Replication factor C (RFC) is a heteropentameric AAA+ protein clamp loader of the proliferating cell nuclear antigen (PCNA) processivity factor. The prokaryotic homologue, γ complex, is also a heteropentamer, and structural studies show the subunits are arranged in a circle. In this report, *Saccharomyces cerevisiae* RFC promoters are examined for their interaction with each other and PCNA. The data lead to a model of subunit order around the circle. A characteristic of AAA+ oligomers is the use of bipartite ATP sites in which one subunit supplies a catalytic arginine residue for hydrolysis of ATP bound to the neighboring subunit. We find that the RFC(3/4) complex is a DNA-dependent ATPase, and we use this activity to determine that RFC3 supplies a catalytic arginine to the ATP site of RFC4. This information, combined with the subunit arrangement, defines the composition of the remaining ATP sites. Furthermore, the RFC(2/3) and RFC(3/4) subassemblies bind stably to PCNA, yet neither RFC2 nor RFC4 bind tightly to PCNA, indicating that RFC3 forms a strong contact point to PCNA. The RFC1 subunit also binds PCNA tightly, and we identify two hydrophobic residues in RFC1 that are important for this interaction. Therefore, at least two subunits in RFC make strong contacts with PCNA, unlike the *Escherichia coli* γ complex in which only one subunit makes strong contact with the β clamp. Multiple strong contact points to PCNA may reflect the extra demands of loading the PCNA trimERIC ring onto DNA compared with the dimeric β ring.

Replicases of cellular chromosom es utilize a circular sliding clamp protein that encircles DNA and tethers the polymerase to the template for high processivity in DNA synthesis (1). An example of this protein class is the *Escherichia coli* β subunit, which confers high processivity onto the chromosomal replicase, DNA polymerase III holoenzyme (2, 3). The eukaryotic equivalent is the PCNA1 ring, which has essentially the same shape and chain fold as β despite lack of sequence similarity between the two (4, 5). These ring-shaped proteins require an ATP-fueled multiprotein clamp loader for assembly onto primed DNA.

The eukaryotic clamp loader is the heteropentameric replication factor C (RFC). The five subunits of RFC are each different proteins, but they are homologous to one another (6, 7) and are members of the AAA+ family of ATPases (8). The recent crystal structure of *E. coli* γ complex, the prokaryotic counterpart of RFC, has facilitated detailed hypothesis regarding RFC structure and mechanism (9, 10). The γ complex (γδδ′χψ) consists of a minimal core of five proteins (γδδ′), which contain the clamp loading activity (11). The remaining two subunits, χ and ψ, are involved in recruiting an RNA primed DNA site from the primase, and they bind single-stranded DNA-binding protein (SSB) to assist polymerase elongation but are not essential to the clamp loading activity of γ complex (12–14). Biochemical studies of γ complex (15–17), combined with crystal structures of γδδ′ (10) and δ-β1 complex (18, 19), reveal a highly detailed view of γδδ′ clamp loader form and function. The five subunits of the γδδ′ core complex are arranged in a circular formation (see Fig. 1). Like RFC, the γ3, δ, and χ′ subunits are members of the AAA+ family; they share a characteristic chain-folding pattern consisting of three domains each. The main intersubunit contacts in the heteropentamer are made via the C-terminal domains, which form a tight circular collar (Fig. 1A, top view). The N-terminal domains contain the ATP binding sites, and there is a gap between the N-terminal domains of the δ and χ′ subunits (Fig. 1A, front view). Only the γ subunits contain ATP binding and hydrolysis activity and therefore serve as the motor of this machine; both δ and χ′ lack a consensus ATP binding sequence, and neither of them bind ATP. The γ and χ′ subunits contain an SRC motif that is highly conserved from γ complex to RFC (20). The arginine residue (Arg finger) within the SRC motif is positioned such that it may participate in hydrolysis of ATP bound to the neighboring subunit (Fig. 1A, back view). Hence, the Arg finger within the δ′ SRC motif functions with ATP bound to γ1 (site 1), the γ1 SRC functions with ATP bound to γ2 (site 2), and the γ2 SRC functions with ATP bound to γ3 (site 3) (see Fig. 1B). Biochemical studies confirm that these conserved Arg residues are catalytic and suggest that ATP must first be hydrolyzed in sites 2 and/or 3 before ATP in site 1 is hydrolyzed (21).

Study of γ complex and its subunits shows that the δ subunit forms the strongest attachment to the β clamp, and in fact can open the ring by itself, leading to unloading of β clamps from closed circular DNA (19). The γ primer can also bind β and unload it, but it is feeble in these actions compared with δ (15). Although δ binds β2 tightly, the γ complex does not bind β3 in
The same elements are also present in ATP sites 1 and 3, forming the three ATPase sites in a diagram illustrating the polarity of the P-loops and arginine fingers complex that exposes subunits promotes tight interaction between the absence of ATP, indicating that one or more subunits of γ complex block the δ-to-βδ interaction (22). ATP binding to the γ subunits promotes tight interaction between γ complex and β, implying that ATP binding induces a conformation change in γ complex that exposes δ, and presumably γ subunits as well, for interaction with β and opening of the β2 ring. The δ subunit appears to be a rigid protein and has been termed the stator, the stationary part of a machine upon which other pieces move (10, 20). Results of mutational studies are consistent with the idea that the ATP-induced conformation change of γ complex requires δ′ and that it may serve as a “backboard” to direct the ATP-induced changes in γ complex (23).

The similarities in RFC and γ complex subunit sequences and their common function in loading circular clamps onto DNA suggest that the RFC subunits may also be arranged in a circular fashion like γδδ′ (10). Electron microscopy and atomic force microscopy studies of RFC are consistent with a circular arrangement of RFC subunits (9, 24). The human RFC1 subunit (p140), like δ′, binds to PCNA (25), leading to the proposal that RFC1 may act to open PCNA just as the δ wrench opens β (9, 18). RFC5, like δ′, contains an SRF motif, and the putative ATP site deviates from the consensus sequence (GKKT instead of GKT) suggesting that if it can bind ATP, the ATP may not hydrolyze efficiently. These similarities suggest that RFC5 may play a similar role as the δ′ stator. On the basis of the γδδ′ structure, it is proposed that the RFC1 (wrench) and RFC5 (stator) subunits may bracket the RFC2, 3, and 4, subunits, which, like γδ′, contain both ATP binding and SRF motifs and thus may act as the motor of the RFC clamp loader (9). However, the order of the RFC2, 3, and 4 subunits within the pentamer and the identity of the subunits that bind RFC5 and RFC1 are not certain.

The aim of this study is to define the arrangement of the subunits within the RFC complex and determine which subunits form the major contact(s) to PCNA. The results indicate the arrangement RFC5:RFC2:RFC3 RFC4:RFC1, and in an orientation looking down the C-terminal plane, RFC5 contributes an arginine finger (SRC) to ATP in RFC2 (site 1), RFC2 contributes an arginine finger to the RFC3 ATP site (site 2), RFC3 contributes an arginine finger to the ATP site in RFC4 (site 3), and RFC4 contributes an arginine finger to the RFC1 ATP site (site 4). Moreover, we find that the RFC(3/4) complex, RFC(2/3) complex, and RFC1 subunit form major contacts to PCNA, unlike the case of the single major contact between δ subunit of γ complex and the β clamp.

**EXPERIMENTAL PROCEDURES**

**Materials**

Radioactive nucleotides were purchased from PerkinElmer Life Sciences. Unlabeled deoxyribonucleoside triphosphates were supplied by Amersham Biosciences. DNA modification enzymes were supplied by New England Biolabs; DNA oligonucleotides were from Integrated DNA Technologies. Protein concentrations were determined using the Bio-Rad Protein stain and bovine serum albumin as a standard. Buffer A is 30 mM HEPES (pH 7.5), 10% (v/v) glycerol, 0.5 mM EDTA (pH 7.5), 1 mM DTT, and 0.04% Bio-Lyte 3/10 ampholyte (Bio-Rad). Buffer B is 20 mM HEPES (pH 7.4), 2 mM DTT, 10% glycerol, and 200 mM NaCl. Buffer C is 20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 40 μg/ml bovine serum albumin, 8 mM MgCl2, 4% glycerol, and 0.5 mM ATP.

**Plasmids**

The *Saccharomyces cerevisiae* RFC genes were cloned into either pET (Novagen) or pLANT (26) vectors. The plasmids containing single genes include pET11a-RFC1, pET11a-RFC2, pLANT (2-RFC2), pET11a-RFC3, pET11a-RFC4, and pET11a-RFC5. Expression plasmids containing two or more genes include pET11a-RFC3[+4], pLANT (2-RFC1[+5], pET11a-RFC2[+3]+4, and pET11a-RFC1[+2]+3+[4]+5).

**Mutations in the RFC1 Gene**—Codons TAT and TTC in pET11a-RFC1 corresponding to residues Tyr-404 and Phe-405 in RFC1 were mutated to GCT and GCC, respectively, by Commonwealth Biotechnologies, changing both hydrophobic residues to alanine. The mutated RFC1 gene was then cloned into the pLANT (2-RFC1[+5]) to replace the wild-type copy of the RFC1 gene.

**Mutations in the RFC3 and RFC4 Genes**—Mutations were introduced into the RFC(3) and RFC(4) genes using the QuikChange method.
(Stratagene). RFC(3/4 SAC) was generated in pET(11a)-RFC(3/4) using the following oligonucleotides: 5′-CAT AAA CTG ACT GTG CTA TTG AGC GCT TGG ACG AGA AGC ATG TTA CCG CAC CTC TTA GAA CCT GAC AGG TGT AAG TTT ATG-3′. RFC(4 SAC) was generated in pET(11a)-RFC(4) using the following oligonucleotides: 5′-GCA GAT CAT TGA GGC GCT GCA AAG GGC TGG TGG GAT TGG GAG GTA TTA TAA AGC GGT ATG GCT CAA TAA CGC CTA AAA TCG CAA CAC CGG GTG CCA GCC GGC ATC TAA TGT CC-3′. The entire open reading frames were then confirmed by DNA sequencing.

Mutant genes were cloned into pET(11a)-RFC[3-4] as follows: the RFC(3/4 SAC) gene was exchanged for the wild-type RFC(3) gene in the pET(11a)-RFC[3-4] plasmid using KpnI. Recombinants were screened with AfeI and the amino acid substitution confirmed by DNA sequencing.

The cells were harvested by centrifugation. A 30-minute interval. The ATPase reactions contained 2 mM ATP (0.5 mM MgCl2) and the final protein concentration was measured by the Bradford assay and the ImageQuant software (Molecular Dynamics).

**Gel Filtration Analysis**

Gel filtration was performed at 4 °C using a 24-ml Superdex-200 column (Amersham Biosciences) equilibrated in Buffer B. Subunit mixtures (protein concentrations indicated in the figure legends) were incubated at 16 °C for 15 min in 250 μl Buffer B and, when present, a 30-mM NaCl. The peak of RFC(2/3) was pooled and diluted with Buffer A to 100 mM NaCl before being further purified over a 6-ml Q-Sepharose Fast Flow column with a 60-ml gradient of 200–400 mM NaCl in Buffer A. The purified protein was dialyzed against Buffer A containing 100 mM NaCl before being stored at −70 °C. The yield was −1 mg RFC(2/3) per liter of cell culture.

**Purification of S. cerevisiae RFC4 Protein—Subcomplex**—The RFC(2/5) subcomplex was coexpressed with pLANT(2- RFC(2/5) and pET(11a)-RFC(5)). The purification protocol for this subcomplex is the same as for the RFC(2/3) complex (refer to above), except that a 150–600 mM NaCl gradient in Buffer A was used with the SP-Sepharose Fast Flow column, and a 100–450 mM NaCl gradient in Buffer B was used with the Q-Sepharose Fast Flow column. Yield was also −1 mg of RFC(2/5) per liter of cell culture.

**Purification of S. cerevisiae RFC2 Protein—Individual subunit**, RFC2, was expressed from the pET(11a)-RFC2 plasmid. The cells were lysed and then the lysate was clarified and diluted to 180 mM NaCl as described above for RFC complex purification. The protein was first fractionated over a 100-ml SP-Sepharose Fast Flow column with a 1-liter gradient of 150–600 mM NaCl in Buffer A. The peak of RFC2 (which eluted at about 300 mM NaCl) was pooled and diluted with Buffer A to ~200 mM NaCl, before being applied to a 50-ml Q-Sepharose Fast Flow column. The flow through fraction containing RFC2 was precipitated by addition of ammonium sulfate (0.5 g/ml). After centrifugation, the pellet was resuspended in Buffer A and dialyzed against Buffer A containing 250 mM NaCl at 4 °C overnight. The yield of protein was ~4 mg of RFC2 per liter of cell culture. Trace contaminants containing ATPase activity were separated away from the RFC(2) protein by gel filtration. Approximately, 700 μg of RFC2 protein was applied to a 24-ml Superdex-75 (Amersham Biosciences) column equilibrated with Buffer A containing 200 mM NaCl. After collecting 240 drops (6 ml), 5-drop (120 μl) fractions were collected (79 fractions). Aliquots (3 μl) from every other fraction were assayed for ATPase activity (refer to protocol below), and 16-μl aliquots from the same fractions were analyzed on an 10% SDS-polyacrylamide gel. ATPase activity was detected in fractions 17–27, whereas RFC2 protein was present in fractions 25–37. RFC2 free from contaminating ATPase activity (fractions 29–33) was used in the ATPase assays reported in this study.

**Purification of S. cerevisiae RFC4 Protein—Subcomplex**—RFC4 was expressed from the pET(11a)-RFC(4) plasmid using EcoRI. Recombinants were screened with AfeI and the amino acid substitution confirmed by DNA sequencing.

The cells were harvested by centrifugation. A 30-minute interval. The ATPase reactions contained 2 mM ATP (0.5 mM MgCl2) and the final protein concentration was measured by the Bradford assay and the ImageQuant software (Molecular Dynamics).

**Analysis of RFC Form and Function**

ATPase Assays

Wild-type and mutant RFC(3/4) subcomplexes were tested for ATPase activity in the presence or absence of a synthetic primed template. The primed template was formed by mixing the following two oligonucleotides: 79-mer, 5′-GGG TAG CAT ATG CTT CCC GGA TTC AGT GCG CTT GGT TAC AGC AGC GGC AGC ATG TTA CCG CAC CTC TTA GAA CCT GAC AGG TGT AAG TTT ATG-3′ and 45-mer (2.4 nmol) were mixed in 100 μl of 5 mM Tris·HCl, 150 mM NaCl, and 15 mM sodium citrate (final pH 8.5). The mixture was brought to 95 °C and then cooled to room temperature over a 30-min interval. The ATPase reactions contained 2 μM RFC(3/4), 2 mM [α-32P]ATP, 500 nm primed template (when present), and 2 μM RFC2 (when present) in a final volume of 70 μl of Buffer C. Reactions were incubated at 30 °C, and 10-μl aliquots were removed at intervals (0 to 4 min) and quenched with an equal volume of 5% SDS/40 mM EDTA mixture. One microliter of each quenched reaction was spotted on a polyethyleneimine cellulose TLC sheet (EM Science) and developed in 1 ml isopropanol–1-thio-β-n-galactopyranoside and then incubated at 15 °C for ~18 h. For RFC(2/3) and RFC(2/5), induction was at 37 °C for 3 h. The cells were harvested by centrifugation.

**Purification of S. cerevisiae RFC Complexes, Subcomplexes, and Individual Subunits**

**Purification of S. cerevisiae RFC3/4 Complex**—RFC3 was overexpressed from the single plasmid, pET(11a)-RFC[1-2+3+4+5]. The RFC3 was over-expressed by co-transformation of pLANT(2- RFC(2)–3+4) plasmid using KpnI. Recombinants were screened with AfeI and the amino acid substitution confirmed by DNA sequencing.

The cells were harvested by centrifugation. A 30-minute interval. The ATPase reactions contained 2 mM ATP (0.5 mM MgCl2) and the final protein concentration was measured by the Bradford assay and the ImageQuant software (Molecular Dynamics).
included 1 μM ATP, 8 mM MgCl₂, and 6.5 μM PCNA (as trimer). Subunit mixtures were applied to the column, and after collecting the first 5.6 ml (void volume), fractions of 180 μl were collected. Column fractions were analyzed in 7.5% SDS-polyacrylamide gels stained with Coomassie Blue. Bovine serum albumin (Sigma) (66 kDa) was added to the protein mixtures to serve as an internal molecular mass marker for each gel filtration analysis. The fraction numbers were normalized to the 66-kDa standard, which was set at peak fraction 49.

Steady-State Fluorescence Measurements

Steady-state fluorescence intensity measurements were performed using a PTI spectrofluorimeter (Photon Technology International). Fluorescence emission spectra were obtained from 500 to 600 nm using an excitation wavelength of 490 nm. The band-pass for excitation and emission was 2 and 4, respectively. All samples were in 10 mM Tris-HCl (pH 7.5), 5 mM DTT, 1 mM EDTA, 8 mM MgCl₂, 1 mM ATP, and 150 mM NaCl, as indicated. Fluorescent measurements utilized PCNA labeled with Oregon Green 488 maleimide (Molecular Probes). Measurements were performed at 100 nM, 200 nM, or 1 μM PCNA, and increasing amounts of RFCwild or RFCmut.

Replication Assays

The primer-template was prepared by annealing a synthetic DNA 30-mer oligonucleotide (M13mp18 map position 6815–6847) to M13mp18 ssDNA as described (2). The large subunit of Pol δ used in this assay was a truncated version of the full-length pol; it contained the N-terminal 867 amino acids (from a total of 1098 amino acids in the full-length Pol δ) and an additional 14 amino acids (LRDPLIIS-PKRNHV). The gene for this subunit of Pol δ was cloned into a pET11a vector, along with the other two subunits of Pol δ, holoenzyme. Induced cell lysate was fractionated over a Q-Sepharose Fast Flow column. The enzyme activity required the presence of both PCNA and yeast RFC and had a specific activity of ~1.0 fmol dNTP incorporated per μg of protein per min. Assays contained 14.1 fmol of primed ssDNA, 3.55 pmol of E. coli [32P]TTP in 12.5 mM Tris-HCl, 5 mM DTT, 1 mM EDTA, 8 mM MgCl₂, 1 mM ATP, and 150 mM NaCl, as indicated. Fluorescent measurements utilized PCNA labeled at its two exposed Cys residues (22 and 62) with Oregon Green 488 maleimide (Molecular Probes). Measurements were performed at 100 nM, 200 nM, or 1 μM PCNA, and increasing amounts of RFCwild or RFCmut.

RESULTS

We have reported previously a compatible two-vector expression plasmid system for coexpression of multiple proteins in E. coli (26). In that study we described the expression and purification of five-subunit yeast RFC in which an unessential N-terminal region of RFC1 is deleted. In the current study we express and purify RFC in which all subunits are full-length and unmodified. The earlier study indicated that expression of individual RFC subunits provided only insoluble protein. In the current study we coexpress two subunits at a time and find that three heterodimeric RFC subcomplexes are soluble and can be purified. In fact, during preparation of an RFC(3/4) complex, isolated RFC4 is also obtained from one of the purification steps. Moreover, we find that RFC2 expression results in the two subunits remaining stably associated with each other during analysis in a gel filtration column. For each heterodimer, induced cells were grown and lysed, and the cell lysate was fractionated using an SP-Sepharose cation exchange column. After this step, methods for the three preparations diverged, as explained under “Experimental Procedures.” Gel analysis of the purified proteins was as follows: ~0.5–1.0 μg of each preparation was analyzed in a 7.5% SDS-polyacrylamide gel stained with Coomassie Blue. The position of each RFC subunit is identified on the right of the gel, which contains a p15A origin and a kanamycin resistance gene. The pLANT vector used in this study includes genes encoding tRNAs that are prevalent in yeast but underrepresented in E. coli (codons AUA (Ile), CUA (Leu), AGG (Arg), and AGA (Arg)) and utilizes the same inducible T7 promoter and cloning sites of the pET11a vector. The two subunit combinations for the five subunits of RFC were tested by placing one gene in pLANT and another in pET. Cells carrying both plasmids were then induced, and the cell lysate was examined for the presence of soluble heterodimer. Using this approach three combinations provided soluble proteins, RFC(2/3), RFC(2/5), and RFC(3/4). These findings are supported by an earlier observation that coexpression in E. coli of RFC2 and 3 and of RFC3 and 4 appeared to increase their solubility (27). In theory, for a circular heteropentamer, there should be five combinations of two subunits that are adjacent to one another. Unfortunately, we observed no soluble complexes containing RFC1, which may account for the two complexes that could not be obtained in soluble form (i.e., RFC1 with either of its two neighboring subunits).

Next we asked whether the three apparently soluble RFC heterodimers can be purified intact, and if so, whether they remain stably associated with each other during analysis in a gel filtration sizing column. For each heterodimer, induced cells were grown and lysed, and the cell lysate was fractionated using an SP-Sepharose cation exchange column. After this step, methods for the three preparations diverged, as explained under “Experimental Procedures.” However, we succeeded in obtaining each of the RFC heterodimers in pure form as illustrated in the SDS-PAGE analysis of Fig. 2 (RFC(2/3), RFC(2/5), and RFC(3/4), lanes 3, 4, and 5, respectively).

The RFC heterodimers were tested for true stable complex formation by asking whether they remain together during analysis in a gel filtration sizing column (Fig. 3). The individual RFC2 and RFC4 subunits were also examined by this approach. Individual RFC2 and RFC4 eluted in fractions 54–58 and 56–58, respectively, slightly later than the 66-kDa size marker, indicating that these subunits are monomeric and consistent with the presence of only one of each of these proteins in the RFC complex (Fig. 3, A and B). Next the RFC(3/4) preparation was analyzed (Fig. 3C). The RFC3 and RFC4 subunits comigrated with one another and eluted earlier than either RFC2 or RFC4 alone, indicating that the RFC(3/4) sub-
units indeed exist as a complex. The two subunits stain with similar intensity suggesting that the complex probably contains one of each subunit. The RFC(2/3) preparation also behaved as an equimolar complex except the complex eluted slightly earlier than RFC(3/4) (Fig. 3). Likewise the RFC(2/5) preparation coeluted earlier than either RFC2 or RFC4 indicating that they form a stable complex, although the two subunits did not resolve well from one another in the polyacrylamide gel because of their very similar mass (Fig. 3).

A Model for Subunit Arrangement in RFC—The fact that RFC(2/3) and RFC(3/4) form complexes indicates that RFC3 binds to both RFC2 and RFC4. If RFC is a circular pentamer in which each subunit has only two neighbors, then the results indicate a subunit order RFC2:RFC3:RFC4 where RFC9 serves as a bridge between RFC2 and RFC4. This arrangement makes two predictions: first that RFC2 will not form a complex with RFC4, and second, that RFC2 and RFC(3/4) will form a RFC2/(3/4) heterotrimer. The test of whether RFC2 and RFC4 interact is shown in Fig. 4A. Although the two subunits appear to coelute, their elution positions in fractions 54–58 is similar to their individual elution positions (compare Fig. 3, A and B) indicating that they do not form a complex. Had they bound to one another the complex should have eluted earlier, as a larger molecular weight complex. Next, the mixture of RFC2 with RFC(3/4) was examined for ability to form an RFC2/(3/4) complex. The results, in Fig. 4B, show that the RFC2 subunit coelutes with RFC(3/4) several fractions earlier than RFC2 alone, indicating formation of the RFC2/(3/4) complex. Hence, the predictions listed above hold true and indicate that the subunit order of these three proteins is RFC2:RFC3:RFC4.

The RFC(2/5) complex, taken together with the formation of RFC(2/3) complex, suggests that RFC2 binds to both RFC5 and RFC3. Thus RFC4, which is positioned on the other side of RFC3 from RFC2, should not form a complex with RFC(2/5). The test of this prediction, in Fig. 4C, demonstrates that RFC4 does not coelute with the RFC(2/5) complex. Taken together with the formation of a RFC2/(3/4) complex described above, the subunit order of the four RFC subunits is RFC5:RFC2: RFC3:RFC4. Consistent with this assignment, when the RFC(2/5) and RFC(3/4) complexes are mixed they coelute at a much earlier position than either of the subcomplexes alone. Given the arrangement of the RFC5/2/3/4 subunits, and the presumed circular arrangement of the RFC pentamer, the RFC1 subunit probably fits between RFC5 and RFC4. We tried to test this hypothesis by co-expression of RFC1 with each of the other subunits, but in no case could we obtain a soluble complex. Nor could we obtain RFC1 alone. We also tested N- and C-terminal truncated versions of RFC1 for solubility and also constructed glutathione S-transferase and maltose-binding protein fusions of RFC1 (and various truncated RFC1 fusion subunits), but what little soluble protein was obtained behaved as an aggregate in gel filtration and did not bind the other subunit preparations. We also tried immobilizing the RFC1 fusion proteins on microtiter plates and on beads but still could not obtain reliable information pertaining to which subunit(s) it binds to. Hence, we were unable to demonstrate directly that RFC1 is adjacent to RFC5 and RFC4. However, protein-protein interaction studies of human RFC have demonstrated that RFC1 (p140) binds to both RFC4 (p40) and RFC5 (p38) subunits (28, 29) thus supporting the above position assigned to RFC1.

**ATP Site Polarity within the RFC Pentamer**—Given the subunit arrangement described above for the RFC pentamer, it may exist in either of two orientations as illustrated in the diagrams of Fig. 4E. These schemes reflect the view looking down the C-terminal plane of the pentamer and result in two very different consequences for how the complex may utilize ATP. The location of ATP sites in AAA+ multimeric machines are at subunit interfaces and require an Arg residue from the adjacent subunit. In the y complex pentamer, this Arg finger is contained within a highly conserved SRC motif in RFC box VII (10). Only four RFC subunits contain the SRC motif, RFC2, 3, 4, and 5 (6). Also, only four subunits contain consensus Walker A motifs for ATP hydrolysis, RFC1, 2, 3, and 4. In the subunit polarity shown in Model 1 of Fig. 4E, the placement of these motifs is maximized so that each ATP binding subunit is placed clockwise next to an SRC-containing subunit. This arrangement could provide four competent ATP hydrolysis sites. The other subunit polarity, in Model 2 of Fig. 4E, produces only three competent ATP sites.

To determine the polarity of the RFC pentamer we first focused on the RFC(3/4) complex and then extended the study to the RFC2(3/4) complex. We find that the RFC(3/4) complex is an ssDNA stimulated ATPase, as illustrated in Fig. 5A. In the experiments to follow, we used this activity to examine the polarity of these two subunits. Two mutant RFC(3/4) complexes were constructed, containing a mutated SRC motif in either RFC3 or RFC4 (R replaced by A and referred to here as SAC). The mutant complexes were then analyzed for ssDNA-dependent ATPase activity. If the subunit polarity is as shown in Model 1 of Fig. 4E, the RFC(3SAC/4) complex should be inactive, whereas the RFC(3/4SAC) mutant should remain active. If the subunits are arranged in the opposite polarity, as in Model 2, then the opposite result should be obtained. The results, in Fig. 5, B and C, are consistent with the polarity of Model 1 in which RFC3 contributes a catalytic Arg to the ATP site of RFC4.

In Fig. 6 we examine RFC2 for ATPase activity and its affect on the RFC(3/4) complex. We find that RFC2 alone lacks ATPase activity ± DNA (data not shown) but provides about 4-fold stimulation to the RFC(3/4) ATPase (see Fig. 6A). The subunit arrangement and polarity of RFC, depicted in Model 1 of Fig. 4E, makes the prediction that RFC2 contributes a catalytic Arg residue to the ATP binding site in RFC3, which may explain how RFC2 stimulates the RFC(3/4) ATPase activity. Namely, RFC(3/4) has only one complete ATP site (site 3), and RFC2 generates a second complete ATP site (site 2). In this case, the RFC(3/4SAC) mutant should still be stimulated by RFC2 to the same extent as wild-type RFC complex. If RFC2 were to bind RFC4, instead of RFC3, addition of RFC2 would not stimulate the activity of the resulting RFC(2/3/4SAC) com-
plex mutant. The results (in Fig. 6B) show that RFC2 indeed stimulates the ATPase activity of the RFC(3/4) mutant, supporting the arrangement of subunits in Model 1. Moreover, if RFC2 provides an arginine finger to the ATP site in RFC3, the inactive RFC(3SAC/4) mutant complex should become ATPase-competent upon addition of RFC2. On the contrary, if the polarity of subunits is as depicted in Model 2, the RFC(3SAC/4) mutant complex should gain no ATPase activity upon addition of RFC2. The results in Fig. 6C uphold the predictions of subunit polarity in Model 1. The level of ATPase in panel C is less than in panels A and B, consistent with only one competent ATP site in the resulting RFC(2/3 SAC/4) complex.

RFC(3/4) and RFC(2/3) Bind PCNA—RFC, like prokaryotic γ complex, binds the clamp in an ATP-dependent fashion (30). ATP binding is sufficient to promote the RFC-PCNA complex, hydrolysis is not required. We examined the individual RFC subunits and heterodimers for ability to form a stable complex with PCNA and tested whether the interaction requires ATP. The RFC-PCNA interaction study of Fig. 7 uses gel filtration analysis, which is a non-equilibrium technique, and only the tightest protein complexes will be observed. PCNA was mixed with the various RFC subunit preparations in the presence of ATP and then the mixture was assayed for complex formation. The result shows an interaction between RFC(3/4) and PCNA, and RFC(2/3) and PCNA, but no interaction between PCNA and RFC(2/5), RFC2, or RFC4. It is interesting to note that neither RFC4 nor RFC2 stay attached to PCNA, yet PCNA stably binds to both the RFC(3/4) and RFC(2/3) complexes. Thus RFC3 may form the main contribution between PCNA and these heterodimeric complexes. This prediction that RFC3 can bind PCNA is supported by studies in the human system indicating that the p36 subunit (yeast RFC3 homolog) of human RFC binds PCNA (31). It is still possible that other RFC subunits bind PCNA but do not form a complex with sufficient

**Fig. 4.** Interaction between RFC subunits and subcomplexes. Panels A–D are protein-protein interaction studies of RFC subunit mixtures analyzed by gel filtration. Approximately 1.75 nmol of each subunit or heterodimer were incubated together and then analyzed on a Superdex-200 gel filtration column. Column fraction numbers are indicated above the top gel, and RFC subunits are identified on the left of each gel. E, two possible models of RFC subunit arrangement in the RFC pentamer, clockwise or counter clockwise, and the corresponding implications for placement of the Arg finger and P-loop, which form the ATPase site at the interface between adjacent subunits. Both diagrams depict the top view looking down onto the C-terminal face of the pentamer (e.g. see Fig. 1A).
stability to be isolated by this nonequilibrium technique.

Study of these same protein mixtures in the absence of ATP revealed no new complexes with PCNA, and in fact the RFC3(3/4)-PCNA complex was no longer observed (Fig. 7A). Possible explanations underlying the ATP dependence of the RFC3(3/4)-PCNA complex and ATP-independent RFC(2/3)-PCNA complex are presented under “Discussion.” We lack the RFC1 subunit in isolation or as a heterodimer with another RFC subunit, but we presume it also forms a major contact to PCNA based on studies of human RFC (25, 32). The next few experiments take a different approach to assess the importance of RFC1 in RFC-PCNA complex formation.

**RFC1 Contributes to PCNA Interaction**—We have no subassembly of RFC that contains RFC1. Therefore, to address whether it contributes to PCNA binding we mutated two hydrophobic residues of RFC1 (Tyr-404 and Phe-405) to alanine and expressed it, along with the other subunits, to produce RFC1mut. These two residues are located between the P-loop of RFC1 and the Arg residue of the SRC motif in either RFC3 or RFC4. The experimentally determined polarity of the RFC2/3/4 subunits and knowledge of the correct structure of RFC complex (9, 33) or three competent ATP sites (Model 1) or three competent ATP sites (Model 2). The correct arrangement of subunits around the ring was determined by exploiting a finding herein showing that the RFC3(3/4) complex contains DNA-dependent ATPase activity, and this ATPase is stimulated further by RFC2. The catalytic Arg residue of the SRC motif in either RFC3 or RFC4 was mutated to alanine, and the effect of these mutations on the ATPase activity of the RFC3(3/4) complex was determined. We observe here that the RFC3(3/4) heterodimer displays ATPase activity was assayed in the presence (closed circles) and absence (open circles) of RFC2. Open squares represent ATPase reactions of RFC3(3/4) in the absence of both RFC2 and synthetic primed template. The scheme at the top of the figure illustrates the conclusion regarding the orientation of the Arg finger and P-loop of RFC2, RFC3, and RFC4.

**Fig. 6. RFC2 contributes an Arg finger to the ATP site in RFC3.** The DNA-stimulated ATPase activity was as follows: panel A, wild-type RFC3(3/4); panel B, RFC3(3/4mut); and panel C, RFC3(3/4mut). ATPase activity was assayed in the presence (closed circles) and absence (open circles) of RFC2. Open squares represent ATPase reactions of RFC3(3/4) in the absence of both RFC2 and synthetic primed template. The scheme at the top of the figure illustrates the conclusion regarding the orientation of the Arg finger and P-loop of RFC2, RFC3, and RFC4.

**PCNAOG reveal that RFC1mut binds PCNAOG (K_d ≈ 3.1 μM) about 10-fold weaker than wild-type RFC (K_d ≈ 0.34 μM), demonstrating that the RFC1 subunit is involved in binding to PCNA. A second experiment was performed to test the above conclusion based on the PCNA-stimulated activity of Pol δ. In Fig. 8, bottom panel, RFC1mut is examined for activity with PCNA on a singly primed M13mp18 ssDNA template using a recombinant derivative of Pol δ. The results show that the RFC1mut is significantly less active than wild-type RFC, consistent with RFC1 forming an important contact site between RFC and PCNA.

**DISCUSSION**

**RFC Subunit Arrangement and ATP Site Architecture**—This study examines the subunit arrangement of the heterotetrameric RFC clamp loader using recombinant heterodimeric subassemblies of RFC and individual RFC subunits to obtain information about subunit-subunit interactions within RFC. These protein reagents also made possible the reconstitution of three- and four-subunit RFC subassemblies. The results are all consistent with the presumed circular subunit arrangement, as no one subunit interacted with more than two other subunits. The five RFC subunits have been proposed to be arranged in a circle by analogy to the crystal structure of the circular pentamer E. coli γββ′ clamp loader (9, 10) and is consistent with electron microscopy and atomic force microscopy studies of RFC (24). The final arrangement of RFC, illustrated in Fig. 9, confirms earlier proposals based on homologies between RFC and γ complex (9, 33) and on previous protein-protein interaction studies of human RFC (28, 29).

The ATP sites of AAA- multimers are located at subunit interfaces, and the adjacent subunit contributes a catalytic Arg residue to facilitate ATP hydrolysis. With this in mind, there are two ways to order the five RFC subunits in a circle, clockwise and counter clockwise (i.e., Models 1 and 2 in Fig. 4E). The two arrangements result in RFC pentamers having either four competent ATP sites (Model 1) or three competent ATP sites (Model 2). The correct arrangement of subunits around the ring was determined by exploiting a finding herein showing that the RFC3(3/4) complex contains DNA-dependent ATPase activity, and this ATPase is stimulated further by RFC2. The catalytic Arg residue of the SRC motif in either RFC3 or RFC4 was mutated to alanine, and the effect of these mutations on the ATPase activity of the RFC3(3/4) complex was determined. The clockwise or counter clockwise arrangements of RFC predict opposite results, and only one of the two results was experimentally observed (i.e., RFC2 contributes an Arg finger to the ATP site of RFC3, and RFC3 correspondingly contributes an Arg finger to the ATP site of RFC4). The experimentally determined polarity of the RFC2/3/4 subunits and knowledge of the positions of RFC subunits clearly revealed Model 1 to be the correct structure of RFC complex (i.e., Model 1 of Fig. 4, and as illustrated in Fig. 9).

**Interaction between RFC and PCNA**—RFC1 is involved in a main connection to PCNA based on previous studies in the human system (25). The current study confirms this interaction in the yeast system and also demonstrates that the Tyr-404 and Phe-405 residues of RFC1 are involved in PCNA binding. The position of these two residues in RFC1 is analogous to that of the two hydrophobic residues in E. coli δ that bind the β clamp and thus were hypothesized to be important to RFC1-PCNA interaction (18). This report also documents that RFC3(3/4) and RFC(2/3) also form a main attachment site between RFC and PCNA. Because neither RFC2 nor RFC4 bind stably to PCNA it is possible that RFC3 alone forms the main contact to PCNA within these complexes (illustrated in Fig. 9).

We observe here that the RFC(3/4) heterodimer displays ATP-
dependent behavior in binding to PCNA, whereas the RFC(2/3) heterodimer binds PCNA in the absence of ATP. We propose that the PCNA binding site(s) within the RFC(3/4) complex is occluded in this heterodimer, and ATP results in a conformation change that makes these sites more accessible to PCNA. The ATP-independent interaction of RFC(2/3) with PCNA indicates that the PCNA binding site(s) of this heterodimer are more exposed to PCNA and thus do not require ATP to power a conformation change for PCNA interaction. However, the intact 5-subunit RFC requires ATP to observe PCNA binding (29), and thus the PCNA binding site(s) of the RFC(2/3) heterodimer must be obscured in the intact RFC, presumably by one or more of the other three subunits, and that ATP is required to expose them for PCNA interaction. The studies of this report do not rule out contact between PCNA and RFC2 or 5, as only strongly interacting complexes are observed by the non-equilibrium gel filtration technique used here. Indeed it has been suggested that all five RFC subunits contact PCNA in the human system (32).

Comparison of RFC with γ Complex—The E. coli γ complex, like RFC, contains five AAA+ subunits that are required for clamp loading activity (10). However, unlike RFC, γ complex has only one strong subunit contact to the β clamp, mediated by the δ subunit (22). Although the γ subunits contact β, they do so only with very weak affinity, and there is no detectable interaction thus far between δ' and β (15). Consistent with δ forming the main contact to β, the γδ'γδ subassembly lacking the δ subunit does not form a stable complex with β (22). The fact that more than one RFC subunit binds PCNA tightly may underlie a divergence in mechanism between prokaryotic and eukaryotic clamp loaders. This difference may simply be a consequence of clamp loaders that function with either a dimer (β) or trimer (PCNA) ring. For example, PCNA dissociates into monomers at low concentrations (34), and RFC may need multiple contact points with the PCNA trimer to prevent it from falling apart when it opens the ring at one interface.

The γ trimer has been referred to as the “motor” of γ complex, as these subunits are the only ones that bind ATP (9, 10, 35). The RFC(2/3/4) subassembly has been proposed to act like the γΔ motor subassembly as they are the only RFC subunits that contain both the ATP site and the SRC arginine finger motifs, like γ (9, 35). Results of this study are consistent with this assignment as they demonstrate that RFC2, 3, and 4 bind one another and contain an inherent DNA-stimulated ATPase activity. Likewise, the analogous human RFC p40/p37/p36 complex contains DNA-dependent ATPase activity (28, 29). Moreover, the current study also confirms the hypothesis that these RFC subunits require the SRC arginine finger for ATP hydro-
lytic activity, as has been shown previously for the \( \gamma \) subunits of \( E. coli \) (21).

The \( E. coli \) \( \delta' \) stator contains an SRC motif that donates a catalytic arginine to the ATP site of \( \gamma_1 \) (21) but lacks the ATP binding site consensus sequence. Similarly, RFC5 contains an SRC motif and has a modified ATP binding site sequence suggesting that even if it binds ATP it may not easily hydrolyze it (6). According to the subunit arrangement determined here, RFC5 contributes a catalytic arginine to the ATP site of RFC2. The \( \delta' \) stator is thought to be a rigid subunit, perhaps acting as a backboard to direct the ATP-induced changes in \( \gamma \) subunits necessary for \( \beta \) to bind \( \gamma \) complex (9, 10). It is not clear at this time whether RFC5 is rigid, and thus assigning a stator role for this subunit is still preliminary.

It has been proposed that RFC1 acts like the \( \delta \) wrench to open the ring, although no clear evidence for this exists at the present time (9, 18). This speculation derives from the fact that RFC1, like \( \delta \), is the most divergent in sequence from the other subunits, lacks an SRC motif, and binds directly to the PCNA ring. Furthermore, as \( \delta \) is positioned between the stator and a motor subunit in \( E. coli \) complex, so is RFC1 positioned in RFC, based upon the results in this report. If RFC1 is the wrench, one may predict that there will be a gap between the N-terminal domains of RFC1 (wrench) and RFC5 subunits, by analogy to the gap between the \( \delta \) and \( \delta' \) subunits of \( E. coli \) \( \gamma_2 \) (see Fig. 9). Unlike \( \delta \), RFC1 contains an ATP site, but mutational analysis demonstrates that this site is not required for clamp loading function and thus may be involved in some other activity (36).

It has been noted previously (18) that RFC1 contains two adjacent hydrophobic residues that are located in the same position as the two hydrophobic residues of \( \delta \) involved in binding to \( \beta \). Studies in this report show that RFC mutated in these residues of RFC1 binds less tightly to the clamp and that the RFC\textsuperscript{1mut} is impaired in replication activity, similar to \( E. coli \) \( \gamma_2 \) complex containing a \( \delta \) subunit that is mutated in the analogous hydrophobic residues (23). However, it also remains possible that the mutations in RFC1 examined here causes some alteration of the RFC structure that causes indirect effects that change PCNA interactions.

The apparent similarity between RFC1 and \( \delta \) may imply that RFC1 opens the clamp like \( \delta \), but until this is demonstrated, other possibilities also exist. For example, it has been pointed out that RFC3 and RFC5 also contain two adjacent hydrophobic residues in a similar position as \( \delta \) and RFC1 (33). Therefore it is also possible that either of these other subunits acts like \( \delta \).
More importantly, RFC may simply function quite differently than γ complex, although the fact that both clamp loaders are composed of five AAA+ proteins suggests that many aspects of their mechanism will be similar. For example, both RFC and γ complex bind ATP to associate with their respective clamps. However, the different oligomeric structures of their rings may require different mechanistic approaches to the problem of binding, opening and closing the rings around DNA. A detailed comparison between the prokaryotic and eukaryotic clamp loaders awaits crystal structure studies of RFC.

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REFERENCES
1. Kelman, Z., and O’Donnell, M. (1995) Annu. Rev. Biochem. 64, 171–200
2. Stukenberg, P. T., Studwell-Vaughan, P. S., and O’Donnell, M. (1991) J. Biol. Chem. 266, 11328–11334
3. Kong, X. P., Onrust, R., and Kuriyan, J. (1992) Cell 69, 425–437
4. Gulbis, J. M., Kelman, Z., Hurwitz, J., O’Donnell, M., and Kuriyan, J. (1996) Cell 87, 297–306
5. Krishna, T. S., Kong, X. P., Gary, S., Burgers, P. M., and Kuriyan, J. (1994) Cell 79, 1233–1243
6. Cullmann, G., Fien, K., Kohayashi, R., and Stillman, B. (1995) Mol. Cell. Biol. 15, 4661–4671
7. O’Donnell, M., Onrust, R., Dean, F. B., Chen, M., and Hurwitz, J. (1993) Nucleic Acids Res. 21, 1–3
8. Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. (1999) Genome Res. 9, 27–43
9. O’Donnell, M., Jeruzalmi, D., and Kuriyan, J. (2001) Curr. Biol. 11, R835–R946
10. Jeruzalmi, D., O’Donnell, M., and Kuriyan, J. (2001) Cell 106, 429–441
11. Onrust, R., and O’Donnell, M. (1993) J. Biol. Chem. 268, 11766–11772
12. Glover, B. P., and McHenry, C. S. (1998) J. Biol. Chem. 273, 23476–23484
13. Kelman, Z., Yuzhakov, A., Andjelkovic, J., and O’Donnell, M. (1998) EMBO J. 17, 2436–2449
14. Yuzhakov, A., Kelman, Z., and O’Donnell, M. (1999) Cell 96, 153–163
15. Leu, F. P., and O’Donnell, M. (2001) J. Biol. Chem. 276, 47185–47194
16. Hingorani, M. M., and O’Donnell, M. (1998) J. Biol. Chem. 273, 24550–24563
17. Stewart, J., Hingorani, M. M., Kelman, Z., and O’Donnell, M. (2001) J. Biol. Chem. 276, 19182–19189
18. Jeruzalmi, D., Yurieva, O., Zhao, Y., Young, M., Stewart, J., Hingorani, M., O’Donnell, M., and Kuriyan, J. (2001) Cell 106, 417–426
19. Turner, J., Hingorani, M., Kelman, Z., and O’Donnell, M. (1999) EMBO J. 18, 771–783
20. Guenther, B., Onrust, R., Sali, A., O’Donnell, M., and Kuriyan, J. (1997) Cell 91, 355–345
21. Johnson, A., and O’Donnell, M. (2003) J. Biol. Chem. 278, 14406–14413
22. Naktinis, V., Onrust, R., Fang, L., and O’Donnell, M. (1995) J. Biol. Chem. 270, 13358–13365
23. Indiani, C., and O’Donnell, M. (2003) J. Biol. Chem. In press
24. Shiomi, Y., Usukura, J., Masamura, Y., Takeyasu, K., Nakayama, Y., Obuse, C., Yoshikawa, H., and Tsurimoto, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14127–14132
25. Foteder, R., Mossi, R., Fitzgerald, P., Rouselle, T., Maga, G., Brickner, H., Messier, H., Kasibhatla, S., Hubscher, U., and Foteder, A. (1996) EMBO J. 15, 4425–4433
26. Finkenstein, J., Antony, E., Hingorani, M. M., and O’Donnell, M. (2003) Anal. Biochem. 319, 78–87
27. Gomes, X. V., Gary, S. L., and Burgers, P. M. (2000) J. Biol. Chem. 275, 14541–14549
28. Uhmann, F., Cai, J., Flores-Rozas, H., Dean, F. B., Finkenstein, J., O’Donnell, M., and Hurwitz, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6521–6526
29. Elssion, V., and Stillman, B. (1998) J. Biol. Chem. 273, 5979–5987
30. Gerik, K. J., Gary, S. L., and Burgers, P. M. (1997) J. Biol. Chem. 272, 1256–1262
31. Mossi, R., Jonsson, Z. O., Allen, B. L., Hardin, S. H., and Hubscher, U. (1997) J. Biol. Chem. 272, 1769–1776
32. Uhmann, F., Cai, J., Gibbs, E., O’Donnell, M., and Hurwitz, J. (1997) J. Biol. Chem. 272, 10058–10064
33. Venclovas, C., Colvin, M. E., and Thelen, M. P. (2002) Protein Sci. 11, 2403–2416
34. Yao, N., Turner, J., Kelman, Z., Stukenberg, P. T., Dean, F., Shechter, D., Pan, Z. Q., Hurwitz, J., and O’Donnell, M. (1996) Genes Cells 1, 101–113
35. Davey, M. J., Jeruzalmi, D., Kuriyan, J., and O’Donnell, M. (2002) Nat. Rev. Mol. Cell Biol. 3, 826–835
36. Schmidt, S. L., Gomes, X. V., and Burgers, P. M. (2001) J. Biol. Chem. 276, 34784–34791