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Molecular cloning and expression of the *Saccharomyces cerevisiae RFC3* gene, an essential component of replication factor C

(DNA replication/DNA polymerase δ/DNA polymerase ε/proliferating cell nuclear antigen)

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ABSTRACT **Yeast replication factor C (RF-C) is a multipolypeptide complex required for processive DNA replication by DNA polymerases δ and ε. The gene encoding the 40-kDa subunit of the *Saccharomyces cerevisiae* RF-C (*RFC3*) has been cloned. The *RFC3* gene is required for yeast cell growth and has been mapped to the left arm of chromosome XIV. The deduced amino acid sequence of the *RFC3* gene shows a high homology to the 36-, 37-, and 40-kDa subunits of human RF-C (also called activator 1), with the highest homology to the 36-kDa subunit. Among the conserved regions are the A motif of ATP binding proteins; the "DEAD box," common to DNA helicases and other ATPases; and the "RFC box," an ≈15-amino acid domain virtually identical in the yeast and human RF-C subunits. Limited homology to the functional homologs of the *Escherichia coli* replication apparatus was also observed. The steady-state mRNA levels of *RFC3* do not change significantly during the mitotic cell cycle of yeast. The intact form of the *RFC3* gene product (Rfc3p) has been overproduced in *E. coli* and purified to homogeneity. Purified Rfc3p has an ATPase activity that is markedly stimulated by single-stranded DNA but not by double-stranded DNA or RNA.**

Chromosomal DNA replication in the eukaryotic nucleus may require the coordinate action of three DNA polymerases (pols), α, δ, and ε (1–4). Pols δ and ε interact with the proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) in their respective holoenzyme forms, which are processive replicases (5, 6). The biochemical function of RF-C has been elucidated in yeast and in mammalian cells: it loads PCNA onto the primer terminus in an ATP-dependent reaction (5, 7–10). Pol δ or pol ε then binds to the DNA–RF-C–PCNA complex to constitute a processive replication complex (6, 11, 12).

Both the mammalian RF-C and the yeast RF-C (yRF-C) proteins are multipolypeptide complexes with a very similar subunit composition. The mammalian RF-C consists of a 145-kDa subunit and four subunits of 36–41 kDa (13, 14). The human genes for the 37- and 40-kDa subunits of RF-C (also called activator 1) have been isolated, and partial clones have been obtained for the 36- and 38-kDa subunits (15–17). They show a high degree of sequence similarity to each other and significant similarity to the functionally homologous prokaryotic DNA replication proteins (17). Similarly, the yRF-C consists of four subunits with apparent sizes of 120, 41, 40, and 37 kDa (5, 10, 18). Crosslinking studies have shown that the 40-kDa subunit binds ATP (18).

The power of genetic manipulation in *Saccharomyces cerevisiae* makes yeast an excellent system to study DNA replication and repair processes. To understand the functionality of all the RF-C subunits in these processes and elucidate genetically possible interactions between these proteins and components of the replication and/or repair machinery, we have started to isolate the genes of several of the subunits of yRF-C. Here we report on the cloning and expression of the *RFC3* gene,* encoding the ATP-binding subunit of RF-C and on the biochemical properties of the *RFC3* gene product (Rfc3p) overexpressed in *Escherichia coli* cells.†

MATERIAL AND METHODS

Strains and Media. The *E. coli* strains were DH5 and K5772 (*gal*$^-$, Str^R, *lacZ*::T7 RNAPol, kindly provided by H. Miller, Genentech). *Saccharomyces cerevisiae* strains were prototrophic diploid NCYC239 (19), CG731 (*MAT***a**, *ade2-1, lys2-1, ura3-52, leu2-3,112, his3-200, trp1-901, can1-100*) (20), isogenic diploid strain W303 (*MAT***a**/*MATα*, *ura3-1/ura3-1, his3-11,15/his3-11,15, trp1-1/trp1-1, leu2-3/leu2-3, ade2-1/ade2-1, can1-100/can1-100*), and its derivative YXL1 (as W303, but *RFC3*/Δ*rfc3*::*URA3*). All rich and minimal media were as described (21).

Enzymes, Proteins, and Nucleic Acids. Yeast pol δ, RF-C, and PCNA were purified as described (5). The DNA sequencing kit was purchased from United States Biochemical. Other enzymes were from New England Biolabs. M13mp18(+) DNA and M13mp18 replicative form were prepared as described (5, 18). All nucleic acids were from Pharmacia. The oligonucleotides used to perform PCR or DNA sequencing were synthesized by the Protein Chemistry Laboratory at Washington University School of Medicine.

Assay of Nucleotide 5′-Triphosphate Hydrolysis. The hydrolysis of ATP catalyzed by the Rfc3p was assayed by measuring the generation of [α-^{32}P]ADP from [α-^{32}P]ATP as described (18). The ATPase activity of Rfc3p was assayed in a 10-μl reaction system containing 50 mM Hepes (pH 7.5), 1 mM dithiothreitol (DTT), bovine serum albumin (0.1 mg/ml), 100 μM [α-^{32}P]ATP (44 cpm/pmol), 5 mM $MgCl_2$, 120 ng of various oligo- or polynucleotides, and 135 ng of Rfc3p. Reaction mixtures were incubated at 37°C for 30 min, terminated, and processed by thin layer chromatography as described (18).

Assay of Pol δ Holoenzyme Activity. The assay for pol δ holoenzyme activity measures DNA synthesis on single-stranded DNA binding protein-coated singly primed single-stranded mp18 DNA as described (5). To determine the inhibitory properties of antibodies against Rfc3p, control or anti-Rfc3p serum was preincubated in a total volume of 25 μl with 60 units of yRF-C in 40 mM Tris·HCl, pH 7.8/5 mM DTT/bovine serum albumin (0.2 mg/ml) at 4°C for 60 min, and then the preincubation mixture was added to a 25-μl

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Abbreviations: RF-C, replication factor C; y (as prefix), yeast; pol, DNA polymerase; PCNA, proliferating cell nuclear antigen; DTT, dithiothreitol.

*Numbering of the *RFC* genes is according to their sizes in the purified complex, with the *RFC3* gene being the third largest in size.

†The sequence reported in this paper has been deposited in the GenBank data base (accession no. L18755).

assay mixture with all the remaining components of the pol δ holoenzyme assay system except for yRF-C. After incubation at 37°C for 30 min, acid-insoluble radioactivity was measured as described (5).

Cloning and DNA Sequencing of the 40-kDa Subunit of yRF-C. Several degenerate oligonucleotides with appropriate sense or antisense directions were designed encoding amino acid sequences conserved between the 37- and 40-kDa subunits of human RF-C (activator 1) (15, 16). Two of these oligonucleotides, corresponding to the amino acid sequences PWEKYRP and IIEPIQSRC of the human 40-kDa subunit, were used in a standard 35-cycle PCR with yeast genomic DNA from strain CG731 as a template. Since no discrete DNA band with the expected size (≈440 bp) was observed, that region was excised from a low-melting-point agarose gel and the DNA recovered was used as a template for another set of PCRs with the original primers and two internal primers (corresponding to the sequences SILCLAR and KGRHKII of the 40-kDa subunit) in all possible combinations. Several discrete bands with expected sizes were detected. The appropriate DNA bands were purified from the agarose gels, made blunt-ended with the Klenow fragment of DNA polymerase I and dNTPs, and cloned into vector Bluescript SKII(+), and partial sequence information was obtained. This analysis indicated that partial clones of possibly three RF-C genes had been obtained.

The partial clone obtained in plasmid pBL401 was used as a probe in a Southern blot analysis of yeast nuclear DNA digested with various restriction endonucleases. A unique 10-kb *Bam*HI fragment was detected by the probe. Accordingly, a genomic library of ≈10-kb *Bam*HI fragments was constructed into vector pUC19 and the positive clone was isolated by colony hybridization using the insert in pBL401 as a probe. Subcloning and restriction enzyme analysis located the gene to a 1.6-kb *Sal* I–*Hin*dIII fragment (in plasmid pBL452), which was sequenced. Both strands of the insert were sequenced using the dideoxynucleotide chain-termination methodology. Additional sequence information upstream of the *Hin*dIII site (one strand only) was obtained using oligonucleotide primers.

Overproduction of Rfc3p in *E. coli*. The bacteriophage T7 expression system (22) was used to overproduce Rfc3p in *E. coli*. The 1.6-kb *Sal* I–*Hin*dIII fragment of pBL452 (see Fig. 3), which contains the first amino acid and the rest of the open reading frame of the *RFC3* gene, replaced the *Nco* I–*Hin*dIII fragment of plasmid vector pPY55, generating the overexpression plasmid pBL456. This cloning step not only put the *RFC3* gene under the control of the strong translation initiation signal of the vector but also perfectly regenerated the translation initiation codon in-frame with the coding sequence of the *RFC3* gene. Therefore, the intact form of Rfc3p could be produced in *E. coli*. Plasmid pBL456 was transformed into *E. coli* K5772, generating *E. coli* OE456.

A single colony of *E. coli* OE456 was grown overnight in 10 ml of LB medium with ampicillin (100 μg/ml) and inoculated into 1 liter of the same medium at 37°C. When the OD_{595} reached 0.15, isopropyl β-D-thiogalactopyranoside was added to the culture to 1 mM, and the culture shaken for another 3 h at 37°C. The culture was then harvested and the cells were suspended in 5 ml of 50 mM Tris·HCl, pH 8.1/10% (wt/vol) sucrose/10 mM EDTA, and an equal volume of 2× lysis buffer was added (lysis buffer is 50 mM Tris·HCl, pH 8.1/2 mM EDTA/0.2 mM EGTA/2 μM leupeptin/0.2 μM pepstatin A/5 mM sodium bisulfite/3 mM DTT). All further steps were carried out at 0–4°C. Lysozyme was added to 0.6 mg/ml and the mixture was stored on ice for 60 min with occasional mixing. Nonidet P-40 and phenylmethylsulfonyl fluoride were then added to 0.05% and 1 mM, respectively, and, after another 10 min on ice, the mixture was sonicated with a Branson Sonifier cell disrupter model 185 to reduce the viscosity. After centrifugation at 27,000 × *g* for 20 min, the supernatant was discarded and the precipitate was washed with 10 ml of wash buffer (lysis buffer except that the pH of the Tris·HCl was reduced to 7.5 and NaCl was added to 2 M). After repeating the wash procedure, the precipitate was homogenized with 2 ml of denaturation buffer (wash buffer containing 400 mM NaCl and 6 M urea). The suspension was shaken for 10 min and then centrifuged for 20 min in a microcentrifuge. The supernatant was diluted to a protein concentration of 150 μg/ml with 6 M urea/denaturation buffer without DTT, dialyzed against 2 M urea in dialysis buffer (50 mM Hepes, pH 7.4/20% glycerol/1 mM EDTA/0.1 mM EGTA/0.4 μM pepstatin A/2 μM leupeptin/10 mM sodium bisulfite/0.1 mM phenylmethylsulfonyl fluoride/200 mM NaCl) for 3 h and then against dialysis buffer overnight at 4°C. The solution was centrifuged and the supernatant was stored at −70°C. Protein concentrations were determined as described by Bradford (23).

Preparation of Polyclonal Antibodies and Immunoblot Analysis. Rabbit polyclonal antibodies against Rfc3p were prepared commercially by Rockland (Gilbertsville, PA), with the homogeneous Rfc3p prepared as described above. The immunoblot experiment was carried out as described (24).

RESULTS

Isolation and Characterization of the *RFC3* Gene. The extensive amino acid sequence similarity between the human 37-kDa and 40-kDa subunits of RF-C (activator 1), coupled to the high degree of structural conservation between human RF-C and yRF-C, prompted us to attempt to isolate the analogous yeast *RFC* genes by a PCR approach. Of the three putative *RFC* genes that were isolated by this method, the analysis of *RFC3* is presented here. The *RFC3* gene contains an open reading frame of 340 aa residues with a calculated molecular mass of 38.2 kDa. The protein encoded by the *RFC3* gene is called Rfc3p.

As expected, the deduced amino acid sequence of the *RFC3* gene shows an extended similarity with the small subunits of human RF-C (activator 1), with the highest similarity to the 36-kDa subunit (51% identity) (17). Fig. 1 shows the alignment of Rfc3p with the amino acid sequences of the hA1-36, hA1-37, and hA1-40 genes. Limited homology was also observed with the phage T4 gene *44* sequence. Among the identifiable motifs that these five polypeptides have in common are the A motif of ATP binding proteins and the "DEAD box" present in many ATPases. It is interesting to note that immediately preceding the DEAD sequence in Rfc3p the IILL amino acid sequence is present. This motif is assumed to form a β-sheet that is part of the substrate binding pocket of many ATP-metabolizing enzymes, whereas the aspartic acid (D117) may be important for magnesium binding (25). The detection of DNA-dependent ATPase activity in the Rfc3p described below further supports the significance of the prediction based on these structural features. In addition, the yeast and human polypeptides show virtual sequence identity in a 15-aa stretch, the "RF-C box." Most of the conserved regions are in the N-terminal 200 aa of the proteins.

Comparisons of the Rfc3p amino acid sequence with those of functional homologs in prokaryotic systems reveal significant similarity not only with phage T4 gene *44* protein (29% identity, Fig. 1) but also to the γ and δ′ subunits of the *E. coli* pol III holoenzyme (24% and 20% identity, respectively) (comparison not shown), with most of the conserved regions in the ATP binding site. However, the RFC box, the largest conserved block among the eukaryotic *RFC* genes, was not present in these prokaryotic homologs, possibly implying that this conserved region is involved in specific interactions with other eukaryotic replication proteins.

FIG. 1. Comparisons of the predicted amino acid sequence of *RFC3* with those of three small subunits of human RF-C (activator 1, designated hA1-36, hA1-37, and hA1-40) and bacteriophage T4 gene *44* protein. The alignment was performed with the Clustal method (DNASTAR programs). The putative NTP binding motifs, the DEAD box region, and the RF-C box region described in text are underlined.

Chromosomal Location and Cell Expression of the *RFC3* Gene. To map the *RFC3* gene to a specific chromosomal location, a genomic Southern blot analysis was carried out with clone filters of a yeast genomic library (kindly provided by L. Riles and M. V. Olson, Washington University) (26). With the *RFC3* probe and a detailed knowledge of the chromosomal location of the different filter clones, the *RFC3* gene was mapped to the left arm of chromosome XIV. A search of the nucleotide sequence data base showed that the *RFC3* gene is ≈0.7 kb upstream of the *HCS26* gene (27).

Most genes involved in DNA replication are periodically expressed during the cell cycle with expression being maximal during late G_1/early S phase. This periodic expression is dependent on the presence of the ACGCGT sequence, identical to the recognition site for the *Mlu* I restriction enzyme, in the upstream regulatory region (28). Although no *Mlu* I site was present up to several kilobases upstream of the *RFC3* gene, a sequence closely resembling it (ACGCGAT) is located ≈110 nt upstream of the translational start site. The steady-state mRNA levels of *RFC3* during the yeast mitotic cell cycle were determined as described (19). Fig. 2 shows that the levels do not change significantly during the cell cycle. The expression pattern is virtually identical to that of the *ACT1* gene, but obviously different from that of *POL30* gene, which is a cell-cycle-regulated replication gene with a *Mlu* I element (19).

The *RFC3* Gene Is Required for Yeast Viability. A null mutant of *RFC3* was made *in vitro* as described in Fig. 3. The resulting plasmid pBL454 was digested with *Eco*RI and *Pvu* II and transformed into diploid yeast strain W303, generating the mutant diploid strain YXL1 (29). The disruption of one of the two copies of the *RFC3* gene was confirmed by genomic Southern blot analysis (data not shown). The strain was sporulated and tetrad analysis was carried out. Of the 11 tetrads dissected from the untransformed diploid strain W303, 9 produced four viable spores, 1 produced three viable spores, and 1 produced two viable spores. However, of the 16 tetrads from strain YXL1, 15 tetrads produced two viable spores and 1 produced one viable spore, and all of the viable spores were Ura$^-$. Therefore, disruption of the *RFC3* gene is lethal to yeast.

Expression of the *RFC3* Gene in *E. coli*. To obtain large quantities of Rfc3p for *in vitro* studies, we put the *RFC3* gene in plasmid vector pPY55, which contains the bacteriophage

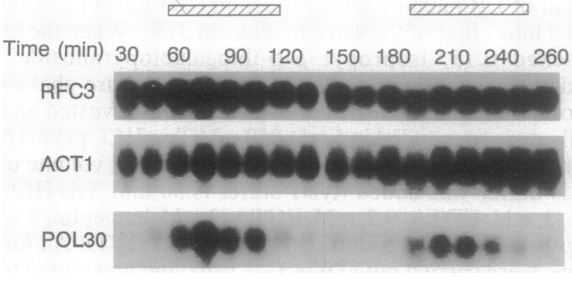

FIG. 2. Steady-state mRNA levels of *RFC3*, *ACT1*, and *POL30* during two mitotic cycles of synchronous growth of NCYC239 cells were determined by a Northern blot analysis as described (19). The hatched bars show the periods during which DNA synthesis occurs (19).

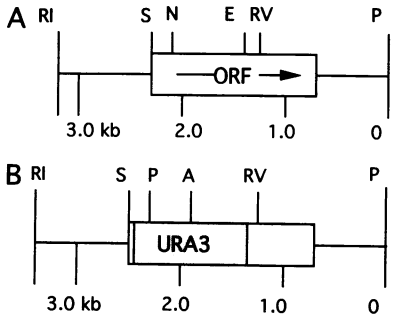

FIG. 3. Restriction maps of the *RFC3* gene region and of a deletion derivative containing an insertion of the *URA3* gene into the *Eco*NI and *Eco*RV sites of the *RFC3* gene. The orientation of the inserts in pUC19 is in a counterclockwise direction. (*A*) *Eco*RI–*Pst* I wild-type fragment in pBL452. (*B*) The disruption construction in pBL454. P, *Pst* I; RV, *Eco*RV; E, *Eco*NI; N, *Nco* I; S, *Sal* I; RI, *Eco*RI; A, *Apa* I.

T7 gene *10* promoter and leader sequence (22). Upon induction, Rfc3p was overexpressed to ≈5% of the total cell protein, but in an insoluble form, presumably in inclusion bodies (data not shown). A modification of the procedure of Claassen *et al.* (30) was used to purify Rfc3p to homogeneity in soluble form after urea extraction of the insoluble fraction. Analysis of the soluble Rfc3p by denaturing PAGE showed a 40-kD polypeptide band (Fig. 4*A*), which comigrated with the 40-kDa subunit of RF-C complex purified from yeast (ref. 5 and data not shown).

Polyclonal Antibodies Against Rfc3p Inhibit yRF-C Activity. Polyclonal antibodies against Rfc3p were raised in rabbit. A Western blot analysis indicated that the polyclonal anti-Rfc3p serum, but not the preimmune serum, specifically recognized the 40-kDa subunit in the various preparations of yRF-C tested (Fig. 4*B*).

Preincubation of RF-C with anti-Rfc3p serum greatly inhibited its activity in a subsequent replication assay, whereas the preimmune serum had no effect on RF-C activity (Fig. 5*A*). The inhibitory effect of the serum is specific for RF-C, since inhibition could be reversed by adding RF-C back to the replication reaction (Fig. 5*B*).

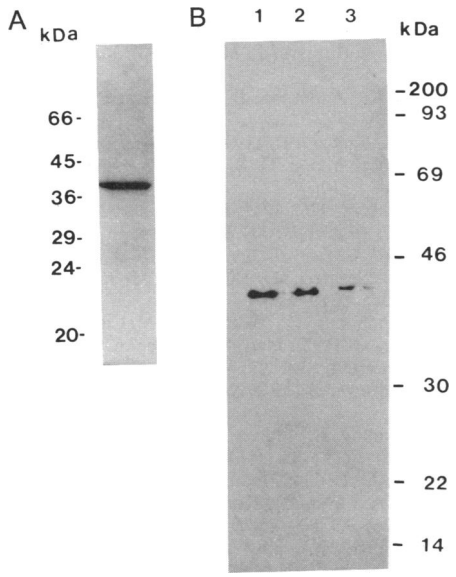

FIG. 4. SDS/PAGE and immunoblot analysis of Rfc3p isolated from *E. coli*. (*A*) Rfc3p (3 μg) was subjected to SDS/PAGE analysis on 12% gels and stained with Coomassie blue. (*B*) Western blot analysis of Rfc3p and of the yRF-C complex with the polyclonal serum against Rfc3p. Lanes 1 and 2 contain 100–200 ng of protein from two preparations of yRF-C, and lane 3 contains 14 ng of Rfc3p. Proteins were transferred to nitrocellulose membrane after SDS/PAGE and probed with antibodies against Rfc3p. Protein molecular mass markers are indicated.

Biochemical Characterization of Rfc3p. The ATPase activity of Rfc3p was determined with various nucleic acids cofactors (Table 1). Rfc3p has an ATPase activity that is markedly stimulated by single-stranded DNA but not by double-stranded DNA or RNA. Unlike the RF-C complex, PCNA has no effect on the ATPase activity of Rfc3p, whether or not the single-stranded mp18 DNA was primed. Coating of the single-stranded DNA with *E. coli* single-stranded DNA binding protein inhibited the ATPase activity.

Recently, Pan *et al.* (31) observed that the bacterially expressed 37- and 40-kDa polypeptides of RF-C (activator 1) interfered with the proper functioning of the native complex isolated from human cells. In particular, a strong inhibition of pol δ holoenzyme activity was observed in the presence of excess human 40-kDa subunit. Addition of 270 ng of Rfc3p (a 10- to 20-fold excess in comparison to the amount of 40-kDa subunit present in yRF-C) caused only a marginal (≈40%) inhibition of pol δ holoenzyme activity and no inhibition of pol ε holoenzyme activity (data not shown).

DISCUSSION

A rapidly growing body of evidence strongly suggests that the conserved functional properties of eukaryotic DNA replication proteins follow from a conservation both at the primary amino acid sequence level and at the subunit structure level

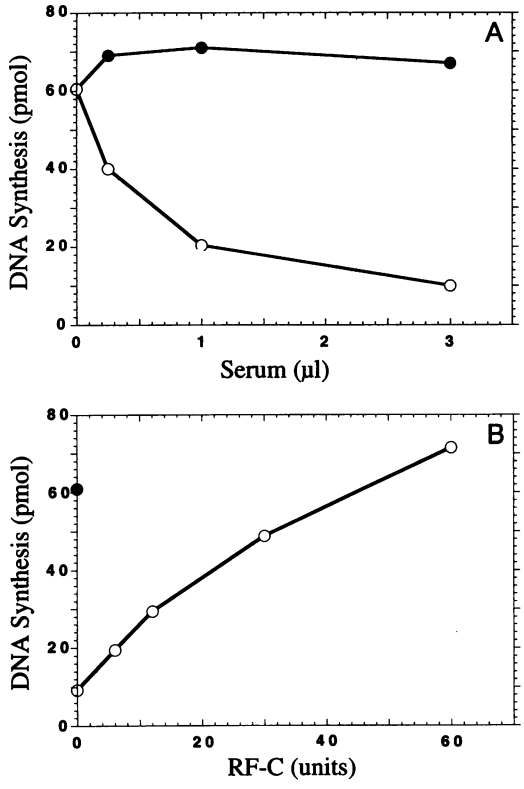

FIG. 5. Polyclonal antibodies against Rfc3p specifically inhibit yRF-C activity. (*A*) Effect of anti-Rfc3p polyclonal antibodies on pol δ holoenzyme activity. (*B*) Effect of adding additional RF-C to reactions inhibited by anti-Rfc3p polyclonal antibodies. After a preincubation of 60 units of RF-C with 3 μl of preimmune serum (●) or 3 μl of polyclonal serum against Rfc3p (○) for 60 min at 4°C, increasing amounts of RF-C were added together with the other components of the pol δ holoenzyme assay system and incubated for an additional 30 min at 37°C.

Table 1. ATPase activity of Rfc3p

Nucleic acid	PCNA	SSB	ADP formed, pmol
None	–	–	24
$(dA)_{16}$	–	–	78
Poly(dA)	–	–	138
Poly(dA)/oligo(dT)(20:1)	–	–	132
Poly(dA)/oligo(dT)(5:1)	–	–	128
$(dT)_{16}$	–	–	143
Poly(dT)	–	–	145
Poly(A)	–	–	29
M13mp18 RF	–	–	47
M13mp18 (+)	–	–	71
	700 ng	–	80
	700 ng	850 ng	38
	–	850 ng	38
Multiply primed ssmp18	–	–	110
	700 ng	–	110
	700 ng	850 ng	40
	–	850 ng	44

SSB, *E. coli* single-stranded DNA binding protein; RF, replicative form; ss, single stranded.

in organisms as far removed evolutionary as yeast and humans (32–35). For some components, this similarity even extends to prokaryotic systems (17, 36). Based on the sequence conservation of two of the small subunits of human RF-C (activator 1) and bacteriophage T4gp44 (16), we used PCR technology to clone the small subunits of yRF-C. Several lines of evidence strongly indicate that the *RFC3* gene product is identical to the 40-kDa subunit of yRF-C. (*i*) The deduced amino acid sequence of the *RFC3* gene reveals high homologies to the corresponding functional homologs in mammalian cells (Fig. 1). (*ii*) Rfc3p comigrates with the 40-kDa subunit of RF-C on a denaturing polyacrylamide gel. (*iii*) Polyclonal antibodies against Rfc3p specifically recognize the 40-kDa subunit in all preparations of purified yRF-C tested (Fig. 4*B*). (*iv*) The anti-Rfc3p serum specifically inhibits RF-C activity in an assay that measures DNA pol δ holoenzyme activity (Fig. 5).

The bacterially expressed Rfc3p exhibits DNA-dependent ATPase activity. Although this ATPase activity of Rfc3p is consistent with the observation that the 40-kDa subunit of yRF-C binds ATP, its specific activity is significantly lower than that of yRF-C (0.02 pmol of ATP hydrolyzed per min per ng of Rfc3p compared to 0.05 pmol per min per ng of yRF-C complex) (5, 18). This discrepancy can be attributed to incomplete renaturation of Rfc3p after its extraction from inclusion bodies by 6 M urea or to the possibility that some of the other subunits of RF-C possess ATPase activity. In addition, unlike that observed with the RF-C complex, the ATPase activity of Rfc3p on primed single-stranded DNA is not stimulated by PCNA (Table 1) (19). Since crosslinking studies with the mammalian RF-C have shown that the primer terminus recognition activity resides in the large subunit of RF-C (9), this lack of stimulation may be due to the inability of Rfc3p to recognize a primer terminus or to a lack of interaction between Rfc3p and PCNA. However, recent experiments with the overexpressed 37-kDa subunit of human RF-C (activator 1) have shown that this protein binds primed single-stranded DNA substrates preferentially (31).

The extensive similarity between the mammalian RF-C (activator 1) subunits suggests that there may be a redundancy in their function in DNA replication or that some of these subunits may have specialized functions—e.g., in DNA repair. From this, one would predict that some of the analogous yeast genes would not be essential for cell growth but rather that deletion mutants would show a phenotype consistent with a repair defect. At least with the *RFC3* gene, that is not the case as it is an essential gene. An analysis of the other *RFC* genes is required to determine whether there is such a functional redundancy in RF-C or whether each polypeptide has its own essential role in the process of DNA replication.

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