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The *RFC2* gene encoding a subunit of replication factor C of *Saccharomyces cerevisiae*

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ABSTRACT

Replication Factor C (RF-C) of *Saccharomyces cerevisiae* is a complex that consists of several different polypeptides ranging from 120- to 37 kDa (Yoder and Burgers, 1991; Fien and Stillman, 1992), similar to human RF-C. We have isolated a gene, *RFC2*, that appears to be a component of the yeast RF-C. The *RFC2* gene is located on chromosome X of *S. cerevisiae* and is essential for cell growth. Disruption of the *RFC2* gene led to a dumbbell-shaped terminal morphology, common to mutants having a defect in chromosomal DNA replication. The steady-state levels of *RFC2* mRNA fluctuated less during the cell cycle than other genes involved in DNA replication. Nucleotide sequence of the gene revealed an open reading frame corresponding to a polypeptide with a calculated Mr of 39,716 and a high degree of amino acid sequence homology to the 37-kDa subunit of human RF-C. Polyclonal antibodies against bacterially expressed Rfc2 protein specifically reduced RF-C activity in the RF-C-dependent reaction catalyzed by yeast DNA polymerase III. Furthermore, the Rfc2 protein was copurified with RF-C activity throughout RF-C purification. These results strongly suggest that the *RFC2* gene product is a component of yeast RF-C. The bacterially expressed Rfc2 protein preferentially bound to primed single-strand DNA and weakly to ATP.

INTRODUCTION

Human Replication Factor C (RF-C) or Activator 1 (A1) has been identified and purified as an auxiliary protein that is required for the elongation of primed DNA templates by DNA polymerase δ along with proliferating cell nuclear antigen (PCNA) (1–6) in an *in vitro* SV40 DNA replication system. RF-C consists of five different subunits of 145, 40, 38, 37, and 36.5 kDa, all of which are copurified with the RF-C activity through various column chromatography and glycerol density gradient centrifugation (4–6). This protein complex interacts with primed DNA to form a complex that binds to PCNA in the presence of ATP. The resulting complex can bind to either DNA polymerase δ or ε to form a complex that elongates primer ends in the presence of dNTPs (3, 7). RF-C has DNA-dependent ATPase activity that is stimulated by PCNA and the human single-stranded DNA binding protein (or RF-A); the hydrolysis of ATP is essential for RF-C dependent DNA polymerase δ or ε activity (6, 8). The RF-C binds to the primer–template junction through the 145-kDa subunit and ATP is bound by the 40-kDa polypeptide (9). Recently, cDNA for all subunits of human RF-C have been cloned and sequenced (10–13). The predicted amino acid sequence of each subunit showed significant homology with each other and T4 gp44 and to a less extent, with the τ and γ subunits of *Escherichia coli* DNA polymerase III (10–13). When expressed in *E.coli*, the 37- and 40-kDa subunits of human RF-C have primed DNA binding and ATP binding activities, respectively (10, 11), similar to these activities in RF-C. Although it is likely that RF-C along with PCNA and RF-A is required for human chromosomal DNA replication, a direct genetic proof has not been obtained with human cells.

Activity similar to human RF-C has been identified and purified from the yeast *Saccharomyces cerevisiae* (14, 15) and calf thymus (16) using singly primed M13 single-stranded circular DNA as a template. This template is relatively inert for either DNA polymerase δ or ε synthesis in the presence of both RF-A (or *E.coli* SSB) and PCNA. The purified activity from yeast consists of several polypeptides ranging from 120 to 37 kDa (14, 15, 16). Although the molecular weight of the largest subunit of the yeast protein complex is considerably different from that of human RF-C, the rest are similar to those from human RF-C. The opportunity to augment biochemical studies with classical genetic and molecular genetic approaches makes yeast attractive for analyzing the *in vivo* functions of enzymatic activities. This has been demonstrated for DNA polymerases α, δ, and ε, RF-A and

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PCNA (18). Here, we describe the isolation and characterization of the *RFC2* gene which encodes the second largest subunit (the 40-kDa polypeptide) of yeast RF-C protein complex. The product of *RFC2* appears to be a homolog of human 37-kDa RF-C subunit, since they share 50% amino acid sequence identity. We also show that Rfc2 protein expressed in *E.coli* has ATP binding activity and DNA binding activity similar to that of human RF-C 37-kDa polypeptide. The *RFC2* gene is essential for yeast cell growth, consistent with the idea that it is required for chromosomal DNA replication in yeast.

MATERIALS AND METHODS

Bacterial and yeast strains

E.coli DH5α (19) was used for the preparation of plasmid DNA. Strain BL21(DE3), which harbors a lysogen containing the T7 RNA polymerase gene under the control of the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible *lac* UV5 promoter, was used for expression of the *RFC2* gene product (10).

The *S.cerevisiae* strains used were S288C (*MATα mal gal2*) (from Yeast Genetics Stock Center, Berkeley, CA), CB001 (*MATa leu2 trp1 ura3 prb pep4::URA3*) (19), CG378 (*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 can1*)(20), CG379 (*MATα leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2*)(20), B-7528 (*MATa cyc1-31 cyc7-67 ura3-52 lys5-10*)(from F. Sherman, Rochester) and diploid strain D778 (*MATa/MATα +/leu2-3,-112 +/trp1-289 ura3-52/ura3-52 +/ade5-1 cyc1-31/+ cyc7-67/+ lys5-10/+*) constructed by mating between CG379 and B-7528.

DNA

A singly primed φX174 single-stranded DNA was prepared as follows. Five times molar excess of the chemically synthesized 39-mer (5′-CGCAAAGTAAGAGCTTCTCGAGCTGCGCAA-GGATAGGTC-3′ which is complementary to nucleotides from 183 to 145 of φX174 single-stranded viral DNA in Ref. 21) was mixed with φX174 single-stranded viral DNA (from GIBCO-BRL) in 10 mM Tris−HCl, pH8.0, 1 mM EDTA, heated for 5 min at 90°C, and incubated for 2 hr at 65°C. Then, the mixture was slowly cooled to room temperature and chilled at 0°C. The sample was applied to a Sephadex 100 column (10 ml) equilibrated with 10 mM Tris−HCl, pH8.0, 1 mM EDTA, 0.1 M NaCl and eluted with the same buffer. Totally excluded fractions were pooled and used as a singly primed φX174 single-stranded viral DNA. Poly(dA)$_{350}$, and oligo(dT)$_{10}$ were purchased from Pharmacia. Poly(dA)$_{350}$.oligo(dT)$_{10}$ was made by incubating the components at 30°C for 60 min in 10 mM Tris−HCl, pH7.5, 1 mM EDTA, 0.3 M NaCl.

Enzymes

Yeast DNA polymerases II*(ε) and III (δ) were the same as previously described (19). Yeast heterotrimeric single-stranded DNA binding protein, RF-A, was purified from *S.cerevisiae* CB001 cells as previously published (22) except that 2.5 M NaCl was used to elute RF-A from ssDNA cellulose column. (When ethyleneglycol was included in the elution buffer, RF-A protein seemed to precipitate in the presence of 0.1 M NaCl and a MonoQ column was not used for purification.) Yeast PCNA was expressed in *E.coli* and purified as published (15). Purity of both yeast RF-A and PCNA was more than 95% judged by SDS-polyacrylamide gel electrophoresis, followed by staining the proteins with either silver or Coomassie brilliant blue.

For the purification of yeast RF-C, 1 kg of *S.cerevisiae* CB001 cells was lyzed and Fraction I was prepared as previously reported (19). Fraction I was applied on 900 ml S-Sepharose Fast Flow column equilibrated with 0.1 M NaCl in Buffer A (50 mM Tris−HCl, pH7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 1 mM PMSF, 1 μg/ml leupeptin) and the column was washed with the same buffer. Proteins were eluted with 0.5 M NaCl in Buffer A and precipitated with 0.31g/ml of ammonium sulfate. The precipitate was collected by centrifugation, resuspended in 30 ml of 0.3 M NaCl and 0.01% NP40 in Buffer A and dialyzed against the same buffer for 4 hr at 4°C. The dialyzed sample was applied on 20 ml Affi-Gel Blue (BioRad) column equilibrated with 0.35 M NaCl, 0.01% NP-40 in Buffer A, the column was washed with 60 ml of the same buffer, and proteins were eluted with 1 M NaCl, 0.01% NP-40 in Buffer A. The active fractions were pooled, dialyzed against 0.1 M NaCl, 0.01% NP-40 in Buffer A for 6 hr, applied on Mono Q (HR 10/10) FPLC column equilibrated with 0.1M NaCl, 0.01% NP-40 in Buffer A, and were eluted with 60 ml of 0.1−0.6 M NaCl linear gradient in Buffer A containing 0.01% NP-40. The active fractions (0.2−0.25 M NaCl) were pooled, dialyzed against 0.1 M NaCl, 0.01% NP-40 in Buffer A for 4 hr and applied on ssDNA cellulose FPLC column (HR5/5) equilibrated with the same buffer. The activity was eluted with 20 ml of 0.1−0.6 M NaCl linear gradient in Buffer A containing 0.01% NP-40. RF-C activity eluted in two separated peaks as published (14). The first peak of activity had higher specific activity (~5×10^5 U/mg protein) than the second (~1×10^5 U/mg protein) (See Fig. 4) and was used in this study. The specific activity of this fraction was similar to Fraction VIA described by Yoder and Burgers (14).

Expression of yeast *RFC2* gene product in *E.coli*

6-HisT-pET11d plasmid DNA containing the *RFC2* gene was constructed as follows. A *Nco*I restriction site was engineered into the *RFC2* ORF and a *Bam*HI site was engineered into the 3′ untranslated region. The 30 mers 5′GGCAATCACTTG-CCATGGTTGAAGGGTTTG3′ ([from nucleotide number 860 to 889 in the nucleotide sequence of *RFC2* (The sequence referred here has been deposited in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the accession no. D28499.)] and 5′ ATTATATAAGGATCCCTGTATATTAGA-GTT3′ (from nucleotide number 1957 to 1928) were used to construct the *Nco*I site and the *Bam*HI site (both underlined), respectively. These two oligonucleotides were used to prime a polymerase chain reaction (94°C for 2 min, 37°C for 2 min, 72°C for 3 min, 35 cycles) containing about 1 ng of pBluescript II KS (+) containing *Hind*III−*Bam*HI fragment of *RFC2* (Fig. 1) and 1 unit of *Taq* DNA polymerase. The resulting 1098-bp product coding for Rfc2 was isolated by agarose gel electrophoresis, digested with *Bam*HI and *Nco*I restriction enzymes, and inserted into a 6-HisT-pET11d vector plasmid DNA digested with *Bam*HI and *Nco*I to construct pET11d-RFC2 plasmid. The generation of the *Nco*I site caused a Phe → Val change at the second amino acid residue of the RFC2 gene. However, the engineered gene could function as a wild-type gene, since it complemented the lethal rfc2Δ::URA3 mutation. *E.coli* BL21(DE3) cells transformed with pET11d-RFC2 plasmid DNA were grown at 37°C in LB + 0.4% glucose + 0.5 mg/ml ampicillin (1 litter) to 3×10^8 cells/ml and 0.4 mM IPTG was added into the cell culture to induce the protein. After 3 hr incubation, cells were collected by centrifugation, suspended in

20 ml of lysis buffer (50 mM Tris−HCl, pH8.0, 10% sucrose, 0.1% lysozyme, 0.6% Brij 58, 10 mM DTT, 10 mM EDTA), incubated at 0°C for 45 min, and sonicated for 2 min at 0°C. The cell lysate was then centrifuged at 12,000 rpm for 15 min in a Beckman JA20 rotor; the precipitate was resuspended in 10 ml of the lysis buffer without lysozyme and Brij 58 and recentrifuged as above. Virtually all of the expressed Rfc2 protein were recovered in the precipitate. To remove other proteins, the precipitate was washed with 10 ml of 6 M urea solution containing 25 mM Tris−HCl, pH7.5, 1 mM EDTA, and 5 mM DTT, centrifuged for 15 min at 12,000 rpm in a Beckman JA20 rotor, and solubilized with 4 ml of 6 M guanidine−HCl containing 25 mM Tris−HCl, pH7.5, 10% glycerol, 1 mM EDTA, and 5 mM DTT. The solubilized precipitate was diluted to 0.7 mg protein/ml with dialysis buffer (25 mM Tris−HCl, pH7.5, 10% glycerol, 1 mM EDTA, 0.5 M KCl, 0.01% NP40, 5 mM DTT), dialyzed against the same buffer for 4 hr, and dialyzed again against the same buffer without EDTA for 4 hr. One fourth of the dialyzed sample (~25 mg protein) was applied on a 15-ml hydroxylapatite (BioRad) column equilibrated with the same buffer, the column was washed with buffer B (20 mM potassium phosphate buffer, 7.0, 10% glycerol, 0.5 M KCl, 5 mM DTT) and the protein was eluted with buffer C (0.5 M potassium phosphate buffer, pH7.0, 10% glycerol, 0.5 M KCl, 5 mM DTT). About 50% of Rfc2 protein applied was recovered in flow through fractions and the remaining protein was eluted by buffer C, both of whose purity was more than 95% based on SDS-polyacrylamide gel electrophoresis followed by staining the gel with Coomassie brilliant blue. When a linear gradient of potassium phosphate buffer was applied to the column, the protein was gradually eluted, suggesting that it was not properly renatured. Therefore, the flow through fractions were used in this study.

Assay for RF-C activity

The column fractions were incubated at 30°C for 15 min in the reaction mixture (15 μl) containing 40 mM Tris−HCl, pH 7.5, 8 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, 100 μM each of dATP, dCTP and dGTP, 25 μM [α-^{32}P]dTTP (specific activity was about 1,600 cpm/pmol), 0.2 mg/ml bovine serum albumin, 50 ng of singly primed φX174 single-stranded DNA, 0.1 unit of yeast DNA polymerase III (δ), 1 μg yeast RF-A and 0.15 μg PCNA. 5 μl of the reaction mixture was used for assay of radioactivity incorporated into DNA (19).

Immunoblotting

Proteins were transferred electrophoretically from SDS-polyacrylamide gels to Immobilon™ transfer membrane filter (Millipore). The membranes were preincubated at room temperature in TBS−2% bovine serum albumin for 1 hr, probed with the Rfc2 antiserum (1/1,000 diluted with TBS−2% bovine serum albumin) for 4−6 hrs at room temperature, washed with TBS−2% bovine serum albumin, and incubated with anti-rabbit IgG conjugated to alkaline phosphatase (Promega).

Antibody inhibition assays

The RF-C fractions (1 μl each) were mixed with various amount of either preimmune serum or polyclonal antibodies against Rfc2 protein expressed in *E.coli* on ice. After incubation at 0°C for 60 min, the other components required for the assay of RF-C activity were added and the mixtures were incubated for 15 min at 30°C. The amount of radioactive dTMP incorporated into acid insoluble material was measured. For immunodepletion experiments, Protein A-agarose beads (Sigma Chemicals) were incubated in 10 mg/ml of bovine serum albumin in PBS (0.1 M sodium phosphate buffer, pH7.5, 0.1 M NaCl) on ice for 30 min. Then, the beads were washed 3 times with PBS. 200 μl of either preimmune serum or polyclonal antibodies was mixed with 100 μl of the beads and incubated at room temperature for 1 hr with constant rotation. Finally, the beads were washed with BufferA−0.1 M NaCl, twice, and 50 μl of the beads were incubated with 50 μl of the RF-C fractions for 1 hr at 4°C. The beads were removed by centrifugation, fresh 50 μl of the beads binding either preimmune serum or the antibodies was added into the supernatant and incubation at 4°C was repeated. The final supernatant was used for the assay of RF-C activity.

DNA binding assay

Nitrocellulose filter DNA binding assays were carried out as described (11). 220 fmol of 5′-^{32}P-labeled DNA (400−500 cpm/fmol) was incubated with either Rfc2 protein, RF-C complex, or RF-A, as indicated, in 0.1 ml binding buffer (25 mM Hepes buffer, pH7.5, 5 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, 0.1 mg/ml BSA) for 30 min at 0°C. The reaction mixture was then filtrated through nitrocellulose filters pretreated with alkaline as described (11), the filters were washed three times with 1-ml binding buffer without BSA and dried. The radioactivity of the filter was measured by a liquid scintillation counter.

Other methods

SDS-polyacrylamide gel electrophoresis, DNA sequencing, ATP binding assays, and yeast cell synchrony were carried out as described (12, 13, 19, 20, 23). Rabbit polyclonal antibodies against Rfc2 protein were prepared commercially with Rfc2 protein eluted from SDS-polyacrylamide gel as described (24). The standard genetic procedures for the yeast *S.cerevisiae* is previously described (20, 25).

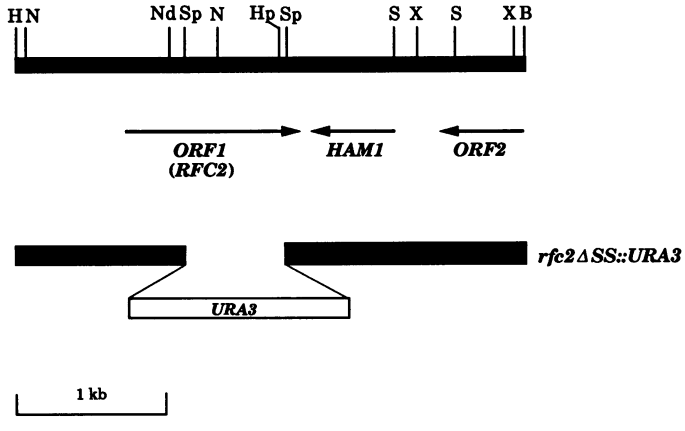

Figure 1. Restriction map of the *RFC2* locus and the construction of *rfc2Δ::URA3* mutations. A solid line represents *S.cerevisiae* chromosomal DNA. An arrow shows the size and directionality of an open reading frame. For construction of *rfc2Δ::URA3* mutations, the *S.cerevisiae URA3* gene was inserted between *Sspl* and *Sspl* sites. H, *HindIII*; N, *NheI*; Nd, *NdeI*; S, *SalI*; Sp, *SspI*; B, *BamHI*; X, *XhoI*; Hp, *HpaI*.

RESULTS

Identification of the *RFC2* gene

While studying the *HAM1* gene which controls 6-N-hydroxylaminopurine sensitivity and mutagenesis in *S.cerevisiae* (25), we identified two unknown open reading frames (ORF) in vicinity of the *HAM1* gene after determining the nucleotide sequence (Fig. 1). The ORF1 encodes a polypeptide consisting of 353 amino acid residues with a calculated Mr 39,716. The predicted polypeptide has regions of consensus to nucleotide-binding proteins and exhibits a significant similarity to subunits of human Replication Factor C (or Activator 1), *E.coli* DNA polymerase III subunits γ and τ, and bacteriophage T4 genes 44 and 46. There is 52.3% identity with the 37-kDa subunit of human RF-C over 321 amino acids, 36.6% identity with the 36-kDa subunit over 322 amino acids (Fig. 2), and 35.2% identity with the 40-kDa subunit over 341 amino acids comparing to the ORF1. Therefore, it is likely that the newly identified gene encodes a yeast homolog of the human RF-C 37-kDa subunit and we tentatively named it *RFC2*. (The *RFC1* gene has been reserved for the largest subunit (110 kDa subunit) of yeast RF-C complex. Based on amino acid sequence homology with human RF-C 145-kDa polypeptide (13), *CDC44* (26) appears to be *RFC1*.)

In order to test whether the *RFC2* gene is essential for yeast cell growth, the *Ssp*I−*Ssp*I fragment that was located in the middle of the ORF of *RFC2* was replaced with the 1.5 kb yeast *URA3* gene and one of the *RFC2* genes in the diploid yeast strain

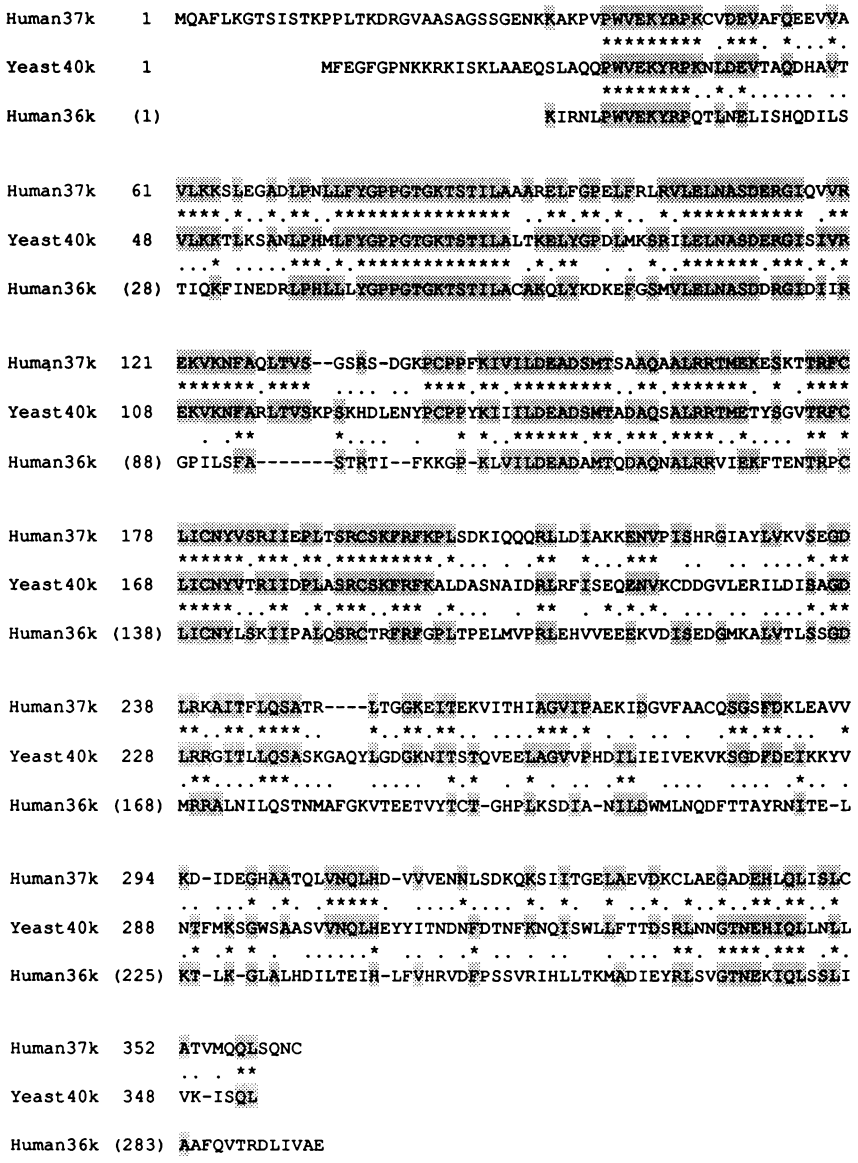

Figure 2. Amino acid sequence alignement between Rfc2, human RF-C 37- and 36-kDa subunits. An asterisk between aligned amino acids indicates an exact match in the sequence; a dot represents a conservative replacement. Dashes within the sequence indicate spaces inserted into one sequence by the ALIGN program to achieve optimal alignment of two protein sequences. Shaded amino acid residues show identical amino acid sequence between the two proteins. Human RF-C 37- and 36-kDa subunit amino acid sequences were obtained from GenBank and ref. 12, respectively. Since the complete amino acid sequence of the 36-kDa subunit has not been determined yet, numbering of the amino acid residues is arbitrary.

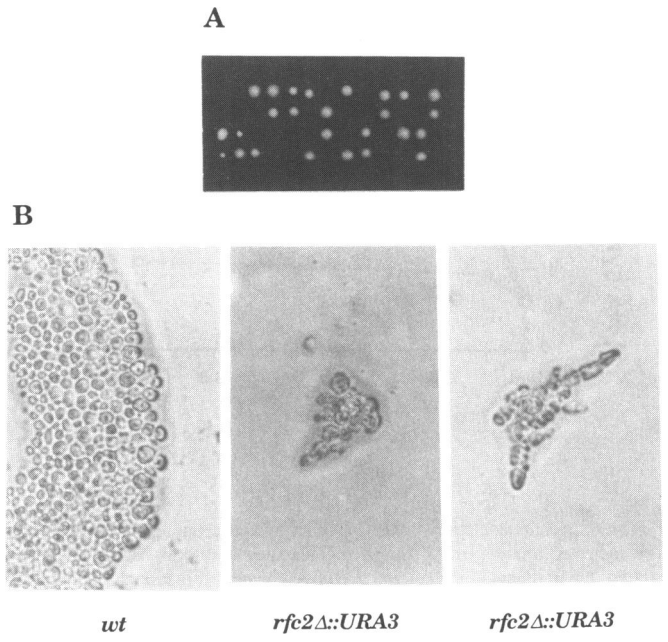

Figure 3. *RFC2* is an essential gene in *S.cerevisiae*. (**A**) Germinated spores dissected from tetrads of the *RFC2/rfc2Δ::URA3* diploid. Only two spores of each tetrad grew, and all viable spores were Ura-, indicating that they contained the *RFC2* gene. (**B**) Photograph of germinated spores. The region where viable and nonviable spores were located was examined by microscope and photographed. wt, viable spores; *rfc2Δ::URA3*, nonviable spores.

D778 was replaced with this disrupted *rfc2* by the one step replacement method (27). The resulting diploid strain was sporulated, the spores were micro-dissected, and germinated on YPD medium at 25°C. As shown in Fig. 3A, only two spores from each set of tetrads were capable of growth and they were all Ura$^-$ (data not shown), indicating that the spores having the disrupted *rfc2* gene are inviable. The inviable spores gave rise to microcolonies with unusual morphology (Fig. 3B). Cells of the microcolonies were separated by a micromanipulator and terminal morphology of the cells was examined. They did not exhibit further divisions. More than 60% of them were dumbbell shaped, 20% single round cells, and the rest were single cells with multiple buds.

The DNA fragment containing the *RFC2* gene was mapped to *S.cerevisiae* chromosome X using nitrocellulose filter containing the separated yeast chromosomes. Genetically, the gene was mapped between *cdc11* and *cdc8* on the right arm of chromosome X (details will be published elsewhere (24)). As no gene has been mapped in this region, *RFC2* is a newly identified gene in *S.cerevisiae*.

The *RFC2* gene product is a subunit of yeast RF-C complex

The *RFC2* gene was expressed in *E.coli* using a T7 promoter-driven expression system and its product was purified by SDS-polyacrylamide gel electrophoresis. The Rfc2 protein was used to raise polyclonal antibodies in rabbits. The antiserum specifically recognized the 40-kDa polypeptide in yeast RF-C preparations as well as the purified Rfc2 protein expressed in *E.coli* (data not shown). Therefore, the antiserum was used to

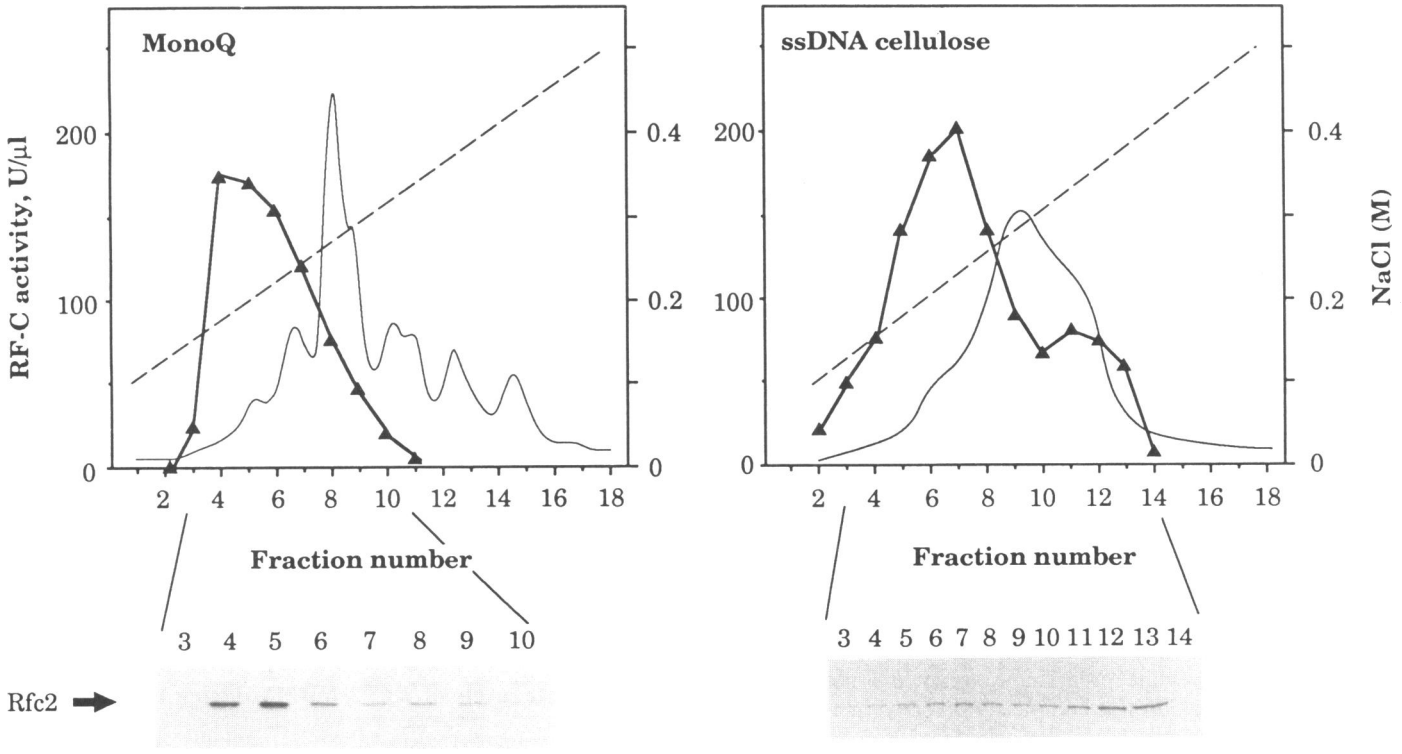

Figure 4. Copurification of Rfc2 protein with RF-C activity. The left pannel shows MonoQ FPLC profile of Affi-Gel Blue pools of RF-C, while the right is the elution profile of MonoQ pools from ssDNA cellulose column. Each fraction was subjected to Western blot analysis with rabbit antiserum against Rfc2 protein (Bottom). A closed triangle represents RF-C activity. The thin line is protein profile recorded by UV-monitor at 280 mm and the dotted line is concentration of NaCl in elution buffer.

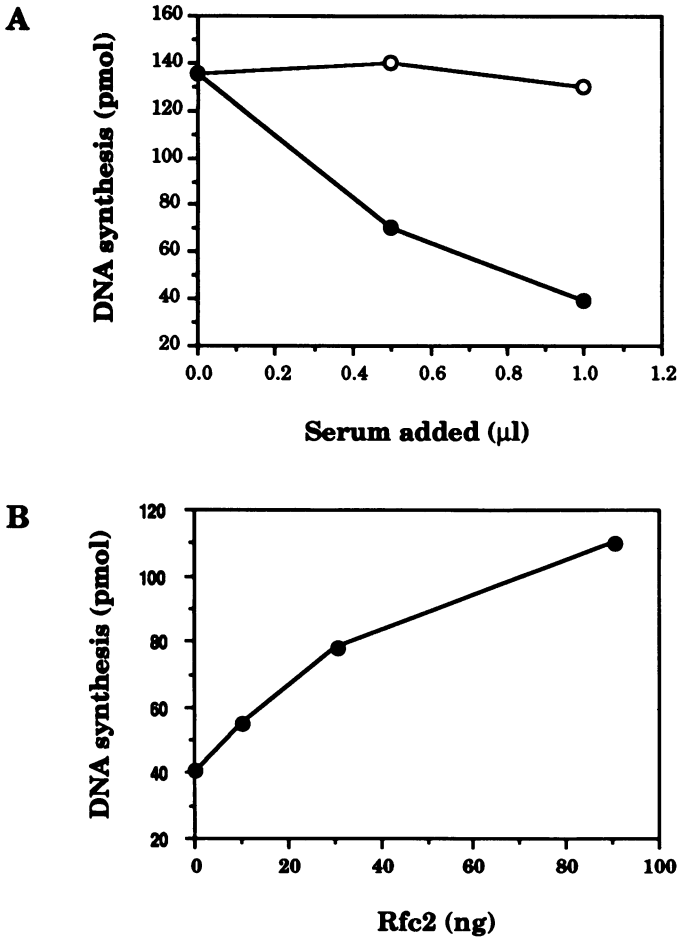

Figure 5. (**A**) Rfc2 antiserum inhibits the yeast RF-C activity. The indicated amount of rabbit antiserum against Rfc2 protein was incubated with the yeast RF-C to measure RF-C-dependent DNA synthesis catalyzed by yeast DNA polymerase III (δ) as described in the MATERIALS AND METHODS. An open circle represents RF-C activity in the presence of preimmune serum and a close circle is the RF-C activity in the presence of Rfc2 antiserum. (**B**) Reversibility of antibody inhibition with purified Rfc2 protein. The indicated amount of the purified Rfc2 protein from *E.coli* (see Fig. 7B) was added to the reaction mixture containing 1 μl of the antiserum and RF-C and the RF-C activity was measured.

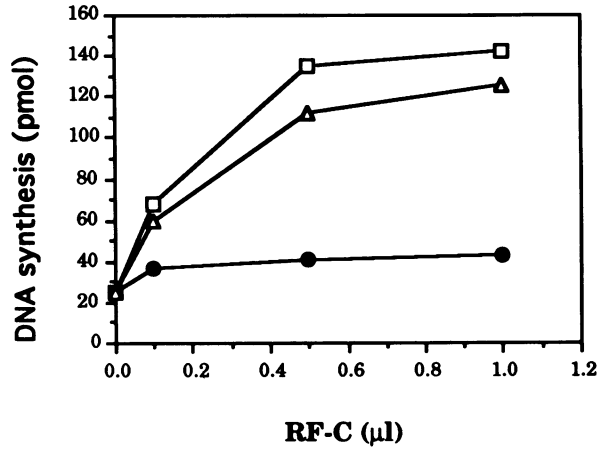

Figure 6. Immunodepletion of RF-C activity by antiserum against Rfc2 protein. The indicated amount of RF-C was preincubated with antiserum against Rfc2 protein, the immunocomplexes were removed by centrifugation after addition of Protein A-conjugated agarose beads, and the remaining RF-C activity was measured. An open square represents the activity without any serum added, open triangle shows the activity after preincubation with preimmune serum, and closed circle is the activity after preincubation with antiserum against Rfc2 protein.

follow the *RFC2* gene product during the purification of yeast RF-C. As shown in Fig. 4, the antiserum reacted with the 40-kDa polypeptide that copurified with the RF-C activity through MonoQ, single-stranded DNA cellulose, and hydroxylapatite column (not shown) chromatography. However, it should be noticed that the major peak of the 40-kDa polypeptide that reacted with the antibodies was coeluted with the second peak of RF-C activity after ssDNA cellulose chromatography (Fig. 4B).

The effect of antiserum on RF-C-dependent DNA synthesis catalyzed by yeast DNA polymerase III (δ) was also examined. As shown in Fig. 5A, the antiserum strongly inhibited the RF-C activity. More than 70% of the activity was inhibited by 1 μl of the antiserum, while preimmune serum did not affect its activity. This inhibition was completely reversed by the addition of 90 ng of the purified Rfc2 protein from *E.coli* (Fig. 5B). Furthermore, the antiserum successfully depleted >80% of the RF-C activity from the RF-C preparation by immunoprecipitation (Fig. 6). Based on these results, the 40-kDa Rfc2 polypeptide appears to be a subunit of yeast RF-C complex.

Biochemical activity associated with Rfc2 protein expressed in *E.coli*

The RFC2 gene was expressed in *E.coli* using the pET expression system (Fig. 7A). However, the expressed Rfc2 protein was not soluble unless extracted with 6 M guanidine−HCl, 0.5 M KCl. After dialysis against 25 mM Tris−HCl, pH7.0, 0.5 M KCl, we succeeded in solubilizing the protein and were able to purify it to near homogeneity (Fig. 7B). As shown in Fig. 7C, the purified Rfc2 protein preferentially bound to poly(dA)$_{350}$-oligo(dT)$_{10}$ and less efficiently to poly(dA)$_{350}$ as does RF-C complex. Approximately 100 molecules of the purified Rfc2 protein are capable of binding to one molecule of primer oligo(dT)$_{10}$. This value is comparable to that of human RF-C 37-kDa subunit expressed in *E.coli* (28). On the other hand, yeast RF-A (single-stranded DNA binding protein) bound to both poly(dA)$_{350}$ and poly(dA)$_{350}$-oligo(dT)$_{10}$ with the same efficiency (data not shown).

Since a consensus sequence of nucleotide-binding proteins can be seen in the *RFC2* ORF, we have tested whether ATP binds to the purified Rfc2 protein. As shown in Fig. 7D, weak, but significant ATP binding to Rfc2 protein could be detected by UV cross-linking. Unlike human RF-C 40-kDa subunit, this binding could be observed in the absence of Mg^{2+}. The binding could be competed out with cold rATP and to a less extent, with other rNTPs (data not shown). The ATP binding activity, however, was less than 1/1,000 of that for the human RF-C 40-kDa subunit expressed in *E.coli* (10).

Steady-state levels of *RFC2* mRNA during the cell cycle

The 5′ upstream region of *RFC2* contains the 5′-TCGCGT between −120 and −125 from the first nucleotide (A) of the translational start codon. The related 5′-ACGCGT is required for the co-ordinate expression of a group of DNA replication genes including *POL1*, *POL2*, *POL3* and *RNR1*, at the onset of

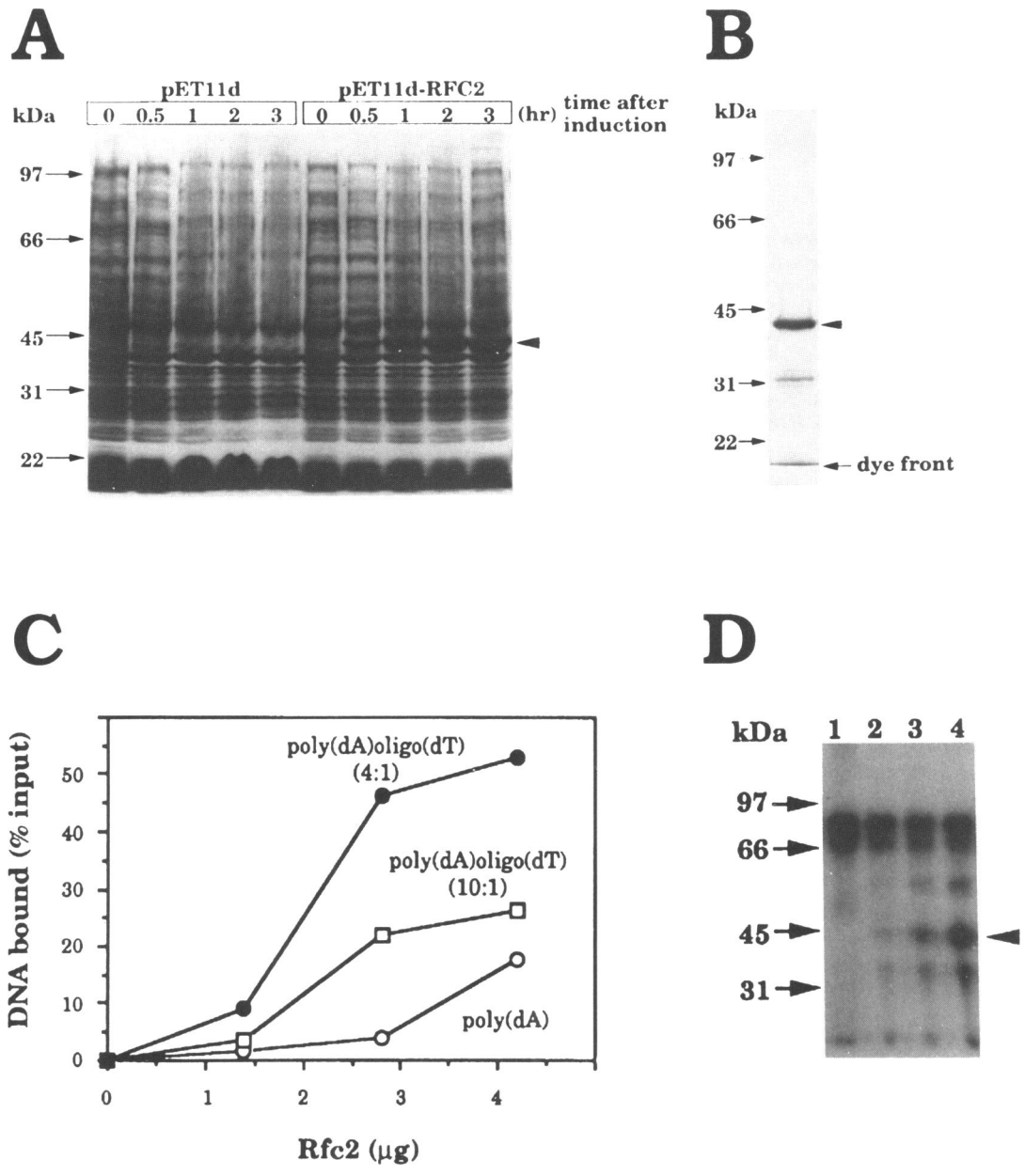

Figure 7. Bacterially expressed Rfc2 protein binds to primed single-stranded DNA and ATP. (**A**) Expression of the *RFC2* gene in *E.coli*. The *RFC2* gene expressed in *E.coli* was analyzed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue. The left panel shows the proteins from *E.coli* with the expression vector. The right hand panel shows proteins from *E.coli* with the plasmid containing the *RFC2* gene. (**B**) SDS-polyacrylamide gel electrophoretic analysis of purified Rfc2 protein. Five micrograms of purified Rfc2 protein was subjected to SDS polyacrylamide gel elecrophoresis and then stained with Coomassie brilliant blue. (**C**) Ability of Rfc2 protein to bind ^{32}P-labeled poly(dA)$_{350}$ with increasing amounts of oligo(dT)$_{10}$ was measured by filter binding assay as described in the MATERIALS AND METHODS. As a control, yeast single-stranded DNA binding protein (yRF-A) was also used. In this case, the same amount of either poly(dA)$_{350}$, poly(dA)$_{350}$-oligo(dT)$_{10}$(4:1), or poly(dA)$_{350}$-oligo(dT)$_{10}$(10:1) bound to yRF-A protein (data not shown). (**D**) Photoaffinity labeling of Rfc2 protein by [α-^{32}P]ATP. The reaction mixture (20 μl) containing indicated amounts of purified Rfc2 protein, 250 μg BSA and 1.25 μM [α-^{32}P]ATP was incubated at 0°C for 30 min. After UV-irradiation, reactions were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography of the dried gel. Lane 1; no Rfc2 protein added, lanes 2–4; 0.7, 1.4, and 2.8 μg of Rfc2 protein were added, respectively. The left side of the panel indicates protein markers in kDa. The right side indicates the position where purified Rfc2 protein was migrated. Two major bands labeled with ATP could be BSA, as the same amount of ATP-labeled bands that coinsided with BSA band stained with Coomassie brilliant blue could be detected without Rfc2 protein. Other bands seen above and below Rfc2 protein could be contaminated *E.coli* proteins in the purified Rfc2 protein.

S-phase (29). Therefore, we measured the steady-state levels of the *RFC2* mRNA during the cell cycle using a synchronized cell culture. As shown in Fig. 8, the levels of 1.1 kb mRNA that hybridized with the *RFC2* probe did fluctuated much less than those of *POL2* and histone H2B.

DISCUSSION

Five lines of evidence described here suggest that the *RFC2* gene product is one of the yeast RF-C subunits, possibly the second largest subunit, and is a homolog of human RF-C 37-kDa subunit.

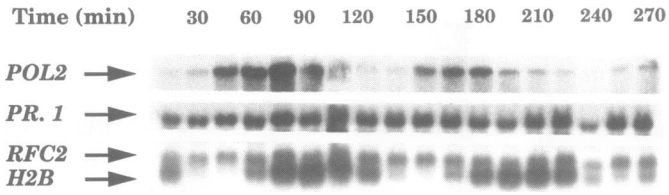

Figure 8. Steady-state levels of the *RFC2* mRNA during the cell cycle. *S.cerevisiae* CG397 cells were synchronized by elutriation as described before (23) and aliquots were withdrawn at the indicated times. Northern blots were hybridized with ^{32}P-labeled *Sspl*−*Sspl* DNA fragment (Fig. 1) along with ^{32}P-labeled Histone H2B, Protein 1, and *POL2* DNA (34, 35). To make sure that the same amount of total RNA from each time point was applied on a gel, the gel was stained with ethidium bromide before RNA transfer to a membrane. Based on intensity of rRNA bands, approximately the same amount of total RNA was used in each time point in this experiment. *PR. 1*; protein 1 mRNA, *H2B*; histone H2B mRNA.

(i) The predicted amino acid sequence from the nucleotide sequence of *RFC2* exhibits a high degree of homology to human RF-C 37-kDa subunit and interestingly, the predicted pI of Rfc2 protein is 6.0, which is very similar to human RF-C 37-kDa subunit (pI=6.2), (ii) the antibodies against bacterially expressed Rfc2 protein inhibit the yeast RF-C activity, (iii) the antibodies successfully depleted the RF-C activity, (iv) the 40-kDa polypeptide that reacted with the antibodies against the Rfc2 protein was copurified with RF-C activity through RF-C purification, and (v) Rfc2 protein expressed in *E.coli* exhibited a preferential binding to a primed template over single-stranded DNA as does human RF-C 37-kDa polypeptide.

It is believed that yeast RF-C complex consists of four different polypeptides (110, 42, 41, and 37 kDa) (15, 30), but the stoichiometry of each subunit of the RF-C complex has not been established (14, 15, 17). Li and Burgers recently identified and cloned the *RFC3* gene that they propose to encode for the 40-kDa polypeptide of the yeast RF-C complex. It consists of 340 amino acid residues (predicted Mr is 38,200) and is highly homologous with human RF-C 36-kDa subunit (30). However, based on the predicted molecular weight from nucleotide sequence, the Rfc3 protein is smaller than Rfc2 protein. Therefore, we suggest that Rfc2 protein is the second largest subunit of RF-C, which corresponds to the 42-kDa polypeptide described by Fien and Stillman (15), while Rfc3 is the third 41-kDa subunit.

While bacterially expressed Rfc2 protein used in this study did not stimulate the reaction catalyzed by yeast DNA polymerase III (δ) (our unpublished results), it exhibited one important activity associated with RF-C complex, preferential recognition and binding to primer−template junctions. Therefore, Rfc2 protein might be in a major function of RF-C and other subunits may give synergistic effect on the activity. The predicted amino acid sequence of Rfc2 protein has regions of consensus to nucleotide-binding proteins as does Rfc3. Therefore, it prompted us to test whether ATP binds to Rfc2 protein. Although ATP binding could be detected in bacterially expressed Rfc2 protein, the activity was rather low (less than 1/1,000 of human 40 kDa RF-C subunit expressed in *E.coli*). This may suggest that the complex formation with other subunits is required for efficient ATP binding, although we can not eliminate the possibility that our harsh solubilization of the protein inactivates the activity. Alternatively, a post-translational modification of Rfc2 protein in yeast may be required for the binding. Interestingly, it has been shown recently that the 40-kDa subunit (possibly Rfc3 protein) of yeast RF-C binds ATP (17) and bacterially expressed Rfc3 protein has a DNA-dependent ATPase activity (30). Therefore, it is possible that Rfc2 and Rfc3 proteins have a different function in RF-C-dependent DNA synthesis, although they share the extensive similarity in the amino acid sequence and this ATP hydrolysis catalyzed by Rfc3 protein may be utilized for Rfc2 protein to search template-primer junctions during DNA synthesis.

As previously observed (14), the yeast RF-C activity could be separated into two peaks after ssDNA cellulose chromatography (Fig. 4). Rfc2 protein copurified with these two activities (Fig. 4). However, the amount of Rfc2 protein did not correlate with RF-C activity. Relative RF-C activity in the first peak was at least 5 times higher than that in the second. Possibly these two different forms of RF-C may have different roles during DNA synthesis in the cell. Alternatively, modification of the Rfc2 protein by phosphorylation could result in different RF-C activities. However, there were no differences in migration of the 40-kDa polypeptide cross-reacted with the antiserum by SDS-polyacrylamide gel electrophoresis analysis (Fig. 4).

The *RFC2* gene is essential for the mitotic growth, consistent with the idea that it is required for chromosomal DNA replication in yeast. This was supported by the terminal morphology (dumbbell) of cells in microcolonies raising from spores with the *rfc2Δ* mutation. Since mutant spores germinated and went through several rounds of the cell division before their death, there appear to be an excess of RF-C complex in the cell as is the case with yeast RF-A. Isolation of temperature-sensitive mutations will help to establish the role of *RFC2* in chromosomal DNA replication.

All known *S.cerevisiae* DNA replication genes have one or more copies of a six-base-pair sequence called the MluI-Cell Cycle Box (MCB) at the 5′ non-translational region of the gene and the levels of the transcript fluctuate during the cell cycle, peaking at the G1/S boundary (29). It has been established that the DSC1 transcription factor (32) consisting of Swi6 and Mbp1 protein recognizes and controls the level of their transcript during the cell cycle (33). The *RFC2* gene also has a copy of MCB-like sequence (-TCGCGT-) upstream from the first ATG codon. However, the transcripts of *RFC2* did not fluctuated as evidently as other DNA replication protein genes during the cell cycle (Fig. 8). Furthermore, it has been shown similarly that the levels of the *RFC3* transcripts do not fluctuate during the cell cycle (30). Possibly Rfc2 and Rfc3 proteins and even the RF-C complex have other roles in DNA metabolism in yeast besides DNA replication, such as DNA repair. In this regard, the *CDC44* gene which may encode the largest subunit of yeast RF-C complex does not have any MCB sequence in the 5′-untranslated region and *cdc44* mutant cells exhibit elevated levels of mitotic recombination, suggesting that the gene product is required for DNA damage repair (26) as well as DNA replication.

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