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Identification of the fifth subunit of *Saccharomyces cerevisiae* Replication Factor C

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ABSTRACT

Yeast replication factor C (RF-C) is a multipolypeptide complex required for chromosomal DNA replication. Previously this complex was known to consist of at least four subunits. We here report the identification of a fifth RF-C subunit from *Saccharomyces cerevisiae*, encoded by the *RFC5* (YBR0810) gene. This subunit exhibits highest homology to the 38 kDa subunit (38%) of human RF-C (activator 1). Like the other four *RFC* genes, the *RFC5* gene is essential for yeast viability, indicating an essential function for each subunit. *RFC5* mRNA is expressed at steady-state levels throughout the mitotic cell cycle. Upon overexpression in *Escherichia coli* Rfc5p has an apparent molecular mass of 41 kDa. Overproduction of RF-C activity in yeast is dependent on overexpression of the *RFC5* gene together with overexpression of the *RFC1–4* genes, indicating that the *RFC5* gene product forms an integral subunit of this replication factor.

INTRODUCTION

DNA replication in the eukaryotic nucleus may require the activities of three essential DNA polymerases, α, δ and ε (for recent reviews see 1,2). Additional replication factors are required for processive DNA synthesis at the replication fork. The proliferating cell nuclear antigen (PCNA) is the processivity factor for DNA polymerases δ and ε (3–6). PCNA is a homotrimer with a subunit molecular weight of 29 kDa and is highly conserved from yeast to mammalian cells. The crystal structure of yeast PCNA shows that the trimer forms a closed ring with the appropriate dimensions and electrostatic properties to encircle double-stranded DNA and to interact with it using non-specific contacts (7). Processivity in DNA synthesis is achieved by protein–protein interactions between PCNA and the polymerase, thereby tethering the DNA polymerase at the primer terminus (8).

Replication factor C (RF-C) is a multipolypeptide complex which loads PCNA onto the template–primer junction in an ATP-dependent manner (9–13). Human RF-C, also called activator 1, consists of a large subunit of 140 kDa and four smaller subunits of 36–41 kDa (14,15). All five known genes have been cloned using a combination of peptide sequence analysis and homology-based PCR (16–19). However, it is not yet known whether these five polypeptides are sufficient to reconstitute the human RF-C complex. Previously the complex from yeast (yRF-C) was known to contain at least four subunits of 100, 41, 40 and 37 kDa (6,13,20). The small subunits of yeast and human RF-C show high sequence similarity, both among themselves and between species. With the exception of the 38 kDa subunit, each human RF-C subunit appears to have a yeast homolog with which it shares particular homology.

In this paper we describe the identification of a fifth RF-C subunit from *Saccharomyces cerevisiae* encoded by the *RFC5* gene, which shows extensive sequence similarity to the 38 kDa subunit of human RF-C. This subunit is essential for viability of yeast. In addition, its overexpression, together with overexpression of the other four *RFC* genes, is necessary and sufficient for overproduction of RF-C in yeast. Therefore, it appears that all of the yeast *RFC* genes have now been identified.

MATERIALS AND METHODS

Strains

Escherichia coli strains used were DH5 and BL21(DE3)pLysS. Yeast strains used were FM113 (MATa, *ura3-52*, *trp1-289*, *leu2-3,112*, *prb1-1122*, *prc1-407*, *pep4-3*) (a gift from M.Johnston), prototrophic diploid NCYC239, PY2 (MATa/α, *leu2-3,112*, *ura3-52*, *trp1* D, *can1*) and its derivative PY61 (as PY2, but *rfc5*::hisG-*URA3*-hisG and containing complementing plasmid pBL605), W303 (MATa/MATa, *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 PY62 (as W303, but *RFC5/rfc5*::hisG-*URA3*-hisG). Strains PY61 and PY62 were created by integrative transformation with the disruption plasmid pBL607, which was previously digested with *Mun*I and partially with *Hin*dIII to yield a 5184 bp fragment (21). Disruption was confirmed by genomic Southern analysis. Except for the specific modifications noted, all yeast protocols and media were as described (22).

Plasmids

Complementing plasmid pBL605 was created by ligating a 1830 bp *Hin*dIII–*Sal*I fragment containing the *RFC5* gene (nt 3552–5382 of GenBank accession no. X78993) with a linker

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*Eco*RI site added to the *Hin*dIII site into the *Eco*RI–*Sal*I site of pRS314 (Bluescript, *TRP1*, *CEN6*, *ARSH4*). pBL606 was generated by ligating a 2076 bp *Hin*dIII–*Cla*I fragment (nt 3552–5628 of X78993) into the *Acc*I–*Hin*dIII site of pUC19. The 1830 bp *Hin*dIII–*Sal*I fragment of pBL606 was inserted into the *Hin*dIII–*Sal*I site of pRS316 (Bluescript, *URA3*, *CEN6*, *ARSH4*) to generate complementing plasmid pBL608. The disruption plasmid pBL607 was created by replacing the central region of *RFC5* (*Mlu*I–*Bgl*II, nt 4142–4811 of X78993) in plasmid pBL606 with the hisG-*URA3*-hisG cassette (21). pBL609 was generated from a PCR fragment containing the entire *RFC5* gene in which a *Bsp*HI site and a *Hin*dIII site were created at the 5′- and 3′-ends of the open reading frame respectively. These sites were generated using oligonucleotide primers which maintained the integrity of the amino acid sequence. The PCR product was digested with *Bsp*HI and *Hin*dIII and ligated into the *Nco*I–*Hin*dIII site of *E.coli* expression vector pPY55, containing the bacteriophage T7 gene10 promoter and leader sequence. The integrity of the insert was confirmed by DNA sequence analysis. Oligonucleotides were synthesized by DNagency. pBL417 contains the *RFC1*, *RFC2*, *RFC3* and *RFC4* genes, each one positioned under transcriptional control of the *GAL1-10* UAS, in a 2 μm based vector, and *URA3* as selectable marker (Impellizzeri,K.J. and Burgers,P.M., manuscript in preparation). pBL419 contains the *RFC5* gene inserted as a *Mse*I–*Sal*I fragment (nt 3985–5382 of X78993) into the *Cla*I (filled)–*Sal*I sites of pRS424-GAL (Bluescript, 2μm ori, *TRP1*, *GAL1-10*). All restriction enzymes were purchased from New England Biolabs.

Overproduction of Rfc5p in *E.coli*

A single colony of BL21(DE3)pLysS containing plasmid pBL609 was inoculated into 5 ml LB medium with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) and grown at 37°C. Once the OD_{595} had reached 0.4–0.6 isopropyl-β-D-galactopyranoside was added to a final concentration of 1 mM. Three hours after induction the cells were harvested, resuspended in 100 μl 50 mM Tris–HCl, pH 7.5, 10% sucrose and frozen at –70°C. Upon thawing an equal volume of 2× lysis buffer was added (100 mM Tris–HCl, pH 8.1, 4 mM EDTA, 0.4 mM EGTA, 2 μM leupeptin, 2 μM pepstatin A, 10 mM sodium bisulfite, 6 mM dithiothreitol). All further steps were carried out at 0–4°C. The samples were stored on ice for 30 min. Nonidet P-40 and phenylmethylsulfonyl fluoride were added to final concentrations of 0.05% and 1 mM respectively. Samples were stored on ice for 10 min with frequent inversion, after which samples were sonicated using a Branson Sonifier cell disrupter model 185 to decrease the viscosity. Following centrifugation for 20 min in a microfuge the supernatant was discarded and the precipitate washed twice with 100 μl wash buffer (lysis buffer containing Tris–HCl, pH 7.5) plus 2 M NaCl. The precipitate was homogenized with 100 μl denaturation buffer (wash buffer containing 400 mM NaCl and 6 M urea). The suspension was agitated for 1 h and then centrifuged for 20 min in a microfuge. The supernatant containing Rfc5p was analyzed.

Overproduction of RF-C in yeast

Strain FM113 containing the appropriate plasmids or control vectors was inoculated to a starting OD_{660} of 0.2–0.3 in 100 ml minimal complete medium containing 2% lactate, 3% glycerol and 0.1% glucose as carbon source. After overnight growth at 30°C 100 ml rich medium containing the same carbon source mixture was added and after 3 h at 30°C 4 g galactose were added and cell growth continued for another 3 h. All breakage and chromatography steps were carried out at 0–4°C. Cells were lysed with glass beads in buffer A (final concentrations 50 mM Tris–HCl, pH 7.8, 5% glycerol, 1 mM EDTA, 3 mM dithiothreitol, 2 μM pepstatin A, 2 μM leupeptin, 10 mM $NaHSO_3$, 0.5 M NaCl) as described (6). The cleared lysate was diluted with buffer B (50 mM Tris–HCl, pH 7.8, 10% glycerol, 1 mM EDTA, 3 mM dithiothreitol, 2 μM pepstatin A, 2 μM leupeptin, 10 mM $NaHSO_3$) to reduce the NaCl concentration to 0.2 M and gently shaken for 1 h with 0.5 ml Affigel Blue. The matrix was then loaded onto a column, washed with 2 ml buffer B plus 0.2 M NaCl, 2 ml buffer B plus 0.3 M NaCl and eluted with 1 ml buffer B plus 1 M NaCl. A Western blot analysis showed that all RF-C cross-reacting material was in the 1 M fraction. RF-C activity was measured in a DNA polymerase δ holoenzyme assay as described below.

MonoS FPLC

FM113 cells containing vector or plasmid pBL417 (*RFC1–RFC4*) were grown on a 2 l scale as described above, resulting in a yield of 15 g wet weight cells each. After cell breakage and chromatography over a 10 ml Affigel Blue column as described above, the 1 M NaCl eluate was dialyzed against buffer C (as buffer B, but 30 mM HEPES–NaOH, pH 7.4, 0.01% Nonidet P-40) until the conductivity had reached that of buffer B plus 100 mM NaCl. The enzyme fraction was loaded onto a 1 ml MonoS FPLC column (Pharmacia, Piscataway, NJ), washed with 2 ml buffer C plus 100 mM NaCl and eluted with a 15 ml linear gradient of 100–600 mM NaCl in buffer C.

DNA polymerase δ holoenzyme assay

The standard 30 μl reaction contained 40 mM Tris–HCl, pH 7.8, 8 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol, 100 μM each of dATP, dCTP and dGTP and 25 μM [^{3}H]dTTP (100 c.p.m./pmol dNTP), 50 mM NaCl, 0.5 mM ATP, 100 ng singly primed single-stranded mp18 DNA (0.04 pmol of circles), 850 ng *E.coli* SSB, 100 ng PCNA, 10 ng polymerase δ and RF-C or 2 μl MonoS fractions. Incubations were at 37°C for 7 min. The reactions were stopped by the addition of 100 μl 50 mM sodium pyrophosphate, 25 mM EDTA and 50 μg/ml calf thymus DNA as carrier and acid insoluble radioactivity determined (6).

ATPase activity

The standard 15 μl reaction contained 40 mM Tris–HCl, pH 7.8, 8 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol, 50 mM NaCl, 100 ng multiple primed single-stranded M13 DNA (~5 primers/circle), 850 ng *E.coli SSB* and 50 μM [α-^{32}P]ATP. The mixture was preheated to 37°C and the reaction started by the addition of 1 μl MonoS column fractions and, if present, 100 ng PCNA. After 10 min at 37°C the reaction was quenched with 5 μl 50 mM EDTA, 1% SDS and 25 mM each of ADP and ATP as markers for UV detection. The products were separated by polyethylene–cellulose TLC in 0.5 M LiCl/1 M HCOOH. Appropriate regions were excised and counted to determine the percentage of ADP formed.

RESULTS

Characterization of the *RFC5* sequence

The *YBR0810* gene (GenBank accession no. X78993) was identified as a result of the yeast genome sequencing project and its similarity to other *RFC* genes was noted (23). We renamed this gene *RFC5* as three other small subunit genes, *RFC2–RFC4*, and most likely also gene *RFC1* (*CDC44*), encoding the large subunit of RF-C, had already been isolated (18,24–27). The *RFC5* gene has an open reading frame of 354 codons, with the protein having a predicted molecular mass of 39.9 kDa. The amino acid sequence was found to be 38% identical to that of the human 38 kDa subunit and 19–22% identical to the other small subunits of yeast RF-C. The most striking sequence similarity is found in the N-terminal halves of these proteins. Figure 1A shows the amino acid sequence comparison of the N-terminal half of the *RFC5* gene with the corresponding regions of other yeast and the human *RFC* genes, as well as bacteriophage T4 gene 44. The latter protein is the functional analog of RF-C (see 28 for a review). Of the five consensus sequence motifs which emerge from a comparison of the 11 proteins scRFC5 and hRFC38 noticeably lack the A motif of ATP binding proteins (Fig. 1A, motif II). In addition, the RF-C box (motif III), which is virtually identical in the other small yeast and human subunits, is not very well conserved in scRFC5 and hRFC38. The same is the case for the DE(A/V)D element (motif IV), which is present in many DNA- or RNA-dependent ATPases. In contrast, the SRC motif (motif V) of unknown function is present in all small subunits. Some, but not all, of these motifs are also present in the large subunits scRFC1 and hRFC140. Remarkably, scRFC5 and hRFC38 share several regions of sequence similarity which are not found in the other RFC subunits (Fig. 1A). Figure 1B shows a phylogenetic tree of the small yeast and human *RFC* genes based on their complete amino acid sequences. Noticeable is the pairwise alignment between separate yeast and human genes, as well as the more distant relationship between the sc*RFC5*/h*RFC38* pair and the other *RFC* genes.

The *RFC5* gene is essential for yeast growth and is constitutively expressed in the cell cycle

A hemizygous *RFC5* null mutation was created in diploid W303 yeast by a one step transformation method using plasmid pBL607 with *URA3* as a selectable marker (see Materials and Methods). Single copy disruption of the diploid was confirmed by Southern analysis prior to sporulation. Of the 11 tetrads dissected eight yielded two viable spores and the remaining three yielded one viable spore. All of the spores were Ura$^-$, indicating that the *RFC5* gene is essential for viability. These results were complemented by similar experiments in a haploid strain. The disruption of *RFC5* on the chromosome in haploid strain PY2 was accomplished in the presence of a complementing centromere plasmid containing the wild-type *RFC5* gene (pBL605, *RFC5*, *TRP1*). With the starting strain PY2 loss of plasmid pBL605 was readily detected after growth on non-selective medium followed by replica plating. However, after disruption of the chromosomal copy of the *RFC5* gene no Trp$^-$ cells were detected after growth on non-selective medium, again demonstrating that *RFC5* is essential for viability of yeast.

Many replication genes are regulated in a cell cycle-dependent manner, with expression levels increasing during late G_1/early S phase. This type of regulation is dependent on the presence of a *Mlu*I cell cycle box (ACGCGT) (29). No such site is present up to 3 kb upstream of *RFC5*. A mitotic cell cycle blot was probed with a DNA fragment containing both the *RFC5* gene and part of the adjacently located *POL30* (PCNA) gene. Whereas *POL30* mRNA levels varied ~10-fold, as previously observed, no significant variation in *RFC5* mRNA levels was detected (data not shown) (29). Therefore, all small *RFC* genes are constitutively expressed during the yeast mitotic cell cycle (24–26).

Overexpression of Rfc5p in *E.coli*

The *RFC5* gene product (Rfc5p) was overproduced to ~2% of the total cell protein by placing its expression under control of the bacteriophage T7 gene 10 promotor in *E.coli* strain BL21(DE3). After cell lysis Rfc5p was found in the insoluble pellet. Upon SDS–PAGE on a denaturing polyacrylamide gel it migrated with an apparent molecular mass of 41 kDa (Fig. 2A). The *RFC3* gene product (Rfc3p) migrates essentially the same as Rfc5p (Fig. 2A) (25). Similarly, the *RFC2* gene product migrates on SDS–PAGE with an apparent molecular weight of 41 kDa (data not shown) (26). Because of the virtual superimposition of these three gene products it is impossible to ascertain unambiguously by SDS–PAGE whether any of these polypeptides is present in purified yeast RF-C. However, the overproduction studies in yeast presented below do indicate that Rfc5p is a *bona fide* subunit of RF-C.

A Western blot analysis revealed that Rfc5p cross-reacts with rabbit polyclonal antibodies generated against Rfc4p (Fig. 2B), but not with anti-Rfc3p antibodies (data not shown). With equal amounts of each protein loaded on the gel the anti-Rfc4p antibodies stain Rfc3p ~3- to 5-fold less well and Rfc5p ~10- to 20-fold less well than Rfc4p. Cross-reactivity of the anti-Rfc4p serum was also observed with Rfc2p (data not shown). These results indicate that the anti-Rfc4p serum contains antibodies elicited against a domain common to all four subunits, perhaps motif I or motif IV (Fig. 1A).

RF-C activity by overproduction in yeast

A plasmid, pBL417, has been constructed which places the *RFC1*, *RFC2*, *RFC3* and *RFC4* genes under control of the inducible *GAL1-10* promoter (Impellizzeri,K.J. and Burgers,P.M., manuscript in preparation; 31). This plasmid was introduced into a galactose-inducible and protease-deficient strain, FM113. A Western analysis showed that when grown on galactose these cells overproduced Rfc1p, Rfc2p, Rfc3p and Rfc4p ~10- to 20-fold. Extracts from cells grown in the presence of galactose were subjected to Affigel Blue chromatography to partially purify RF-C and permit accurate quantitation of RF-C activity (see Materials and Methods). No significant increase in RF-C activity was observed in the Affigel Blue eluate when *RFC1–RFC4* were overexpressed (see below). As a Western analysis showed that all RF-C subunits were quantitatively recovered in the 1 M Affigel Blue eluate, the lack of overproduction of RF-C must be attributed to the need for another factor, rather than dissociation of this overproduced complex during chromatography (data not shown). Upon further fractionation of the Affigel Blue eluate on MonoS FPLC the overproduced four subunit complex showed a different elution pattern from native RF-C, as shown by Western analysis (Fig. 3). All subunits

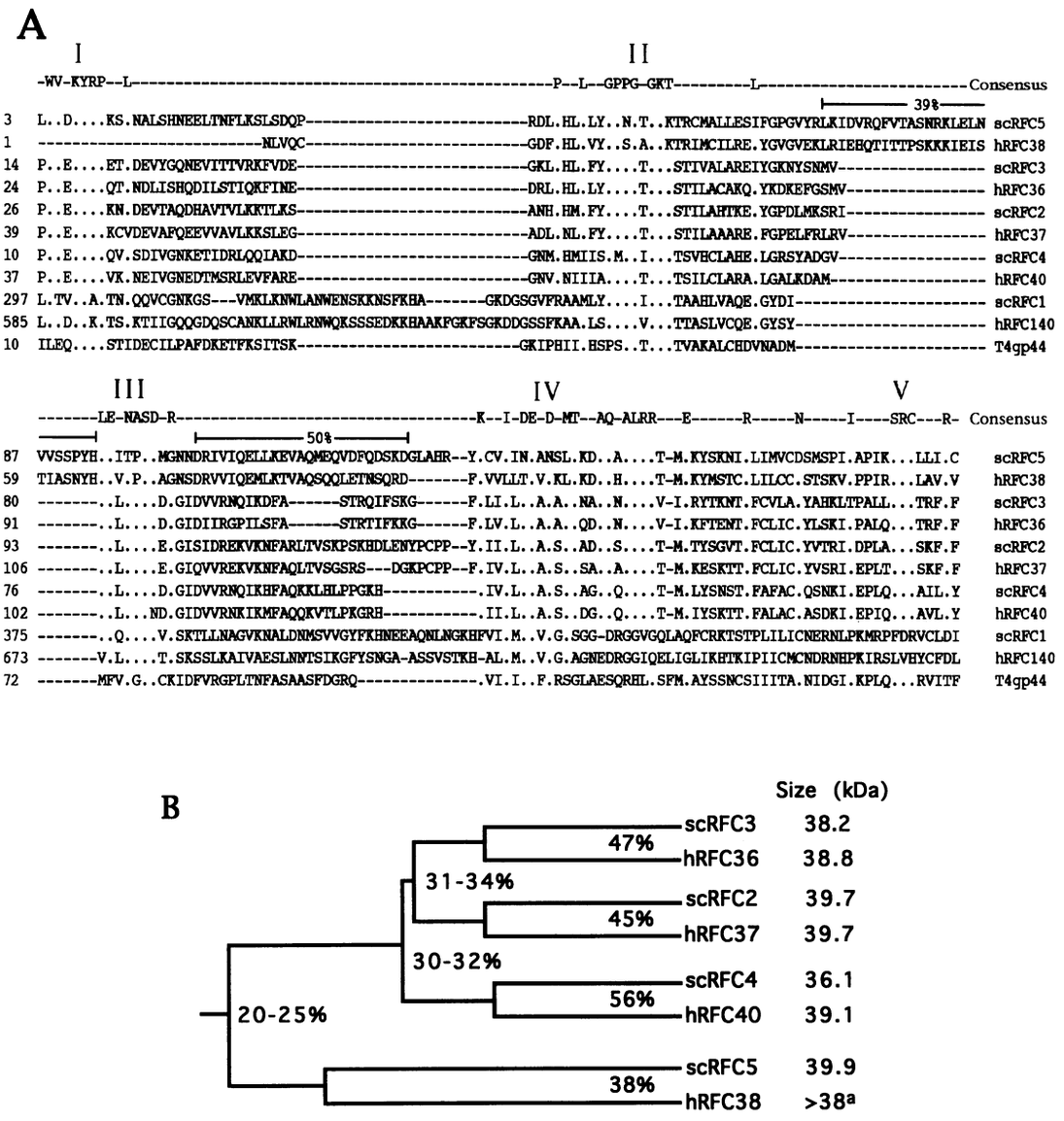

Figure 1. Comparison of the subunits of RF-C. (**A**) Alignment of amino acids 1–190 of yeast *RFC5* (scRFC5) with the corresponding sequences of the human (hRFC140, etc.) and yeast (scRFC1, etc.) genes and with bacteriophage T4 gene 44 protein. A consensus amino acid occurs in at least eight out of 11 sequences. Identity to the consensus in each individual sequence is indicated by a period. Gaps in the sequences are indicated by a dash. The two motifs unique to scRFC5 and hRFC38 are indicated by bars above the scRFC5 sequence. The program CLUSTAL V was used to generate the alignment (32). (**B**) Phylogenetic tree of the human and yeast small RF-C subunits. Sequence similarities in percent between pairs and groups are shown. [a]The entire sequence of hRFC38 is not known. Based upon comparison with scRFC5 an estimated 25 amino acids at the N-terminus are missing.

of this four subunit complex co-purified on the MonoS column. This complex was completely inactive for RF-C activity in the DNA polymerase δ holoenzyme assay (Fig. 3B). On the other hand, DNA-dependent ATPase activity was increased substantially in fractions 14–17, containing the overproduced four subunit complex, indicating that at least one activity of RF-C was present (compare Fig. 3A with B). However, this ATPase activity was not stimulated by PCNA, whereas the ATPase activity of native RF-C in fractions 22–23 was stimulated ~3-fold upon addition of PCNA (data not shown). All of these results show that *RFC1–RFC4* form a complex which, because of the lack of one or more subunits, fails to load PCNA onto the primer terminus.

The missing factor proved to be Rfc5p, as an ~6-fold overproduction of RF-C activity resulted when cells contained plasmid pBL417 as well as plasmid pBL419, which places *RFC5* under control of the *GAL1* promoter (Fig. 4). No overproduction resulted with plasmid pBL419 alone.

DISCUSSION

Several lines of evidence indicate that *RFC5* encodes the fifth subunit of yeast RF-C. (i) *RFC5* exhibits sequence similarity with the small subunits of yeast and human RF-C, having 38% identity with the human 38 kDa subunit. From the phylogenetic tree it is

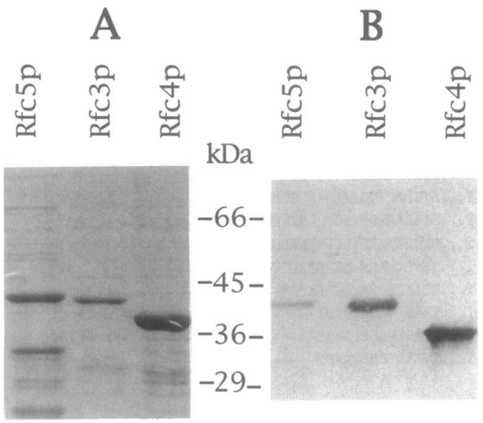

Figure 2. SDS–PAGE and immunoblot analysis of Rfc5p. (**A**) The urea extracts of cell pellets of strains overproducing Rfc5p, Rfc3p and Rfc4p were subjected to 10% SDS–PAGE and the proteins visualized by Coomassie blue staining. (**B**) Rfc5p, Rfc3p and Rfc4p (50 ng each) were subjected to 10% SDS–PAGE and proteins detected with polyclonal antibodies to Rfc4p. Molecular weight markers are indicated.

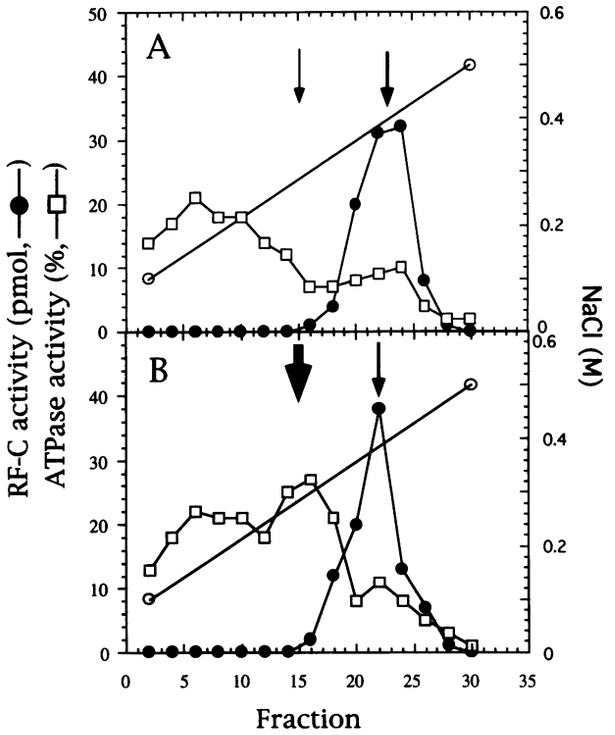

Figure 3. MonoS chromatography of a four subunit subcomplex of RF-C. (**A**) Strain FM113 (vector pRS424); (**B**) strain FM113 (pBL417). Column fractions were analyzed for RF-C activity in a DNA polymerase δ holoenzyme assay and for DNA-dependent ATPase activity (see Materials and Methods for details). The arrows point to elution positions of different RF-C complexes as determined by Western analysis. Arrow thickness is proportional to Western signal obtained. Note that in the wild-type strain (no overproduction) two species of RF-C are observed, only one of which is active.

obvious that each human subunit has a yeast counterpart (Fig. 1B). In the light of the overall conservation between

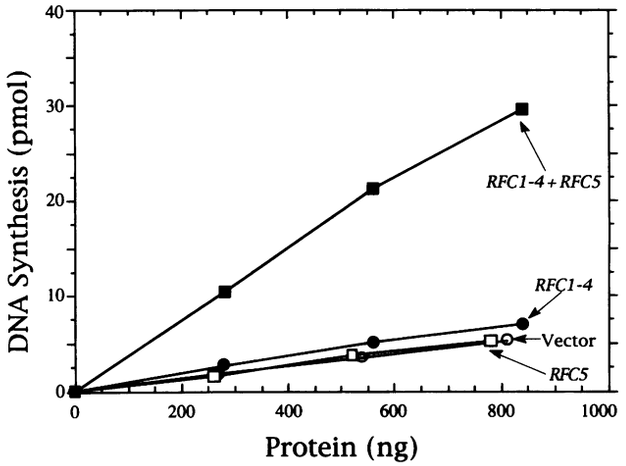

Figure 4. Overproduction of RF-C requires *RFC5*. Strain FM113 contained two plasmids, either the appropriate *URA3* plus *TRP1* vectors (vector) or pBL417 plus the *TRP1* vector (*RFC1–4*) or pBL419 plus the *URA3* vector (*RFC5*) or pBL417 plus pBL419 (*RFC1–4* + *RFC5*). Extracts were partially purified through the Affigel Blue step and assayed for RF-C activity in a DNA polymerase δ holoenzyme assay (see Materials and Methods for details).

replication proteins in the yeast and human systems, the existence of a fifth yeast subunit does not come as a surprise. (ii) Like the other *RFC* genes, *RFC5* is essential for yeast viability. (iii) When *RFC5* is present on a plasmid in conjunction with another plasmid carrying the *RFC1*, *RFC2*, *RFC3* and *RFC4* genes a 6-fold increase in RF-C activity is observed. This demonstrates that *RFC5* is a necessary component of RF-C. Overproduction of RF-C activity *per se* does not adversely affect yeast growth, as cells containing control plasmids and cells containing plasmids which overproduce part or all of RF-C grow equally well on the inducing carbon source galactose.

Preparations of purified RF-C had been reported to consist of a large subunit and only three small subunits (6,13,20). Considering that Rfc2p, Rfc3p and Rfc5p have virtually the same electrophoretic mobility, it is easy to see how this misassignment arose. Is it possible that there are additional small subunits of RF-C with similar molecular weights which remain undetected? The overproduction studies make this possibility extremely unlikely. Overexpression of only five *RFC* genes is required to overproduce RF-C activity. Considering that all of these subunits are expressed to a similar level, it is also unlikely that a putative sixth subunit would be present in the cell at very high levels, thereby obviating the need for its overexpression in order to overproduce RF-C.

With all five *RFC* genes identified and the method available to produce RF-C in large quantities the means to study this complex factor in detail should now be accessible. Specific functions, structural or otherwise, could be attributed to each of the subunits. Interactions between various RF-C subunits, either individually or in combination, and other replication factors can be studied by a combination of biochemical and genetic methods.

While this manuscript was under review a paper by Stillman and co-workers reported cloning of the *RFC5* gene based upon peptide sequence analysis of purified RF-C (33). Their data on the essential function of *RFC5* are completely in accord with our results.

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