 4.6A, containing both PCNA and primer-template DNA. Introduction of an arginine finger mutation in RFC-B causes hydrolysis in site D to increase compared to the level in the RFC D(SAC) complex. The scheme at the top summarizes this finding. Other ATP sites in wild-type RFC are presumably active, but they are colored in gray to draw attention to site D.

## MATERIALS AND METHODS

*Materials* --Unlabeled ADP and ATP were purchased from Sigma; unlabeled ATP $\gamma$ S and eosin-5-maleimide, Roche Diagnostics; radioactive nucleoside triphosphates, Perkin Elmer Life Sciences, Inc. Unlabeled deoxyribonucleoside triphosphates, Pharmacia LKB Biotechnology Inc. Nitrocellulose membrane circles were purchased from Schleicher and Schuell and polyethyleneimine-cellulose TLC plates were purchased from EM Science. Streptavidin-coated Dyna-Bead M-280 magnetic beads were purchased from Dynal Biotech. Bio-Gel A-15m and P6 resins were purchased from Bio-Rad.

*Proteins*--Proteins were purified as described:  $\alpha$ ,  $\epsilon$ ,  $\gamma$ ,  $\tau$  (Studwell and O'Donnell, 1990),  $\beta$  (Kong et al., 1992),  $\delta$  and  $\delta'$  (Dong et al., 1993),  $\chi$  and  $\psi$  (Xiao et al., 1993),  $\theta$  (Studwell-Vaughan and O'Donnell, 1993), SSB (Yao et al., 2000b), PCNA and PCNA (Cys81-Only) (Ayyagari et al., 1995); *E. coli* SSB (Yao et al., 2000a).. Core polymerase (Studwell-Vaughan and O'Donnell, 1993), and  $\gamma$  complex (Onrust and O'Donnell, 1993) were reconstituted from pure subunits and purified as described. Mutant subunits were purified by the same methods as wild-type proteins. The  $\gamma$  complex containing mutant subunits was reconstituted and purified using the same procedure as wild-type  $\gamma$  complex. Samples of purified complex were analyzed on a 14 % SDS-polyacrylamide gel stained with Coomassie Brilliant Blue G-250 and each lane was scanned by laser densitometer (Molecular Dynamics).

$\beta^{\text{PK}}$  is  $\beta$  containing a 6 residue C-terminal kinase recognition site (Naktinis et al., 1996), and was labeled to a specific activity of 10 dpm/fmol with  $\gamma^{32}\text{P}$ -ATP

using the recombinant catalytic subunit of cAMP-dependent protein kinase produced in *E. coli* (a gift from Dr. Susan Taylor, Univ. of California at San Diego). PCNA<sup>HK</sup> is a PCNA construct with a His-tag and a N-terminal 6 residue kinase recognition site (Kelman et al., 1995). PCNA<sup>HK</sup> was purified in one step by nickel chelate chromatography. PCNA<sup>HK</sup> was labeled to a specific activity of 100 dpm/fmol with  $\gamma$ -<sup>32</sup>P-ATP using the recombinant catalytic subunit of cAMP-dependent protein kinase.

$\gamma$  complex, PCNA, and RFC concentrations were determined by absorbance at 280 nm in 6 M guanidinium hydrochloride using extinction coefficients calculated from the Trp and Tyr content ( $\gamma$  complex,  $\epsilon_{280} = 223770 \text{ M}^{-1} \text{ cm}^{-1}$ ; PCNA,  $\epsilon_{280} = 5120 \text{ M}^{-1} \text{ cm}^{-1}$ ; RFC,  $\epsilon_{280} = 158,880 \text{ M}^{-1} \text{ cm}^{-1}$ ). All other protein concentrations were determined by Bradford assay reagent (Bio-Rad) using BSA as a standard.

*DNA*s--The following oligonucleotides were synthesized and gel purified by Integrated DNA Technologies (IDT): 79 mer, 5'-GGG TAG CAT ATG CTT CCC GAA TTC ACT GGC CGT CGT TTT ACA ACG TCG TGA CTG GGA AAA CCC TGG CGT TAC CCA ACT T-3'; 45 mer, 5'-GGG TTT TCC CAG TCA CGA CGT TGT AAA ACG ACG GCC AGT GAA TTC-3'; 30-mer, 5'-GCA ATA ACT GGC CGT CGT TTG AAG ATT TCG-3'; 66-mer, 5'-CCA TTC TGT AAC GCC AGG GTT TTC GCA GTC AAC ATT CGA AAT CTT CAA ACG ACG GCC AGT TAT TGC-3'. A 102-mer 3' biotinylated oligonucleotide was synthesized and gel purified by the W. M. Keck Facility at Yale University: 5'- CCA TTC TGT AAC GCC AGG GTT TTC GCA GTC AAC ATT CGA AAT CTT CAA ACG ACG GCC

AGT TAT TGC TCT TCT TGA GTT TGA TAG CCA AAA CGA CCA TTA TAG - 3' {Biotin}. To form synthetic primed template, the 30-mer or 45-mer primer oligonucleotide was mixed with the 79-mer (for 45-mer) or 66-mer or 102-mer (for 30-mer) template strand in a 1:1.2 ratio of primer to template in 50  $\mu$ l of 5 mM Tris-HCl, 150 mM NaCl, 15 mM sodium citrate (final pH 8.5), then incubated in a 95°C water bath which was allowed to cool to room temperature over a 30 min interval. M13mp18 ssDNA was purified as described (Turner and O'Donnell, 1995) and primed with a 30-mer DNA oligonucleotide as described (Studwell and O'Donnell, 1990).

*Buffers*—Buffer A is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Buffer H is 30 mM Hepes-NaOH (pH 7.5), 0.5 mM EDTA, 2 mM DTT, and 10% glycerol (v/v). Gel Filtration Buffer is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM DTT, 10 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA, and 4% glycerol (v/v). Reaction Buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 4% glycerol (v/v), and 40  $\mu$ g/ml BSA. ATPase Buffer is 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, and 10% glycerol (v/v). ). Loading Buffer (LB): 30 mM Hepes-NaOH (pH 7.5), 7 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM CHAPS. Membrane Wash Buffer is: 20 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 150 mM NaCl.

*Equilibrium Gel Filtration*—Analysis of ATP binding to wild-type and mutant  $\gamma$  complexes was performed by equilibrium gel filtration as described (Hummel and Dryer, 1962). Wild-type and either  $\gamma$ (R169A)  $\gamma$  complex or  $\delta'$ (R158A)  $\gamma$  complex (8.3  $\mu$ M  $\gamma$  complex in 60  $\mu$ L; 6.6  $\mu$ M  $\gamma$ (R169A) complex in 150  $\mu$ L; 6.6  $\mu$ M

$\delta'$ (R158A)  $\gamma$  complex in 150  $\mu$ l) were incubated in Gel Filtration Buffer + 100 mM NaCl containing  $\alpha$ - $^{32}$ P ATP at the indicated concentration (0.1-10  $\mu$ M) for 15 min. at 25°C. Samples were then applied to a 5 mL Bio-Gel P-6 column (Bio-Rad) at 25°C pre-equilibrated in Gel Filtration Buffer + 100 mM NaCl having the same concentration of  $\alpha$ - $^{32}$ P-ATP as the respective sample. Thirty-five fractions of 240  $\mu$ L each were collected, and 100  $\mu$ L of each fraction was analyzed by liquid scintillation to determine the total amount of ATP ( $[\text{ATP}]_{\text{TOTAL}}$ , see below). 50  $\mu$ L of the peak fractions were also analyzed for total protein concentration by the Bradford assay (Bio-Rad) using  $\gamma$  complex as a standard. Scatchard analysis from equilibrium gel filtration data was as described (Davey and Funnell, 1997).

*Gel Filtration Analysis of  $\gamma$  complex •  $\beta$  Interaction*—Ability of wild-type and mutant  $\gamma$  complex to associate with  $\beta$  was analyzed by gel filtration on a FPLC Superose 12 column (Pharmacia).  $\beta$  (30  $\mu$ M, 480  $\mu$ g) was incubated alone or with  $\gamma$  complex (25  $\mu$ M, 1.25 mg) for 15 min. at 15°C in 200  $\mu$ L Buffer A + 100 mM NaCl containing 1 mM ATP and 10 mM  $\text{MgCl}_2$ . The mixture was then injected onto a 24 mL Superose 12 column equilibrated in the same buffer at 4°C. After collecting the first 5.8 mL (void volume), fractions of 155  $\mu$ l were collected and analyzed in a 14% SDS-polyacrylamide gel.

*ATPase Assays with *E. coli* clamp loader complexes*—Wild-type and mutant clamp loader complexes were tested for ATPase activity in the presence of the synthetic primed template, with or without  $\beta$ . ATPase assays contained 50 nM clamp loader complex, 1 mM  $\alpha$ - $^{32}$ P ATP, 200 nM  $\beta$  dimer (when present), and 500 nM



synthetic primed template DNA in a final volume of 60  $\mu$ L ATPase Buffer. The synthetic primer / template DNA is linear, and thus allows  $\beta$  to slide off the ends after it is loaded. Thus  $\beta$  is continuously recycled during these assays as demonstrated previously (Bloom et al., 1996). Reactions were brought to 37°C and initiated upon addition of clamp loader. Aliquots of 5  $\mu$ l each were removed at intervals (0-10 min.) and quenched with an equal volume of 0.5 M EDTA (pH 7.5). 1  $\mu$ L of each quenched aliquot was spotted on a polyethylenimine cellulose TLC sheet (EM Science) and developed in 0.6 M potassium phosphate buffer (pH 3.4). The TLC sheet was dried and  $\alpha$ - $^{32}$ P ATP and  $\alpha$ - $^{32}$ P ADP were quantitated using a PhosphorImager (Molecular Dynamics).

*Clamp Loading Assay with E. coli clamp loader complexes*—Clamp loading was measured by separating  $^{32}$ P- $\beta^{PK}$  on DNA from free  $^{32}$ P- $\beta^{PK}$  using Bio-Gel A-15m, a large pore resin that excludes large DNA substrates but includes protein.  $^{32}$ P- $\beta^{PK}$  (13.3 nM as dimer) was incubated for 10 min. at 37°C either alone or with mutant or wild-type clamp loader complex (10.7 nM) in 75  $\mu$ L of Reaction Buffer containing primed M13mp18 ssDNA (13.3 nM), SSB (3.2  $\mu$ M as tetramer), 1 mM ATP, and 10 mM  $MgCl_2$ . The reaction was applied to a 5 mL Bio-Gel A-15m column (Bio-Rad) equilibrated in Gel Filtration Buffer + 50 mM NaCl at 25°C. Thirty-five fractions of 180  $\mu$ L each were collected and 100  $\mu$ L was analyzed by liquid scintillation.  $^{32}$ P- $\beta^{PK}$  bound to the DNA elutes early (fractions 11-15) while the free  $^{32}$ P- $\beta^{PK}$  elutes later (fractions 17-28). The amount of  $\beta$  in each fraction was determined from its known specific activity.

*Replication Activity Assays*—Activity of wild-type and mutant *E. coli* clamp loader complexes was assayed by the requirement to load  $\beta$  onto a primed circular M13mp18 ssDNA template in order to observe nucleotide incorporation by the core polymerase ( $\alpha\epsilon\theta$  subunits). The reaction mixture contained core polymerase (5 nM),  $\beta$  (10 nM as dimer), SSB (420 nM tetramer), primed M13mp18 ssDNA (1.1 nM), 60  $\mu$ M each of dATP, dCTP, and dGTP, 20  $\mu$ M  $\alpha$ - $^{32}$ P TTP, 1 mM ATP, and 10 mM  $\text{MgCl}_2$  in 25  $\mu$ L Reaction Buffer (final volume). Replication was initiated upon addition of either wild-type or mutant clamp loader (0-1.28 nM titration) and incubated at 37°C for 5 min. Reactions were quenched upon addition of 25  $\mu$ L 1 % SDS, 40 mM EDTA. Quenched reactions were spotted onto DE81 (Whatman) filters, then washed and quantitated by liquid scintillation as described (Studwell and O'Donnell, 1990).

*Reconstitution of mixed  $\gamma/\tau$ (SAC) clamp loader complexes*—*E. coli* clamp loader complexes with a mixture of  $\gamma$  and  $\tau$ (SAC) subunits were reconstituted using the procedure described below.  $\tau$ (SAC) and a 2.5-fold excess of  $\gamma$  were incubated for 1 h at 16°C in Buffer A. Subsequently,  $\delta$  and  $\delta'$  (5.25-fold excess each),  $\chi$  (10.5-fold excess), and  $\psi$  (7-fold excess) were added and the mixture was incubated for a further 30 min. The mixture was then brought to 100 mM NaCl in 10 mL and loaded onto a 1 mL MonoS (Amersham Biosciences) column equilibrated in Buffer A containing 100 mM NaCl. The column was washed with Buffer A + 100 mM NaCl and bound protein was eluted with a linear 16-mL NaCl gradient from 100-500 mM. 10  $\mu$ L of each fraction was analyzed by 14% SDS-PAGE and Coomassie Brilliant Blue (G250) staining.

Aliquots of fractions 7-26 were analyzed by Bradford reagent. 8000-fold dilutions of each fraction were made in Buffer A containing 40  $\mu\text{g/mL}$  BSA, yielding a concentration of  $\sim 250$  ng/mL for the peak fraction (fraction 15). 5  $\mu\text{L}$  aliquots of each dilution were added to reactions containing core polymerase (5 nM),  $\beta$  (10 nM as dimer), SSB (420 nM tetramer), primed M13mp18 ssDNA (1.1 nM), 60  $\mu\text{M}$  each of dATP, dCTP, and dGTP, 20  $\mu\text{M}$   $\alpha\text{-}^{32}\text{P}$  TTP, 1 mM ATP, and 10 mM  $\text{MgCl}_2$  in 25  $\mu\text{L}$  Reaction Buffer (final volume). Reactions were incubated at 37°C for 5 min. and quenched upon addition of 25  $\mu\text{L}$  1 % SDS, 40 mM EDTA. Quenched reactions were spotted onto DE81 (Whatman) filters, then washed and quantitated by liquid scintillation as described (Studwell and O'Donnell, 1990).

Fractions 13-17 were pooled and termed " $\gamma_2\tau(\text{SAC})_1$  complex". Wild-type  $\gamma_2\tau_1$  complex and  $\gamma_2\tau(\text{SAC})_1$  complex were titrated (0-64 fmol) into a similar assay to that described above, and analyzed as above.

*Construction of the  $\gamma - \gamma - \delta'$  fusion construct*--PCR was performed (using Vent Polymerase) on a plasmid containing the *dnaX* gene that over-expresses both  $\gamma$  &  $\tau$ . Two ORFs were generated, both of which encode the  $\gamma$  subunit, which is normally produced by translational frameshift of the *dnaX* gene transcript that encodes the  $\tau$  subunit. A stop codon was introduced at the end of the  $\gamma$  sequence. One ORF contained an Nde I site overlapping the initiating ATG and one ORF did not contain this Nde I site. The ORF lacking the overlapping Nde I site was additionally mutated near the C-terminus to change an Asc I site to Mlu I, resulting in the alteration of a single amino acid near the C-terminus of  $\gamma$ .

For  $\gamma$ (no Nde I/+Mlu I), the following PCR primers were used to generate PCR product #14:  $\gamma$ (no Nde I).N (35-mer): 5'-C CCT CTA GAA GGA GAT ATA AAT ATG AGT TAT CAG G-3';  $\gamma$ -Mlu I PCR.C (57-mer): 5'-CAC GGA TCC CTA CAT ATG ACG CGT CGC CAG CAC CTG GCT GGT GGT TTC CGG GAG CGG-3'. The Nde I site could be avoided by including an Xba I site followed by a Shine-Delgarno sequence, the appropriate space, and then the start codon in this  $\gamma$ (no Nde I).N PCR oligo.

For  $\gamma$ (start Nde I) the following PCR primers were used to generate PCR product #12:  $\gamma$ (ATG Nde I).N (35-mer): 5'-C CCT CTA GAA GGA GAT ATA CAT ATG AGT TAT CAG G-3';  $\gamma$  only.C (48-mer): 5'-AGC GGC GGA TCC TTA TTC CTT CTT CGC TTT GGT TGC TCC CTG CAC GCG-3'.

The following two oligos were annealed to form the “pET sites destroy linker”: pET sites destroy.a (31-mer): 5'-AAT TCT CAT GTT TGA CAG CTT ATC ATC GAC C-3'; pET sites destroy.b (31-mer): 5'-AG CTG GTC GAT GAT AAG CTG TCA AAC ATG AG-3'.

The Aat II site of pET(11a) was destroyed by linearizing the vector with Aat II, treating with klenow in the presence of dNTP's to blunt the ends, and religating. This plasmid is referred to as pET(11a)(A-). pET(11a)(A-) was digested with EcoR I/Hind III; the “pET sites destroy linker” was ligated in (100-200-fold molar excess) to destroy the Cla I and Hind III sites, resulting in a vector called pET(11a)(ACH-).

PCR product #12 was digested with Nde I/BamH I and was cloned into pET(11a)(ACH-), digested with the same enzymes. Clones were confirmed by sequencing. PCR product #14 was digested with Xba I/BamH I and was cloned

into pET(11a)(ACH-) digested with the same enzymes. Clones were confirmed by sequencing.

$\gamma$ (start Nde I)-pET(11a)(ACH-) was digested with Asc I/BamH I. The  $\gamma$ - $\delta'$  linker was ligated in (oligo at 100-fold molar excess over vector). The  $\gamma$ - $\delta'$  linker was made by annealing the following two oligos: g417-dp linker.a (33-mer): 5'-CG CGC CAG ATG AGA TGG TAT CCA TGG GGA TGA G-3'; g417-dp linker (corrected).b (33-mer): 5'-GA TCC TCA TCC CCA TGG ATA CCA TCT CAT CTG G-3'. The new construct was called  $\gamma$ ( $\gamma$ - $\delta'$  linker)-pET(11a)(ACH-). It was digested with Nco I/BamH I; the large fragment was then ligated with the small fragment of  $\delta'$ -pET(11a)ACH-) cut with the same enzymes. This generated ( $\gamma$ - $\delta'$ )-pET(11a)(ACH-).

$\gamma$ (no Nde I; + Mlu I)-pET(11a)(ACH-) and ( $\gamma$ - $\delta'$ )-pET(11a)(ACH-) were both digested Nde I/BamH I. The large fragment was purified from the former; the small fragment from the latter. These two fragments were ligated together to form ( $\gamma$ - $\gamma$ - $\delta'$ )-pET(11a)(ACH-). Subsequently, the Mlu I site in the pET(11a)(ACH-) vector was destroyed and this vector was ultimately used to generate ( $\gamma$ - $\gamma$ - $\delta'$ )-pET(11a)(MACH-).

Mutagenesis was done by the QuikChange (Stratagene) method as directed. An Arg169 to Ala mutation was made in the full-length *dnaX* gene with an Nde I site at the start codon. To swap into first position, this mutant construct was cut Xba I/Asc I and cloned into the fusion construct ( $\gamma$ - $\gamma$ - $\delta'$ )-pET(11a)(MACH-) cut with Xba I/Mlu I (Asc I and Mlu I have the same sticky end; neither enzyme re-cuts new sequence that results from ligation). To swap

into the 2nd position, the mutant was cut Nde I/ Asc I and cloned into the fusion construct cut Nde I/ Asc I.

*Purification of  $\gamma$ - $\gamma$ - $\delta'$ — $\gamma$ - $\gamma$ - $\delta'$  constructs with and without arginine finger mutations* were purified as follows. Plasmids were transformed into *E. coli* strain BLR(DE3) and grown in 12 L LB media containing 100  $\mu$ g/mL ampicillin at 37°C. Upon reaching an OD of 0.6, IPTG was added to 1 mM, followed by a 2-3 h incubation with shaking at 37°C. Cells were harvested by centrifugation, resuspended in 100 ml 10 mM Tris-HCl (pH 8.0), 500 mM NaCl, 30 mM spermidine, 10% sucrose; lysed using a French Press at 22,000 psi; and clarified by centrifugation. Solid ammonium sulfate was added to the supernatant in the amount of 0.16 g/mL supernatant and the ammonium sulfate was dissolved by sitring. Precipitated protein was isolated by centrifugation and the pellet was washed in Buffer A containing 500 mM NaCl and 0.125 g/mL ammonium sulfate. The washed pellet was dissolved in 30 mL Buffer A and dialyzed against Buffer A. After dialysis, sample was loaded onto a 15 mL Fast Flow Q (Amersham Biosciences) column equilibrated in Buffer A containing 150 mM NaCl. A 105 mL 150-500 mM NaCl linear gradient was used to elute the  $\gamma$ - $\gamma$ - $\delta'$  construct at ~350 mM NaCl. Typical yield was 12 mg at ~80% purity.

*Reconstitution of  $\gamma$ - $\gamma$ - $\delta'$  complex*—A clamp loader containing the  $\gamma$ - $\gamma$ - $\delta'$  construct,  $\tau$ ,  $\delta$ ,  $\chi$ , and  $\psi$  in an equimolar stoichiometry was reconstituted and purified as follows. Reconstitution was performed under dilute concentrations of all protein components (< 0.3 mg/mL) to promote intramolecular interactions within  $\gamma$ - $\gamma$ - $\delta'$ .

$\tau$  subunit was mixed with a two-fold excess of  $\gamma$ - $\gamma$ - $\delta'$  and subsequently with 1.5-fold excess  $\delta$ , the mixture was then incubated at 16°C for 2 h. To this mixture was added a four-fold excess of  $\chi$  and a three-fold excess of  $\psi$  subunit and the mixture was incubated for a further 30 min. at 16°C. The mixture was brought to 100 mM NaCl and applied to a pre-packed 1 mL MonoS column (Amersham Biosciences) equilibrated in Buffer A containing 100 mM NaCl. A linear gradient of 16 mL from 100-500 mM NaCl was performed. The complex containing equimolar  $\tau$ ,  $\gamma$ - $\gamma$ - $\delta'$ ,  $\delta$ ,  $\chi$  and  $\psi$  eluted at ~200 mM NaCl.

*Construction and purification of RFC (SAC) mutants*--Individual RFC-B (RFC4), RFC-C (RFC3), RFC-D (RFC2), and RFC-E (RFC5) genes were mutated using the Quik-Change method (Stratagene). The following mutations were made: RFC-B (RFC4) Arg157 to Ala, RFC-C (RFC3) Arg160 to Ala, RFC-D (RFC2) Arg183 to Ala, RFC-E (RFC5) Arg184 to Ala. All *S. cerevisiae* five-subunit RFC complexes used in this study were expressed in *E. coli* using a two-plasmid system described previously (Finkelstein et al., 2003). In all RFC constructs, the first 283 residues of the RFC-A (RFC1) subunit were deleted and RFC-A contained an N-terminal His-kinase tag (Kelman et al., 1995). All RFC complexes were purified as follows: Plasmids were transformed into *E. coli* strain BL21(DE3) and grown in 12 L LB media containing 100  $\mu$ g/ml ampicillin and 50  $\mu$ g/ml kanamycin at 37°C. Upon reaching an OD of 0.6, cells were brought to 15°C and IPTG was added to 0.5 mM, followed by an 12 h incubation with shaking at 15°C. Cells were harvested by centrifugation, resuspended in 100 ml Buffer H containing 150 mM NaCl, and lysed using a French Press at 22,000 psi. 2 mM PMSF was added

to the cell lysate and the lysate was clarified by centrifugation. The supernatant was applied to a 100 mL SP-Sepharose (Amersham Pharmacia) column equilibrated in Buffer H containing 150 mM NaCl. Protein was eluted in a 750 mL gradient from 150-600 mM NaCl. The five-subunit RFC complex eluted at approximately 510 mM NaCl, separating from RFC subcomplexes that eluted in earlier fractions. Fractions from the SP-Sepharose column containing five-subunit RFC were pooled, diluted in Buffer A to a conductivity equal to 100 mM NaCl, and loaded onto a 15 mL Fast Flow Q-Sepharose column (Amersham Pharmacia) equilibrated in Buffer A containing 90 mM NaCl. Protein was eluted using a 80 mL gradient of Buffer A from 90-500 mM NaCl. Fractions containing RFC eluted at approximately 250 mM NaCl and were pooled, aliquoted and stored at  $-80^{\circ}\text{C}$ . The protein yield was approximately 50 mg from 12 L of *E. coli* culture.

*ATP $\gamma$ S Binding Assays*--Nitrocellulose membrane circles (25 mm) were washed with 0.5 M NaOH, rinsed immediately with water and equilibrated in membrane wash buffer before use. Reactions contained 1  $\mu\text{M}$  RFC and  $^{35}\text{S}$ -ATP $\gamma$ S (0-25  $\mu\text{M}$ ) in Buffer B + 160 mM NaCl and 10 mM  $\text{MgCl}_2$  in a total volume of 15  $\mu\text{L}$ . After 1 min incubation on ice, 10  $\mu\text{L}$  of the reaction was filtered through a pre-treated nitrocellulose membrane on a glass microanalysis filter assembly (Fisher) at a rate of 60  $\mu\text{L}/\text{min}$ . The membrane was immediately washed with 150  $\mu\text{L}$  of ice-cold membrane wash buffer at  $\sim 1 \text{ mL}/\text{min}$ . Membrane circles were dried and radioactivity was measured by liquid scintillation counting. An apparent  $K_d$  was



determined using the simple binding model,  $\text{RFC} + \text{ATP}\gamma\text{S} \leftrightarrow \text{RFC-ATP}\gamma\text{S}$ , to fit the data by KaleidaGraph (Synergy Software).

*Clamp Loading Assays Using Primed M13mp18 ssDNA*--Clamp loading was measured using  $^{32}\text{P}$ -PCNA<sup>HK</sup> (29.4 nM as trimer) which was incubated for 10 min. at 30°C with RFC BCDE(SAC), or wild-type RFC (16.7 nM), in 60  $\mu\text{L}$  of Reaction Buffer containing primed M13mp18 ssDNA (17 nM), SSB (4.2  $\mu\text{M}$  as tetramer), 0.5 mM ATP, and 10 mM  $\text{MgCl}_2$ . The reaction was applied to a 5 mL Bio-Gel A-15m column (Bio-Rad) equilibrated in Gel Filtration Buffer containing 100 mM NaCl at 23°C. Thirty-five fractions of 180  $\mu\text{L}$  each were collected and 100  $\mu\text{L}$  was analyzed by liquid scintillation.  $^{32}\text{P}$ -PCNA<sup>HK</sup>-DNA complex elutes in the early fractions (fractions 9-15) while the free  $^{32}\text{P}$ -PCNA<sup>HK</sup> elutes later (fraction 18-31). The amount of PCNA in each fraction was determined from its known specific activity.

*Magnetic Bead PCNA Clamp Loading Assay*--The 30-mer/102-mer biotinylated DNA was conjugated to Dynabeads M-280 Streptavidin (DynaL Biotech), and the DNA-bead conjugate was incubated with 5 mg/ml BSA in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl at room temperature for 30 min. with agitation and washed three times in the same buffer until no BSA remained in the supernatant. Typical yield was ~100 pmole DNA/mg Dynabeads, as analyzed by comparing DNA ethidium bromide staining intensities in a polyacrylamide gel using known amounts of primed DNA as a standard. Clamp loading reactions were performed at 16°C in Loading Buffer (LB) containing 130 mM NaCl. Mixtures containing final concentrations of 0.5 mM ATP, 170 nM DNA,

510 nM *E. coli* SSB (tetramer), 700 nM  $^{32}\text{P}$ -PCNA<sup>HK</sup> (trimer), but lacking RFC were agitated by pipette and incubated for 1 min. at 16°C, then clamp loading was initiated upon adding RFC to a final concentration of 100 nM. Reactions were quenched with a final concentration of 21 mM EDTA (pH 7.5) at the indicated timepoints. Quenched reactions were placed on ice for 1 min., then the DNA-beads were isolated with a magnetic concentrator (1 min) at 4°C. Beads were washed twice in LB containing 300 mM NaCl at 4°C. Protein was stripped from the beads using 1 % SDS and counted by liquid scintillation. A similar reaction was performed using unlabeled PCNA in 120  $\mu\text{L}$  final volume and incubated for 2 min. with wild-type RFC. The bead-bound protein was stripped, separated by a 10% SDS-polyacrylamide gel, and subjected to Western analysis using INDIA HRP (Pierce), as directed, to follow histidine-tagged RFC and PCNA. Results are shown in Appendix Figure A.1.

*PCNA Ring Opening Assay*--The following residues in the *S. cerevisiae* PCNA gene were mutated by the Quik-Change procedure (Stratagene): Cys22 to Ser, Cys30 to Ser, Cys62 to Ser. One buried native cysteine residue (Cys81) at each trimer interface remained in this PCNA construct, referred to as PCNA (Cys81-Only). PCNA ring opening was tested by treatment with eosin-5-maleimide, a thiol-reactive dye. Ring opening reactions contained 20 mM Tris-HCl (pH 7.1), 200 mM NaCl, 0.5 mM EDTA, 10 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  ATP $\gamma\text{S}$ , 3  $\mu\text{M}$  PCNA(Cys81-Only), 3  $\mu\text{M}$  RFC, and 30  $\mu\text{M}$  eosin-5-maleimide (final concentrations in 15  $\mu\text{L}$ ). Reactions were incubated without eosin-5-maleimide for 1 min on ice, initiated with eosin-5-maleimide, and quenched after 30 s with 5  $\mu\text{L}$  1 M DTT. Control assays

without RFC contained 1.1 mg/mL BSA. Quenched reactions were analyzed in the dark on a 10 % SDS-polyacrylamide gel, and labeled PCNA was visualized in a UV scan (305 nm) on a Fluor-S MultiImager (Bio-Rad).

*Fluorescent DNA Binding Assays for RFC mutants*--RFC binding to DNA was measured using a fluorescently-labeled primed-template DNA. A synthetic 30-mer oligonucleotide, modified at the 3' hydroxyl with a C6-spacer primary amino group, was reacted with a Rhodamine-Red-X-NHS-ester (IDT) to form a Rhodamine Red-X-conjugated 30-mer. "Rh-P/T" primed-template DNA was prepared by mixing 1.0 nmol of Rhodamine Red-X-conjugated 30-mer (IDT) with 1.2 nmol unlabeled 66-mer in 100  $\mu$ L 5 mM Tris-HCl, 150 mM NaCl, 15 mM sodium citrate (final pH 8.5) and annealed as described above for unlabeled templates. RFC complexes were titrated from 0-420 nM into reactions of 60  $\mu$ L final volume containing 20 mM Tris-HCl (pH 7.5), 175 mM NaCl, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.5 mM EDTA, 100  $\mu$ M ATP $\gamma$ S or ADP, and 35 nM Rh-P/T DNA. When present, PCNA trimer was at a concentration of 810 nM. The fluorophore was excited at 570 nm and a 580-680 nm scan was taken. Relative intensity of the peak at 588 nm was plotted versus RFC concentration. Apparent K<sub>d</sub> measurements were determined using a simple model (RFC + DNA  $\leftrightarrow$  RFC-DNA) to fit the data using the KaleidaGraph program (Synergy Software). Attempts to measure primer-template association by a change in fluorescence anisotropy were complicated by a low signal of RFC that bound nonspecifically to regions other than the primer-template junction. Measurement of fluorescence

intensity change of the probe at the primer-template junction avoids the influence of this nonspecific binding activity.

*Steady-State ATP hydrolysis of RFC (SAC) mutants*—ATPase assays contained 500 nM RFC, 1 mM  $\alpha$ -<sup>32</sup>P-ATP, 2  $\mu$ M PCNA trimer (when present), and 1  $\mu$ M synthetic 30/66 DNA (when present) in a final volume of 30  $\mu$ L ATPase Buffer containing 150 mM NaCl. The synthetic primer/template DNA is linear and allows PCNA to slide off the ends after it is loaded. Hence, PCNA is continuously recycled during these assays. Reactions were brought to 30°C and initiated upon addition of RFC. Aliquots of 5  $\mu$ l were removed at one minute intervals from 0-3 min (for “+DNA” and “+PCNA+DNA” samples) or at five minute intervals from 0-20 min (for “alone” and “+PCNA” samples) and quenched with an equal volume of 0.5 M EDTA (pH 7.5). 1  $\mu$ L of each quenched aliquot was spotted on a polyethylenimine cellulose TLC sheet (EM Science) and developed in 0.6 M potassium phosphate buffer (pH 3.4). The TLC sheet was dried and  $\alpha$ -<sup>32</sup>P-ATP and  $\alpha$ -<sup>32</sup>P-ADP were quantitated using a PhosphorImager (Molecular Dynamics). Three independent experiments were performed and the standard deviation is indicated in the figure chart. A similar procedure was used when PCNA was titrated into a fixed concentration of either wild-type RFC or RFC E(SAC), except that each reaction was analyzed at a fixed 2 min timepoint.

*Measurement of Pre-steady-state ATP Hydrolysis by RFC Single (SAC) Mutants*--To measure a single turnover of ATP by the single RFC (SAC) mutants we designed a pre-steady state assay to measure one cycle of ATP hydrolysis. RFC was mixed with  $\alpha$ -<sup>32</sup>P-ATP, PCNA, and synthetic primed-template DNA in the absence of

magnesium, then the reaction was incubated for 5 seconds and subsequently mixed with magnesium and excess ATP $\gamma$ S. Final concentrations of the reaction were 1  $\mu$ M RFC, 2  $\mu$ M PCNA (trimer), 2  $\mu$ M 30/66 DNA, 10 mM MgCl<sub>2</sub>, 20  $\mu$ M  $\alpha$ -<sup>32</sup>P-ATP, and 1 mM ATP $\gamma$ S in 20  $\mu$ L ATPase Buffer containing 160 mM NaCl. After 5 seconds the reaction was quenched with 20  $\mu$ L 0.5 M EDTA (pH 7.5). Quenched reactions were spotted on TLC plates, developed, and quantitated as described above for steady-state ATPase assays.

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