Overproduction and Affinity Purification of *Saccharomyces cerevisiae* Replication Factor C*

(Received for publication, September 10, 1996, and in revised form, October 30, 1996)

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Yeast replication factor C (RF-C) is a heteropentamer encoded by the *RFC1–5* genes. RF-C activity in yeast extracts was overproduced about 80-fold after induction of a strain containing all five genes on a single plasmid, with expression of each gene placed under control of the galactose-inducible *GAL1–10* activator sequence; ATP∂ and DNA polymerase ε. The complex of Pol δ, RF-C, and PCNA is called Pol δ holoenzyme. Yeast RF-C consists of a large subunit of 94 kDa and four smaller subunits of 36–40 kDa (3, 4). All five known genes have been cloned using a combination of complementation cloning, peptide sequence analysis, genome data base searching, and homology-based polymerase chain reaction. All are essential for yeast cell growth (3–8). The four small *RFC* genes share significant sequence identity (20–34%). Human RF-C, also called Activator 1, has a similar subunit structure, and each of the human genes shows extended sequence similarity (38–56%) with a specific yeast RF-C gene (3, 4, 9, 10).

Biochemical studies of RF-C have established that the complex has a preferential binding affinity for template-primer junctions and has a single-stranded DNA-dependent ATPase activity which is further activated by the presence of primer termini and PCNA (9, 11–14). Unfortunately, more detailed biochemical studies of this complex have been hampered by its scarcity and the difficulty in isolating homogeneous and fully active preparations. Previously, from 1000 g of yeast we routinely obtained about 50–200 μg of RF-C after a seven-step purification protocol, with a purity of about 50–90%, and similar yields were obtained by others (13, 14). Biochemical studies have also been less informative and reproducible, because the different subunits were often present at above or below stoichiometric levels. As a result, its activity varied substantially between preparations. These various problems have precluded a thorough biochemical analysis until now.

In this paper we describe two important improvements to obtaining relatively large quantities of pure RF-C, which should make a thorough study of this loading factor feasible. The first improvement is the simultaneous overexpression of all five *RFC* genes in yeast, which provides an almost 100-fold higher level of RF-C. Second, a novel purification step has been developed which depends on the strong binding of RF-C to PCNA-agarose beads in the presence of Mg-ATP and weak binding in the absence of the nucleotide. This step allows the rapid purification of RF-C essentially to homogeneity. Because PCNA can be overproduced in *Escherichia coli* in large quantities, this affinity purification procedure should also be useful for the purification of RF-C from other organisms for which the PCNA gene is available.

**MATERIALS AND METHODS**

**Strains and Plasmids**—The yeast strain used in this work is the protease-deficient galactose-inducible strain BJ2168 (MATa, ura3–52, trpl1–289, leu2–3, 112, pyr1–1122, prc1–407, pep4–3). The overproduction plasmids used in this study are based upon the pRS420 series plasmids into which the *GAL1–10* upstream activating sequence (GAL1–10 UAS), including the transcriptional start sites for the GAL1 and GAL10 genes, as a 678-nt *BamHI-EcoRI* fragment, was inserted into the corresponding plasmid polylinker sites, resulting into vectors pRS424-GAL (TRP1), pRS425-GAL (LEU2), and pRS426-GAL (URA3) (15, 16). All vectors have in addition the yeast 2 μm origin for replication.
high copy maintenance in yeast and the Bluescript SKII® backbone for propagation in E. coli (16). The transcriptional start site of the GAL1 gene is 60 nucleotides upstream of the BamHI cloning site and the transcriptional start site of the GAL10 gene is 10 nucleotides upstream of the EcoRI cloning site. Both promoters are of similar strength. Using conventional subcloning procedures, all five downstream of the relevant transcriptional start sites. Upstream cloning sites were used for RfC for RfC1, BamHI for RfC2, HindIII for RfC3, MluI for RfC4, and MseI for RfC5. These restriction sites are 90, 60, 50, 135, and 10 nucleotides, respectively, from transcriptional start sites. Recombinant RfC plasmids are listed in Fig. 1. pMTL4 is a centromere plasmid with LEU2 as selectable marker and with the GALA gene placed under control of the GAL1 UAS (a gift of Mark Johnston, Washington University).

Media and Buffers—SCG medium contains per liter: 1.7 g yeast nitrogen base without amino acids and ammonium sulfate, 5 g of ammonium sulfate, 30 ml of glycerol, 20 ml of lactic acid, 1.0 g of glucose, 20 g of agar for solid media, 20 mg of each of adenosine, uracil, histidine, tryptophan, proline, arginine, and methionine, 30 mg each of isoleucine, tyrosine, and lysine, 50 mg of phenylalanine, and 100 mg each of leucine, glutamic acid, aspartic acid, valine, threonine, and serine. Uracil, tryptophan, and/or leucine were omitted when necessary to ensure selective maintenance of plasmids. Prior to autoclaving, the pH of the media was adjusted to 5–6 with concentrated sodium hydroxide. YPG medium contained per liter: 30 g of Bacto-agar, 20 g of yeast extract, 30 ml of glycerol, 50 ml of lactic acid, 2 g of glucose, and 20 ml of mannitol. Prior to autoclaving, the pH of the medium was adjusted to 5–6 with concentrated sodium hydroxide.

Buffer A contains: 50 mM Tris-HCl, pH 7.8, 5% (v/v) glycerol, 2 mM EDTA, 3 M sodium acetate, 5 mM pepstatin A, 5 μM leupeptin, 0.5 mM p-methyl-phenylsulfonyl fluoride, 10 mM NaHSO3. Buffer B contains: 50 mM Tris-HCl, pH 7.7, 0.5 mM EDTA, 10% glycerol, 8 mM magnesium acetate, 1 mM ATP, 3 mM DTT, 5 mM pepstatin A, 5 μM leupeptin, 5 mM NaHSO3. Buffer C contains: 30 mM Tris-HCl, pH 7.7, 2 mM EDTA, 10% glycerol, 3 mM DTT, 5 mM pepstatin A, 5 μM leupeptin, 5 mM NaHSO3. Buffer D contains: 30 mM HEPES-NaOH, pH 7.4, 0.5 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 3 mM DTT, 1 μM pepstatin A, 1 μM leupeptin. Salt concentrations (as NaCl) are indicated by a suffix, e.g. Buffer A500 = Buffer A + 500 mM NaCl.

PCNA-Agarose Beads—All steps were carried out at 0–4°C. PCNA was purified to about 95% purity from E. coli cells containing the overexpression plasmid pBL228 as described (17). 800 mg of PCNA (10 mg/ml) in a 200 ml of 20 mM Tris-HCl, pH 7.8, 500 mM NaCl buffer (500 mM NaCl, 10 mM MgCl2, 0.2 mg/ml BSA, 1 mM DTT) were mixed with 175 ml of glass beads (0.4–0.5 mm), and the mixture was immersed in a container filled with ice water. The cells were blended with 10,000 units of RF-C in 200 ml binding buffer (50 mM Tris, 7.8, 10% glycerol, 5 mM DTT, 1 mg/ml of BSA) and EDTA, magnesium acetate, ATP, and NaCl as indicated, was gently agitated with 10 μl of PCNA-agarose beads in a 0.5 ml thin-walled microcentrifuge tube (polymerase chain reaction tube). When extracts or Affi-Gel blue fractions were incubated with the beads, protease inhibitors (0.5 mM p-methyl-phenylsulfonyl fluoride, 10 mM NaHSO3, 5 mM MgCl2, and 10 mM NaHCO3) were also added. After 1 h at 0–4°C, the beads of the tube was pierced with a 25-gauge needle and the solution spun through at 500 rpm for a few seconds, taking care not to let the beads become dry. 50 μl of the same buffer was layered onto the beads, and after an equilibrium time of 5 min, spun through. This washing was repeated one more time. Bound RF-C was eluted with 2 × 50 μl of binding buffer containing 2 mM EDTA and 1 mM NaCl. RF-C activity was determined in the 1 × NaCl eluate and the percent of activity recovered in comparison with starting activity to represent percent of bound RF-C. Control assays showed that when RF-C was not bound to the beads by this criterion, activity was recovered in the unbound fraction. In general, total recoverable activity (bound + unbound) was 80–90%.

RF-C Activity Assay—The standard 30-μl reaction contained 40 mM Tris-HCl, pH 7.8, 5, 8 mM MgCl2, 0.2 mg/ml BSA, 1 mM DTT, 100 μM each of dATP, dCTP, and dGTP, and 25 μM of [α-32P]dATP (100 cpn/μmol dNTP). 0.5 mM ATP, 100 ng of singly primed single-stranded mp18 DNA (0.04 pmol of circles), 850 ng of E. coli single-stranded DNA-binding protein, 50 mM NaCl, 100 ng of PCNA, 10 ng of Pol δ, and RF-C. Incubations were at 37°C for 4 min. The reactions were stopped by the addition of 5 μl of 500 mM sodium acetate, 1 mM EDTA, and 50 μg/ml of calf thymus DNA as carrier, and acid-insoluble radioactivity was determined. One unit of RF-C stimulates the incorporation of 1 pmol of nucleotide into acid-precipitable radioactivity by Pol δ under the standard assay conditions, i.e. using primed single-stranded mp18 DNA. During the time period of the assay, Pol δ, PCNA, and RF-C form a holoenzyme complex on a primed circle, and the holoenzyme completely passes the viral primer. However, turning over the template circle is minimal. For this reason, the unit definition depends on the length of replicable template available (about 7210 nucleotides for the primed mp18 template).

Overproduction and Affinity Purification of RF-C—Strain BJ2168 containing plasmids pBL420 and pMTL4 was grown and induced with galactose as described above. The preparation was carried out with a total of 120 g of frozen cells. All steps were carried out at 0–4°C. The cells were thawed and mixed with an equal volume of 2 × A260 buffer. The suspension was loaded into the large chamber of a bead-beater (Biospec products) containing 175 ml of glass beads (0.4–0.5 mm), and the chamber was filled to the top with additional buffer A260 and immersed in a container filled with ice water. The cells were blended for 45 s on, 90 s off, for a total blending time of 6 min. The lysate was poured off, and the beads were washed with 2 × 50 ml of buffer A260. The combined lysate was spun at 13,000 rpm for 30 min and the supernatant diluted with an equal volume of buffer A0. This fraction I (520 ml) (Table I).

Fraction I was gently agitated for 45 min with 50 ml of Affi-Gel blue beads (Bio-Rad) equilibrated in buffer A0. The beads were spun down at 1000 rpm, and the supernatant poured off. The beads were loaded into a column and washed with 200 ml of buffer A260. Protein was eluted with buffer A0 (with EDTA lowered to 0.5 mM). 40-ml fractions were collected and RF-C activity determined. The bulk of RF-C eluted in fractions 2 through 5 (Fraction II, 164 ml).

ATP and magnesium acetate were added to Fraction II to and 1 mM, respectively, and the enzyme fraction was dialyzed against 500 ml of 0.1 M triethanolamine hydrochloride, pH 7.5. The preparation was filtered through a capillary grade 6 nitrocellulose filter (Millipore).
buffer B_<sub>300</sub> until the conductivity had reached that of buffer B_<sub>200</sub>. The dialyzed fraction was loaded, at a rate of 1 ml/min, onto a 15-ml PCNA-agarose column, equilibrated in buffer B_<sub>400</sub>. The column was washed with 30 ml of buffer B_<sub>200</sub> followed by 30 ml of buffer B_<sub>300</sub>. The flow direction was then reversed and the column eluted with buffer C_<sub>200</sub>. Fractions of 8 ml were collected and RF-C analyzed enzymatically and by SDS-PAGE. Fractions 2 through 4 contained RF-C (Fraction III, 24 ml).

Fraction III was dialyzed against 100 ml of buffer D_<sub>0</sub> until the conductivity reached that of buffer D_<sub>200</sub>. The flow direction was then reversed and the column eluted with buffer C_<sub>100</sub>. Fractions of 8 ml were collected and RF-C eluted at about 0.35 M NaCl.

### RESULTS

**Inducible Overexpression of RFC Genes**—An inducible system for the overexpression of RFC genes was chosen to allow normal cell growth prior to induction, in case overproduction of any or all of the subunits would be deleterious to cell growth. The strain used in this study is the protease-deficient strain BJ2168. In this strain, the expression of genes placed under control of the GAL1–10 UAS is appropriately induced by addition of galactose to the medium, but the strain grows very poorly on galactose as the sole carbon source. Adequate cell growth was obtained on media containing a carbon source 3% glycerol, 2% lactate, and a nonrepressing concentration of glucose (0.1%). Galactose was added to this medium to induce the appropriate one-subunit or the analogous vector. Extracts were made from induced cells carrying the five-subunit plasmid pBL420 (Fig. 3). Because there might be insufficient Gal4p in the cell to saturate all Gal4p binding sites on the multicopy plasmid, we also introduced a Gal4p overproduction plasmid in the strain. The strain with both plasmids overproduced RF-C activity about 80-fold (Fig. 3). These expression studies show that the five known RFC genes are sufficient for forming an active RF-C complex.

**Subunit Requirement of RFC**—All five RFC genes are essential for yeast growth (3–8). From these observations it does not necessarily follow that all five subunits are necessary for forming a functional RF-C complex as defined by our assay, i.e. loading of PCNA on singly primed mp18 DNA to allow processive DNA synthesis by Pol δ. To test whether one of the subunits could be omitted, we systematically designed overexpression plasmids lacking one of the genes at a time (Fig. 1). Strain BJ2168 was then transformed with a four-subunit plasmid together with a second plasmid containing the gene for the fifth subunit or the analogous vector. Extracts were made from induced cells and passed over an Affi-Gel blue column as described under “Materials and Methods” and outlined in Fig. 1.

Strains carrying plasmids containing one single RFC gene under galactose control greatly overproduced the appropriate RFC-C subunit after galactose induction as shown by Western analysis (results not shown). A 4-h incubation in galactose medium was optimal. The consequence of overproduction of a single RF-C subunit on cell growth was monitored by growing cells under selective conditions on glycerol-lactate medium, followed by plating on selection plates containing either raffinose or galactose. Both are fermentable carbon sources, but raffinose does not induce genes placed behind the GAL1–10 UAS. The plating efficiency on galactose plates versus raffinose plates was reduced about 10-fold when the strain contained the RFC1 overproducing plasmid, pBL409 (Fig. 2A). In addition, the colonies that did appear on the galactose plate were very small, whereas the colonies on the raffinose plate were normal in size (Fig. 2B), indicating that overproduction of Rfc1p is detrimental. The reduction in plating efficiency and colony size is largely due to increased plasmid loss and slow growth of overproducing cells, but not to a specific cell cycle arrest in these cells (results not shown).

Five plasmids were constructed which contained four RFC genes each, all genes being under galactose control (Fig. 1). Of those, only the strain with the plasmid lacking RFC3 (pBL425) had a low plating efficiency and produced small colonies when grown on galactose (Fig. 2A). When the appropriate one-subunit and four-subunit plasmids were combined in yeast, poor growth was in general observed, except for the combinations pBL412 + pBL424 (RFC1, RFC3, RFC4, RFC5 + RFC2) and pBL419 + pBL417 (RFC1, RFC2, RFC3, RFC4 + RFC5), which showed normal growth (Fig. 2A). Coincidentally, these two combinations of plasmids gave the lowest overproduction of RF-C activity, suggesting that overproduction of the complex is deleterious to cell growth (Fig. 1 and below). As with overproduction of Rfc1p, the reduction in plating efficiency and colony size is largely due to plasmid loss and cell death and not to specific cell cycle arrest in the cells overproducing RF-C. As expected from these previous results, when a plasmid containing all five RFC genes (pBL420) was transformed into yeast, the strain also grew poorly on galactose media (Fig. 2).

### Yeast RF-C Overproduction and Affinity Purification

**TABLE I**

| Fraction | Step          | Protein | Activity (units/mg) | Yield (%) |
|----------|---------------|---------|---------------------|-----------|
| I        | Lysate        | 2900    | 96                  | 0.033     |
| II       | Affi-Gel blue | 265     | 78                  | 0.29      |
| III      | Affi-Gel blue | 3.5     | 70                  | 20        |
| IV       | Mono S        | 3.0     | 57                  | 19        |

The purification started with 120 g of yeast paste (see “Materials and Methods” for details). Activity in the cleared lysate was set at 100%. Protein concentrations were determined as described by Bradford (24).
to varying levels (Fig. 1). The variation in RF-C levels in the strains with two RFC plasmids may be due to plasmid copy number effects and has been observed previously for the combination RFC1, RFC2, RFC3, RFC4 + RFC5 (4). This series of experiments allows the conclusions that (i) with the possible exception of Rfc1p, all subunits are present in yeast at comparable levels, and (ii) all subunits are necessary for a fully functional RF-C. Again, these experiments do not exclude the possibility that a subcomplex of RF-C may be formed with a very weak activity, which is at least 1 order of magnitude lower than that of normal RF-C.

ATP Promotes Complex Formation between PCNA and RF-C—Previously, we have shown by kinetic studies that the most favorable pathway of loading PCNA at a primer terminus requires the DNA-independent formation of a complex between PCNA and RF-C (18). In a separate study we are investigating the parameters that govern the interaction between PCNA and RF-C using several techniques, including PCNA-agarose beads. Using PCNA-agarose beads, a stable complex between PCNA and RF-C was detected, but only in the presence of Mg-ATP. The data in Fig. 4 show that binding of RF-C to the PCNA beads is unstable at 200 mM NaCl when carried out in a buffer containing either magnesium (without ATP) or ATP (with EDTA). In contrast, when both magnesium and ATP are present in the binding buffer, stable binding is observed at 400 mM NaCl, and substantial binding is still observed at 700 mM NaCl (Fig. 4). ATP supported complex formation with half-maximal binding observed at about 1 mM. This defines an apparent KD value for ATP of about 1 mM. The formation of such a stable complex requires ATP binding, but not its hydrolysis. Both ATPγS and AMP-PNP as competitive inhibitors of the ATPase activity of RF-C with Ki values of 1.8 and 130 μM, respectively (13). GTP at 100 μM and CTP near 1 mM concentration also supported strong complex formation, whereas ADP was ineffective (Fig. 5). However, none of these nucleotides can stimulate the loading of PCNA by RF-C onto a primed template as determined in a Pol δ holoenzyme assay, indicating that the hydrolysis of the bound nucleotide during the loading reaction is a distinct step which shows both base and phosphate specificity and can only be carried out by ATP (Refs. 9 and 13 and data not shown).3

Affinity Purification of RF-C—The great difference in interaction strength between PCNA and RF-C in the presence or absence of Mg-ATP was exploited in an improved purification scheme for RF-C. To test the possible value of this purification step, extracts from a protease-deficient yeast strain were incubated with PCNA beads in a buffer containing magnesium acetate and 0.4M NaCl, with or without 1 mM ATP. After washing the beads with the same buffer, again with or without ATP, protein was eluted with 1M NaCl in EDTA buffer and analyzed by SDS-PAGE. A large number of proteins, which bind to the PCNA-agarose, beads can be detected. Strikingly, the only difference between the two lanes is the clear presence of the RF-C polypeptides in the Mg-ATP lane, indicative that this complex can be specifically bound to the beads from crude extracts (Fig. 6). The PCNA-agarose step purified RF-C to a purity of about 1–5% from crude extracts. This corresponds to a 1000–5000-fold purification in this one step. Together with the overproduction of RF-C in yeast, the affinity purification step was applied to obtain homogeneous RF-C in high quantity and yield. Affi-Gel blue batch chromatography was used as a first step in the purification procedure

2 In these studies, bound RF-C is defined as the percent of the starting activity recoverable from the beads by elution with buffer containing 1 mM NaCl and 2 mM EDTA (see “Materials and Methods” for details).

3 At 1 mM GTP a weak stimulatory activity (20% of maximal) was observed in the Pol δ holoenzyme assay. This correlates poorly with the stimulatory activity of GTP in complex formation (Fig. 5) and may well be caused by a minute contamination of GTP by ATP.
to remove endogenous PCNA and to allow for a cleaner handling of the PCNA beads so they could be re-used. After the Affi-Gel blue step, the RF-C-containing fraction was loaded onto the PCNA-agarose bead column in a buffer containing Mg-ATP and 0.3M NaCl. The column was washed with 0.4M NaCl in the same buffer, and RF-C was quantitatively eluted from the column by switching from Mg-ATP to 2 mM EDTA in the same buffer (Fig. 7). RF-C eluting from this column contained trace amounts of PCNA and was therefore passed over a small Mono S FPLC column which removed PCNA and also served to concentrate the complex. The yield from 120 g of cells was about 3 mg of pure RF-C (Table I). Unlike previous preparations of RF-C, no DNA helicase activity could be detected after the PCNA-agarose step (19).

**RF-C activity is measured in a DNA polymerase**

**holoenzyme assay.** Loading of PCNA by RF-C on singly primed single-stranded mp18 DNA allows processive DNA synthesis by Pol δ. One unit of RF-C activity has been defined as that amount which stimulates 1 pmol of DNA synthesis by Pol δ when PCNA and ATP are also present. During this assay, which is for 4 min at 37 °C, holoenzyme complex formation and complete replication of the phage DNA is easily achieved, but turnover of the holoenzyme to another phage DNA molecule is slow (18). Therefore, in practice this is a single turnover assay. If we assume that RF-C consists of a monomer of each subunit in the heteropentamer, its molecular weight would be 249,000, close to the previously measured value of 240,000 (13). The maximum theoretical specific activity obtainable by this assay would be $10^9/240,000$ (picomoles of RF-C/mg of protein) × 7210 (nucleotides per mp18 circle) = $29 \times 10^6$ units/mg. Previous preparations of RF-C typically had a specific activity of about $5 \times 10^6$ units/mg (13). The affinity-purified material had a specific activity of $20 \times 10^6$ units/mg.

**DISCUSSION**

Apart from the technical advances described in this study, two conceptual conclusions can also be drawn: (i) overexpression of the five RFC genes is both necessary and sufficient for overproduction of RF-C, and (ii) PCNA forms a distinct, salt-resistant complex with RF-C in the presence of Mg-ATP.

Interactions between the clamp and the clamp loader proceed similarly in prokaryotes and in eukaryotes. Using a gel filtration technique to detect stable complexes, Naktinis et al. (20) deter-
mined that co-migration on the gel filtration column of the E. coli β subunit (clamp) with the γδ complex (clamp loader) required the presence of Mg-ATP. The same conclusion follows from the PCNA-agarose bead studies in this paper. The sensitivity of the PCNA complex to even moderate salt levels, and the resistance of the PCNA complex to high levels of salt suggest that non-ionic interactions contribute very strongly to the latter complex. Possibly, in the ternary complex, the PCNA ring may already be opened up in advance of loading around the template-primer junction, with the subunit-subunit interface of PCNA stabilized by hydrophobic interactions with RF-C. After loading, hydrolysis of the bound ATP would be required in a subsequent step, either for closing of the clamp around the DNA or for altering the interaction of PCNA with RF-C to allow a proper interaction with Pol δ ATPγS, which supports formation of the strong ternary complex (Fig. 5), does not stimulate processive replication by Pol δ holoenzyme under normal assay conditions. However, when RF-C and PCNA are incubated with primed DNA in the presence of ATPγS, and then excess ATPγS removed by filtration over a Biogel A5 m column, the isolated complex contains bound PCNA and RF-C and supports processive DNA replication upon addition of Pol δ (18, 21). However, this complex is different from the analogous complex made with ATP. It binds Pol δ more weakly than the ATP-derived complex. Furthermore, both the PCNA RF-C complex and the Pol holoenzyme complex, as well as DNA synthesis by these complexes, are sensitive to added ATPγS, whereas elongation of a holoenzyme complex formed with ATP is resistant to ATPγS (18). This is summarized in Fig. 8. Distinct ATPγS-mediated complexes of the clamp and the clamp loader with DNA have also been documented in the phage T4 and the mammalian systems (12, 22, 23). Together, these results indicate that ATP binding to PCNA-RF-C is sufficient to load PCNA onto the DNA, but its hydrolysis is required to stabilize the clamp.

The unique properties ATP in stabilizing interactions between PCNA and RF-C allowed us to design an efficient purification step for RF-C. The affinity chromatography step purified RF-C 1000–5000-fold from crude extracts. If preceded by an Affi-Gel blue batch chromatography step, which gives approximately a 10-fold purification of RF-C, it should be possible to obtain relatively pure RF-C by a two-column procedure, even when the complex has not been overproduced. The PCNA gene has been cloned from a large number of organisms, and so far the large scale expression of the protein in E. coli has been simple. Therefore, the utility of the affinity purification step should make the isolation and purification of RF-C from those organisms very feasible.

Fig. 5. Nucleotide requirement for the formation of a stable PCNA-RF-C complex. PCNA-agarose bead assays were as described in the legend to Fig. 4, except that the buffer contained 5 mM magnesium acetate, 350 mM NaCl, and nucleotide at the indicated concentration.

Fig. 6. Binding of crude extracts to PCNA-agarose beads. Strain BJ2168 was grown in rich medium, and extracts were made as described under Materials and Methods. The extracts were spun at 100,000 × g for 1 h. 25 μl of PCNA-agarose beads were incubated with 5 mg of extract that had been adjusted with binding buffer to a final volume of 5 ml and to a final concentration of 400 mM NaCl and either 5 mM magnesium acetate (first lane) or 5 mM magnesium acetate plus 1 mM ATP (second lane). The beads were washed with 2 × 50 μl of the same buffer and bound protein eluted with 2 × 50 μl of 1 mM EDTA, 1 x NaCl buffer. Protein in the eluate was precipitated with 80% acetone and the pellet dissolved in loading buffer and analyzed by 10% SDS-PAGE. A silver staining of the gel is shown. Migration positions of the RF-C subunits are indicated.

Fig. 7. Purification of RF-C. 10% SDS-PAGE of fractions after different steps of the purification procedure. Staining was with Coomassie Brilliant Blue.
Acknowledgments—We thank John Majors and Tim Lohman for critical discussions during the progress of this work.

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