

Overproduction in *Escherichia coli* and Characterization of Yeast Replication Factor C Lacking the Ligase Homology Domain*

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Eukaryotic replication factor C (RF-C) is a heteropentameric complex that is required to load the replication clamp proliferating cell nuclear antigen onto primed DNA. *Saccharomyces cerevisiae* RF-C is encoded by the genes *RFC1–RFC5*. The *RFC1* gene was cloned under control of the strong inducible bacteriophage T7 promoter, yet induction did not yield detectable Rfc1p. However, a truncated form of *RFC1* deleted for the coding region for amino acids 3–273, *rfc1-ΔN*, did allow overproduction. The other four *RFC* genes were cloned into the latter plasmid to yield a single plasmid that overproduced RF-C to moderate levels. Overproduction of the complex was further enhanced when the *Escherichia coli* *argU* gene encoding the rare arginine tRNA was also overproduced. The enzyme thus produced in *E. coli* was purified to homogeneity through three column steps, including a proliferating cell nuclear antigen affinity column. This enzyme, as well as the enzyme purified from yeast, is prone to aggregation and inactivation, and therefore, light scattering was used to determine conditions stabilizing the enzyme and preventing aggregation. Broad-range carrier ampholytes at about 0.05% were found to be most effective. In some assays, the Rfc1-ΔN containing RF-C from *E. coli* showed an increased activity compared with the full-length enzyme from yeast, likely because the latter enzyme exhibits significant nonspecific binding to single-stranded DNA. Replacement of *RFC1* by *rfc1-ΔN* in yeast shows essentially no phenotype with regard to DNA replication, damage susceptibility, telomere length maintenance, and intrachromosomal recombination.

DNA replication in eukaryotes is a complex process involving a large number of proteins. In yeast, processive DNA synthesis is performed by DNA polymerase δ (Pol δ)¹ and DNA polymerase ϵ . Two accessory factors are also required for processivity, the proliferating cell nuclear antigen (PCNA) and replication

factor C (RF-C) (1, 2). Yeast PCNA is a ring-shaped homotrimer with a monomer mass of 29 kDa (3). It functions by encircling the DNA and interacting with Pol δ or Pol ϵ to maintain highly processive DNA replication (4–7). PCNA also interacts with several other replication and repair proteins, including the replication inhibitor p21, the flap-specific endonuclease FEN-1, and DNA ligase 1 (reviewed in Ref. 8).

RF-C is a multisubunit complex that binds preferentially to the 3'-end of template-primer junctions and is essential for loading PCNA onto DNA (9). RF-C has an associated single-stranded DNA-dependent ATPase activity that is stimulated by the presence of primer termini and PCNA (9–11). The mechanism by which RF-C loads PCNA onto DNA is not well understood. RF-C may recognize and bind to template-primer junctions and subsequently load PCNA in an ATP-dependent manner (12). Alternatively, RF-C may form an ATP-dependent complex with PCNA, open the trimeric ring, and, upon binding a template-primer junction, close the ring around the DNA with hydrolysis of ATP (13).

Yeast RF-C consists of a large subunit with a molecular mass of 95 kDa and four smaller subunits of 36–40 kDa (10, 11). The genes encoding all five subunits have been cloned, and all are essential (14–19). All five subunits show sequence similarity to each other, and in fact, the sequences of all Rfc subunits are conserved among eukarya. This homology is localized in seven regions known as RF-C boxes II–VIII (Fig. 1A) (reviewed in Ref. 18). RF-C boxes III and V contain sequences that show homology to nucleotide-binding proteins (20). *RFC1* contains an additional box (I) in the N-terminal region that shows homology to prokaryotic DNA ligases and poly(ADP)-ribose polymerases (21). The role of the other RF-C boxes is unknown. The C termini of all five subunits are unique and are required for complex formation (22, 23).

Deletion studies with human Rfc1 (p140) have identified at least two DNA-binding domains, one in an N-terminal domain of the protein (amino acids 369–480, analogous to *Saccharomyces cerevisiae* Rfc1p amino acids 150–230) that contains the ligase homology box I and one broadly mapped to the C-terminal half of the protein between homology box IV and the C terminus (Fig. 1A) (23, 24). Although binding to double-stranded DNA by the C-terminal domain is much weaker than that by the N-terminal domain, only the C-terminal activity is required for RF-C function *in vitro*. In fact, deletion of the N-terminal domain (amino acids 1–555, approximately analogous to *S. cerevisiae* amino acids 1–275) results in a human RF-C preparation with increased replication activity, indicating an inhibitory contribution of the N-terminal domain, perhaps by nonspecific binding to non-template-primer junctions (23, 25).

The role of the N-terminal domain remains unclear. Substrate specificity binding studies with human Rfc1(1–555) show that this domain preferentially binds to partially double-

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¹ The abbreviations used are: SS, single-stranded; SSB, single-stranded binding protein; Pol δ , DNA polymerase δ ; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C; RF-C-1ΔN, RF-C with the *rfc1-ΔN* subunit; dAMP-PNP, 2'-deoxyadenyl-5'-yl imidodiphosphate; MMS, methylmethane sulfonate; kb, kilobase pair; PCR, polymerase chain reaction; TEMED, *N,N,N',N'*-tetramethylethylenediamine; dAMP-PNP, 2'-deoxyadenyl-5'-yl-imidodiphosphate; ATP γ S, adenosine 5'-O-(thiotriphosphate).

stranded DNA substrates in which at least one of the 5'-ends is phosphorylated and the phosphate is either recessed or at a blunt end, suggesting functional significance of this domain in Okazaki fragment maturation or at DNA ends (26). Human Rfc1 shows a significant binding preference to 5'-phosphorylated double-stranded human telomeric repeat DNA, and a deletion analysis implicates homology domain I in this activity, again suggesting the possible importance of this domain for DNA end metabolism (27).

In this paper, we describe the overexpression in *Escherichia coli* of yeast RF-C lacking the ligase homology domain of Rfc1p. Optimization of the overproduction conditions allowed us to obtain, after purification, 1 mg of pure RF-C from 1 liter of cells, as well as 5 mg of the four-subunit Rfc2-5p subcomplex lacking the large subunit. In addition, we have analyzed the phenotype of a yeast mutant carrying the truncated *RFC1* gene as the only source of *RFC1*. Our *in vitro* data are in agreement with those obtained by others for human RF-C, but, surprisingly, no significant DNA metabolic defect could be ascribed to the lack of the ligase homology domain *in vivo*.

EXPERIMENTAL PROCEDURES

Enzymes and DNA—Wild-type RF-C and Pol δ were purified from yeast overproduction strains, and PCNA and replication protein A were purified from *E. coli* overproduction strains as described (13, 28, 29). *E. coli* SSB was a gift from Dr. T. Lohman of this department. All other enzymes and oligonucleotides were obtained commercially. Single-stranded mp18 DNA was singly primed with a synthetic 36-mer (position 6330–6294). Plasmid pSBETa (pA15-ori, kanR, ArgU) was a gift from Dr. Hans-Henning Steinbiss (30). dAMP-PNP was a gift from Dr. Bruce Alberts.

Plasmids—The proper sequences of all plasmids obtained by ligation of PCR products or synthetic oligonucleotides were confirmed by DNA sequence analysis. Plasmid pBL472 (colE1-ori, Amp^R, T7-*RFC2*, T7-*RFC3*, T7-*RFC4*, T7-*RFC5*) was assembled from four plasmids (pBL456, pBL555, pBL565, and pBL609), each of which has a small *RFC* gene cloned into expression vector pPY55 (colE1-ori, Amp^R, T7 promoter-leader sequence cassette) in such a way that the native amino acid sequence was maintained and the methionine was optimally located behind a cassette containing a tandem repeat of the bacteriophage T7 gene 10 promoter followed by a single copy of the gene 10 ribosome binding site and leader sequence. All four *RFC* genes in pBL472 are arranged in a counterclockwise orientation and have retained the T7 expression cassette. pBL456 (pPY55, T7-*RFC3*), pBL555 (pPY55, T7-*RFC4*), and pBL609 (pPY55, T7-*RFC5*) have been described (14, 15, 19). The *RFC2* gene was amplified with primers RFC2-1 (CCTTGACATGTTTGAAGGGTTTGGTCC) and RFC2-2 (GGGAAGCTTG-TATATTAGAGTTGGGATATT), which introduce an *Afl*III site at the initiating methionine and a *Hind*III site at the stop codon, respectively. The PCR product was digested with *Afl*III and *Hind*III and ligated into vector pPY55, in which the single *Afl*III site in a nonessential region had previously been destroyed by filling in and digested with *Nco*I and *Hind*III to give pBL565 (pPY55, T7-*RFC2*).

A 0.38-kb fragment of the *RFC1* gene was amplified with primers RFC1-1 (GAGACCATGGTCAATATTCTGATTTTC), which introduces a *Nco*I site at the initiating methionine, and RFC1-2 (GAACACCTGTGAAGACAATTGTTAG) just past an internal *Bam*HI site in the *RFC1* gene. The PCR product was made blunt-ended with DNA polymerase I, Klenow fragment plus dNTPs, digested with *Nco*I, and ligated into vector pPY55 that had been digested with *Cla*I, filled in with DNA polymerase I, Klenow fragment plus dNTPs and then digested with *Nco*I. The resulting plasmid was cut with *Aat*II, filled in with DNA polymerase I, Klenow fragment plus dNTPs, and then digested with *Bam*HI, and the remaining portion of the *RFC1* gene was ligated in as a *Bam*HI to filled-in *Xho*I fragment obtained from plasmid pBL411 to give plasmid pBL614 (pPY55 colE1-ori, Amp^R, T7-*RFC 1*) (13). To obtain an expression plasmid with a truncated *RFC1* gene, pBL614 was digested with *Nco*I and *Hind*III, and the linear fragment was isolated. A double-stranded oligonucleotide made by hybridization of oligonucleotides CATGGTGCATCATCACCATCACCACCATA and AGCTTATGGTGGTGGTGGTGGTGCAC was ligated into the linearized vector. The resulting plasmid, pBL480 (pPY55 colE1-ori, Amp^R, T7-*RFC1*- Δ N) contains a deletion of residues 3–273 of *RFC1* and an addition of seven histidines between amino acids 2 and 274.

Plasmid pBL472 was digested with *Hind*III and *Pvu*II and treated with DNA polymerase I, Klenow fragment plus dNTPs, and the resulting fragment containing the four small *RFC* genes was ligated into plasmid pBL480 digested with *Sma*I. The resulting plasmid, pBL481, contains all five *RFC* genes in a counterclockwise orientation, each under the control of the T7 cassette (Fig. 1B).

Plasmids pBL641 (pRS316, *RFC1*, *URA3*) and pBL642 (pRS314, *RFC1*, *TRP1*) are complementing centromere plasmids containing the entire *RFC1* sequence (31). Plasmid pBL642-2 (pRS314, *RFC1*, *TRP1*) was created using site directed mutagenesis in order to introduce a *Nco*I restriction site at the N terminus of *RFC1*. This mutation did not alter the amino acid sequence of the *RFC1* gene. Plasmid pBL642-3 (pRS314, *RFC1*- Δ N, *TRP1*) was constructed by replacing the 1.5-kb *Nco*I-*Bgl*II *RFC1* fragment in pBL642-2 with the 0.8-kb *Nco*I-*Bgl*II truncated *RFC1* fragment from plasmid pBL480. This swap deletes residues 3–273 and adds seven histidine residues between amino acids 2 and 274.

Strains and Genetics—The *E. coli* strain used for general cloning purposes was DH5 α , whereas BL21(DE3) was used for overproduction studies. Diploid yeast strain W303 (*MAT α /MAT α ade2-1/ade2-1 ura3-1/ura3-1 his3-11/his3-11 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100*) was used for the genetic studies with *RFC1*. The entire *RFC1* gene was disrupted by transformation of strain W303 with a PCR-generated fragment spanning from ~500 nucleotides upstream to ~500 nucleotides downstream of the *RFC1* gene and in which the entire *RFC1* coding sequence was replaced by the KanMX6 resistance marker (32). G418 resistant transformants were examined by PCR to verify disruption of the *RFC1* gene in one of the two *RFC1* alleles. The G418 resistant cells were transformed with plasmid pBL641 (pRS316, *RFC1*, *URA3*) and sporulated. Haploid progeny carrying the disruption of the *RFC1* gene and complementing plasmid pBL641 were identified by standard genetic techniques and PCR analysis. Strain PY171 (*MAT α rfc1- Δ :KanMX6 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 + pBL641 (pRS316, *RFC1*, *URA3*)*) was transformed with the pBL642 series of plasmids, and transformants that had lost pBL641 were identified by growth on 5-fluoroorotic acid medium to give strain PY173-X (*MAT α rfc1- Δ :KanMX6 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 + pBL642-x* (pRS314, *rfc1-x*, *TRP1*)).

Standard media were used (33). YPDA is YPD plus 20 μ g/ml of adenine. DNA damage sensitivity measurements were carried out on YPD solid media to which was added, after autoclaving, 0.05% of methylmethane sulfonate (MMS) or 110 mM hydroxyurea.

Strains PY173 and PY173-X were transformed with *Hpa*I-cut pRS305-*URA3* (Bluescript *LEU2 URA3*) to target integration in the *LEU2* locus (34). To determine *URA3* pop-out frequencies, a fluctuation analysis was carried. Two transformants of each strain were grown to saturation in complete minimal SC medium, diluted in SC to 100 cells/ml, divided into 10 cultures for each transformant, and grown for 3 days to saturation. 4×10^6 cells were plated on SC medium plus 5-fluoroorotic acid to score for *URA3* pop-outs. Median values were obtained and averaged over the two independent transformants of each strain. Frequencies were corrected for residual growth on the selection plates.

Telomere Length Determination—The telomere analysis was performed essentially as described (35). Colonies from two successive 5-fluoroorotic acid selection plates, as described above, were diluted in YPDA medium and grown to saturation at 30 °C. This corresponds to approximately 30 generations of cell growth of the *RFC1* mutants. Successive 1000-fold dilutions in YPDA and growth to saturation corresponded to additional steps of 10 generations each. Genomic DNA was isolated from cultures after 30, 70, and 120 generations of growth, digested with *Xho*I, and subjected to Southern blot analysis. The probe was a *Xho*I-*Sa*I fragment from plasmid pJH345 (a gift of Michael McAlear), which contains a telomere-associated Y' sequence and ~300 nucleotides of poly(G₁₋₃T) sequences.

Buffers—HEG buffer contains 30 mM HEPES-NaOH (pH 7.5), 1 mM dithiothreitol, 0.5 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 5 μ M pepstatin A, 5 μ M leupeptin, and 0.5 mM *p*-methylphenylsulfonfyl fluoride. Where indicated, the buffer was supplemented with 7 mM magnesium acetate and 1 mM ATP. Added NaCl is indicated in mM with a subscript, e.g. buffer HEG₂₀₀ contains 200 mM NaCl. Ampholytes 3.5–9 (0.05%) are added to all steps after the PCNA-agarose column, and *p*-methylphenylsulfonfyl fluoride was omitted from the MonoS step.

Overexpression of RF-C in *E. coli*—Plasmid pBL481 was transformed into BL21(DE3) cells and selected on LB + Amp plates. If the strain also contained pSBETa, selection was on LB + Amp + Kan plates. A few colonies were used to inoculate a 50-ml culture of "terrific" broth (containing, per liter, 12 g of tryptone, 24 g of yeast extract, 0.4% glycerol, and

50 mM potassium phosphate, pH 7.2, and required antibiotics at 50 μ g/ml). Standard media were used for yeast genetics and grown for 8–10 h at 24 °C with vigorous shaking (300 rpm). A 5–10-ml inoculum was used to inoculate 1 liter of terrific broth ($A_{600} = 0.01$), and the flask was incubated overnight at 24 °C with vigorous shaking (300 rpm) to $A_{600} = 2$. The cells were then induced with 0.4 mM isopropyl β -D-thiogalactopyranoside, and shaking was continued for an additional 4–6 h. The cells were harvested and resuspended in 10 ml of HEG₂₀₀ buffer and stored frozen at –70 °C.

Purification of RF-C and the Four-subunit Rfc2–5p Complex—All steps were carried out at 0–4 °C unless indicated otherwise. The thawed cells were lysed by sonication, polymin P was added to 0.5% to precipitate nucleic acids, and after stirring for 5 min, cell debris and the precipitate were removed by centrifugation at 18,000 rpm for 30 min. Ammonium sulfate (0.28 g/ml) was added to the supernatant, and after 30 min, the precipitated proteins were collected at 38,000 rpm for 60 min and resuspended in 10 ml of HEG buffer. The fraction was then dialyzed against HEG buffer to a conductance of HEG₁₀₀ and loaded onto a 10-ml S-Sepharose column equilibrated in HEG₁₀₀. The column was washed subsequently with 30 ml of HEG₂₀₀, and eluted with a 75-ml linear gradient of HEG₂₀₀–HEG₆₀₀, followed by a 10-ml wash with HEG₁₀₀₀. The four-subunit Rfc2–5p complex elutes at ~HEG₂₅₀, and RF-C-1 Δ N elutes at ~HEG₄₀₀. Further purification was only carried out with complexes overproduced in pSBETA-containing cells.

ATP and magnesium acetate were added to RF-C containing fractions to final concentrations of 1 and 7 mM, respectively, and the fraction was diluted with HEG buffer to a conductance of HEG₂₀₀ and loaded onto a 15-ml PCNA-agarose column equilibrated in buffer HEG₂₀₀. The column was washed with 30 ml of buffer HEG₃₀₀ containing magnesium acetate-ATP, and the bound proteins were eluted with HEG₃₀₀ buffer containing 3 mM EDTA. To fractions containing RF-C-1 Δ N, ampholytes 3.5–9 were added to a final concentration of 0.05%.

The RF-C-1 Δ N containing fractions were diluted with HEG buffer containing 0.05% ampholytes to a conductance of HEG₁₅₀ and injected onto a 1-ml Mono S column (Amersham Pharmacia Biotech) equilibrated in HEG₁₅₀, washed with 5 ml of HEG₁₅₀ buffer, and eluted with a 10-ml linear gradient from 150 to 600 mM NaCl in HEG buffer containing 0.05% ampholytes. RF-C-1 Δ N eluted at ~350 mM NaCl. The yield of RF-C was ~0.8 mg/liter of culture.

The Rfc2–5 complex from the S-Sepharose column was dialyzed against HEG buffer to a conductivity of HEG₁₀₀ and loaded onto a 1-ml MonoQ column. The column was washed with 3 ml of HEG₁₀₀ and eluted by a 10-ml gradient of HEG₂₀₀–HEG₅₀₀. Rfc2–5p eluted at ~150 mM NaCl. The RF-C subcomplex containing fractions were diluted 2-fold with HEG buffer and loaded onto a 1-ml MonoS column. The column was washed with 3 ml of HEG₁₀₀ and eluted by a 10-ml gradient of HEG₁₀₀–HEG₅₀₀. The Rfc2–5 complex eluted at ~250 mM NaCl. The yield of Rfc2–5 was ~5 mg/liter of culture.

SDS-Polyacrylamide Electrophoresis—A 16 cm \times 20 cm \times 0.75 mm, 7.5% acrylamide, 0.25% bisacrylamide, 0.1% SDS gel was cast containing 0.16% *N,N,N',N'*-tetramethylethylenediamine (TEMED). The gel was run in the cold room with prechilled buffer at 25 mA constant current until the dye front had progressed $\frac{2}{3}$ of the way through the stacking gel. The current was then increased to 50 mA until the dye front had entered the resolving gel by 1–2 cm. At this time, the gel was run at a constant voltage of 260–300 V.

Replication Assays—Standard 30- μ l replication assays contained 40 mM Tris-HCl, pH 7.8; 8 mM magnesium acetate; 50 mM NaCl; 0.2 mg/ml bovine serum albumin; 1 mM dithiothreitol; 100 μ M each of dATP, dCTP, and dGTP; 25 μ M [³H]dTTP (100 cpm/pmol dNTP); 1 mM ATP; 100 ng of singly primed single-stranded mp18 DNA (0.04 pmol of circles); 0.85 μ g of *E. coli* SSB or 0.6 μ g of yeast replication protein A; 100 ng of PCNA (1.1 pmol of trimers); 25 ng of Pol δ (0.05 pmol); and RF-C as indicated. Incubations were at 37 °C for 4 min, and acid-precipitable radioactivity was determined. In some assays dNTP-PNPs replaced dNTPs, meaning that 100 μ M dAMP-PNP replaced dATP in an otherwise identical assay. [α -³²P]dTTP (1000 cpm/pmol nucleotide) replaced [³H]dTTP when products were analyzed by alkaline agarose gel electrophoresis. After electrophoresis, the gel was neutralized, dried, and exposed to film for autoradiography or exposed to a phosphor screen for phosphor imaging and quantitation.

Replication of poly(dA) with an average chain length of 300 nucleotides was carried out in a 30- μ l assay in the same buffer system containing 200 ng of poly(dA)-(dT)₂₂ (40:1 nucleotide ratio); 1.7 pmol of template molecules and 1.7 pmol of primers) with 40 μ M [³H]dTTP, 1 mM ATP, 50 mM NaCl, 1.3 μ g of SSB, 2.2 pmol of PCNA, 0.1 pmol of Pol δ , and RF-C as indicated. Incubations were at 30 °C for 8 min, and the reactions were processed as described above.

Biogel A-5m Filtration of Complexes—Complexes were formed in a 60- μ l reaction containing 30 mM Tris-HCl, pH 7.8, 8 mM MgAc₂, 50 mM NaCl, 100 μ g/ml bovine serum albumin, 1 mM dithiothreitol, 1.65 μ g of singly primed SS mp18 DNA (0.7 pmol of circles), 14 μ g of *E. coli* SSB, 0.5 mM ATP, 100 μ M each dCTP and dGTP, 10 pmol of PCNA, 2 pmol of RF-C or RF-C-1 Δ N, and 1 pmol of Pol δ . The SSB-coated DNA was incubated with PCNA and RF-C or RF-C-1 Δ N in the assay mix for 30 s at 30 °C, Pol δ was added, and the reaction was incubated further for 30 s. The reaction was then cooled on ice and filtered through a 2-ml Biogel A-5m (Bio-Rad) column, equilibrated in 30 mM Tris-HCl, pH 7.5, 5% glycerol, 0.1 mM EDTA, 8 mM MgCl₂, 2 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 50 mM NaCl, and 25 μ M dCTP and dGTP. Three drop fractions were collected. DNA predominantly eluted in fractions 8–10, free protein in fractions 15–22, and free nucleotides in fractions 25–30.

Replication Activity of Isolated Complexes—The void volume fractions (fractions 8–10) from the Biogel column were pooled, and 40 μ l of complex, corresponding to approximately 0.1 pmol of DNA complexes, were assayed in a 65- μ l reaction containing 30 mM Tris-HCl, pH 7.8; 8 mM magnesium acetate; 100 μ g/ml bovine serum albumin; 100 μ M each of dCTP, dGTP, and dATP; and 12.5 μ M [α -³²P]dTTP. The reaction was incubated at 14 °C for 1 min, at which time 1.5 pmol of RF-C or RF-C-1 Δ N was added to the reaction or RF-C storage buffer was added in a control assay. At various time intervals, 20- μ l aliquots were removed into 5 μ l of 50 mM EDTA, 50% glycerol, and 2% SDS. The products were separated on a 1% alkaline agarose gel as described above.

PCNA Loading and Unloading Assays—PCNA containing the N-terminal phosphorylatable tag (phPCNA, MRRASVGS-PCNA) was ³²P-labeled using cAMP-dependent protein kinase and purified as described (34). Complexes were assembled with RF-C or RF-C-1 Δ N and isolated by Biogel A-5m filtration as described above, except that 300 fmol of ³²P-phPCNA replaced PCNA. The void volume fractions (fractions 8–10) from the Biogel column were pooled, and 55 μ l of complex, corresponding to approximately 0.1 pmol of DNA, were assayed in a 65- μ l reaction containing 30 mM Tris-HCl, pH 7.8; 8 mM magnesium acetate; 100 μ g/ml bovine serum albumin; 100 μ M each of dCTP, dGTP, dAMP-PNP, and dTTP; and 0.5 mM ATP as indicated. The reaction was incubated at 14 °C for 1 min, at which time 1.5 pmol of RF-C or RF-C-1 Δ N was added to the reaction or RF-C storage buffer was added in a control assay. After 20 min at 14 °C the reaction was cooled on ice and filtered through a second 2-ml Biogel A-5m (Bio-Rad). Three-drop fractions were collected and counted in a scintillation counter.

Light Scattering Experiments—Measurements were performed using a DynaPro-801 molecular sizing instrument. Approximately 1 mg/ml of RF-C or RF-C-1 Δ N in HEG₃₀₀ buffer was filtered through a Whatman Anaplot 10 filter (0.1 μ m) and measured at 20 °C in the absence or presence of various osmolytes. Twenty independent measurements were determined from each sample. The data were analyzed with the software provided by the manufacturer to obtain values for the hydrodynamic radius and polydispersity.

Surface Plasmon Resonance—The BIAcore apparatus was used for this analysis. About 500 response units of a 71-mer 5'-biotinylated oligonucleotide (mp18 nucleotides 6230–6300) was immobilized on the surface of a streptavidin SA chip in 10 mM sodium acetate buffer at pH 5.5. The protein-DNA interactions were measured by injecting a 10 nM solution of either wild-type RF-C or RF-C-1 Δ N over the immobilized DNA substrate at a flow rate of 30 μ l/min. The running buffer used in the analysis contained 30 mM Hepes-NaOH (7.5), 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 0.1% ampholytes 3.5–9, 8 mM MgCl₂, 125 mM NaCl, and 0.2 mg/ml bovine serum albumin.

RESULTS

Experimental Rationale for Overexpression of RF-C with Truncated Rfc1p in *E. coli*—Previously, we have reported an overproduction strategy for RF-C in yeast, in which all genes were placed in a multicopy plasmid under control of the galactose-inducible *GAL1-10* promoter (13). Although this strategy yielded sufficient protein for many biochemical studies, about 2 mg per 100 g of cells, it was subject to several disadvantages and restrictions. First, overproduction of RF-C was still too limited for extended structural studies of RF-C that we intend to carry out. Second, the overproduction of partial complexes, e.g. Rfc2–5p, in yeast was very cumbersome. Because subcomplexes, unlike the complete RF-C, do not form an ATP-dependent complex with PCNA, a PCNA-affinity column as a one-step

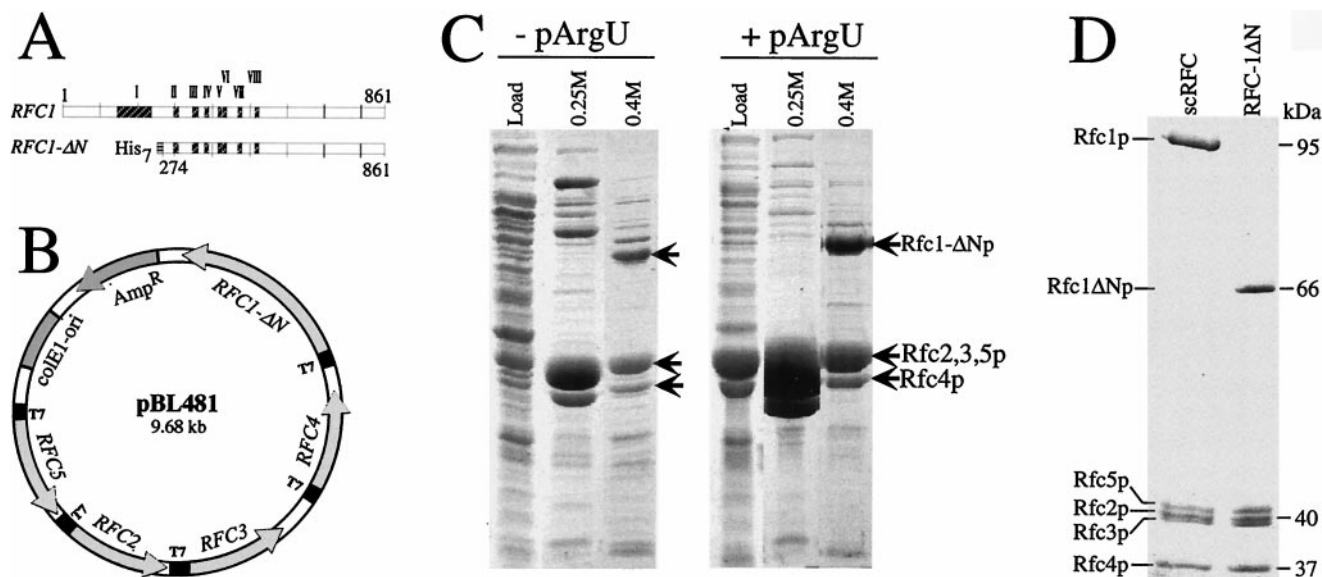


FIG. 1. Overproduction of RF-C in *E. coli*. A, schematic representation of the *RFC1* gene with the consensus homology boxes from Ref. 18. Box I, the ligase homology domain, is deleted from *RFC1-ΔN*, and a His₇ tag has been added. B, schematic of plasmid pBL481 for overexpression of the entire RF-C complex. Each *RFC* gene is placed under control of the bacteriophage T7 promoter and the gene 10 leader sequence (T7). C, overproduction of RF-C from *E. coli* with or without plasmid pSBETa overproducing the *argU* tRNA. The load and the 0.25 and 0.4 M NaCl fractions of a S-Sepharose column were separated on a SDS-10% polyacrylamide gel. Proteins were visualized by Coomassie staining. D, comparison of RF-C purified from yeast with RF-C-1ΔN purified from *E. coli*. Proteins were separated as described under "Experimental Procedures" and stained with colloidal Coomassie.

purification tool could not be employed, and extensive chromatography with associated loss of material was necessary. Most importantly, however, the homologous overexpression system limited mutational studies to those mutants that were viable in yeast. Therefore, *E. coli* was attempted as an overproduction host.

Overexpression of the small *RFC* genes in *E. coli* had previously been carried out in our laboratory and by others (14, 15, 16, 19). When overexpressed individually, all the small subunits were produced in insoluble form. However, when the genes were co-expressed, the solubility of the subunits increased as complexes were formed. For instance, overexpression of *RFC2* together with *RFC3* yielded a soluble complex, and so did to some degree co-expression of the *RFC3* and *RFC4* genes. Co-expression of the *RFC2*, *RFC3*, and *RFC4* genes also yielded a soluble complex, although it was not stable and dissociated upon chromatography. In contrast, the analogous three-subunit complex from human RF-C is extremely stable (36). However, when all four small *RFC* genes in plasmid pBL472 were co-expressed, a stable soluble complex was produced in high yield in *E. coli* (data not shown).

Initial attempts to obtain expression of the full-length *RFC1* gene from the T7 promoter in *E. coli* carrying pBL614 were completely unsuccessful. No polypeptide corresponding to Rfc1p could be detected by Western analysis. As the N-terminal domain of human Rfc1 is not essential for function *in vitro*, we truncated the *RFC1* gene and added a His₇ tag to the N terminus. The start point of the *RFC1-ΔN* gene at amino acid 274 corresponds approximately to the start point of the truncated human Rfc1 subunit at amino acid 555, but as the two proteins lack significant sequence similarity in this region, no precise comparison can be made. Expression of the truncated gene from plasmid pBL480 yielded marginal amounts of insoluble Rfc1-ΔNp, detectable only by Western analysis (data not shown). As it was possible that the synthesized Rfc1-ΔNp polypeptide could be subject to rapid degradation in *E. coli*, and stabilization might occur in the presence of the other RF-C subunits, we cloned all *RFC* genes into a single plasmid, pBL481. To avoid possible expression problems dealing with

colliding transcription complexes originating from strong promoters arranged in an opposing direction, all genes were arranged into a counterclockwise orientation (Fig. 1B). Although Rfc1-ΔNp could still not be identified as a unique Coomassie-stained band in extracts from induced cells carrying pBL481, a Western analysis indicated a greatly increased yield of this polypeptide, with a major fraction detectable in the soluble extract (data not shown). Fractionation of the soluble extract by S-Sepharose chromatography allowed us to unambiguously identify the desired Rfc1-ΔNp containing RF-C complex, designated RF-C-1ΔN, in the 0.4 M NaCl eluate from the S-Sepharose column (Fig. 1C). Additionally, a large excess of the four-subunit Rfc2-5p complex eluted in the 0.25 M NaCl fraction.

The coding sequences of the *RFC* genes contain an unusually large number of the rare arginine codons AGA and AGG, which pose translational problems in *E. coli*, particularly if they occur in tandem, because of the low abundance of the *argU* tRNA for those codons (37, 38). In the *RFC1* gene, such a tandem repeat occurs at codons 476 and 477, and tandem rare arginine codons also occur in *RFC2*, *RFC3*, and *RFC4*. Therefore, we carried out overexpression in the presence of a plasmid, pSBETa, which overproduces the *argU* tRNA (30). This strategy was very successful, as overproduction of both the Rfc2-5p complex and of RF-C-1ΔN increased 2-3-fold (Fig. 1C). Consequently, this system was applied to our studies of RF-C.

In order to obtain maximum expression levels of soluble RF-C-1ΔN, various conditions were altered, including temperature, media, and aeration. Lowering the temperature to 24 °C dramatically increased the levels of all the subunits of RF-C, including Rfc1-ΔNp. The best results were obtained when cells were cultured in rich media, such as terrific broth with vigorous shaking (see under "Experimental Procedures").

Purification and Electrophoretic Analysis of RF-C-1ΔN—Cleared lysates were subjected to ammonium sulfate fractionation and S-Sepharose chromatography. After this step, the four- and five-subunit complexes were generally >80% pure (Fig. 1C). However, an imbalance in the stoichiometry of the subunits of RF-C-1ΔN often occurred. As it was important to obtain only active heteropentameric complexes we applied a

PCNA-affinity chromatography step as described previously (13). Briefly, binding of RF-C to PCNA-agarose under high salt conditions occurs only in the presence of magnesium and ATP. As this represents a step in the catalytic pathway of PCNA loading, only active RF-C binds to the matrix under these conditions. Subsequent elution of the complex is achieved with EDTA. A MonoS step is then carried out in order to remove traces of ATP. As expected, the four-subunit Rfc2–5p complex did not specifically bind to PCNA-beads and was instead purified by successive MonoQ and MonoS columns.

Three of the four RF-C subunits, Rfc2p, Rfc3p, and Rfc5p, comigrate during standard SDS-polyacrylamide electrophoresis. In order to obtain separation, we surveyed different denaturing electrophoresis systems, including high resolution electrophoresis in the presence of Tricine buffers, but without success (39). However, separation of the subunits could be achieved by increasing the concentration of the cross-linking catalyst TEMED in the gel from the usual 0.06% to 0.16% and running the gel in the cold room (Fig. 1D). Presumably, the increased TEMED concentration shortens the chain length of the acrylamide polymer, thereby altering its sieving properties. A further increase of TEMED to 0.4% did not further increase the resolution. A comparison of RF-C purified from yeast with RF-C-1ΔN from *E. coli* showed an exact comigration and proper stoichiometry of the small subunits (Fig. 1D). The relative migration positions of Rfc2p, Rfc3p, and Rfc5p were obtained by comparison with the individually overproduced subunits (data not shown).

Stability of RF-C and RF-C-1ΔN—Problems with RF-C stability had been noted previously (10). In part, inactivation of RF-C was caused by aggregation, as could be demonstrated by loss of RF-C protein after filtration through an 0.1 μm filter (data not shown). Similar problems were encountered with RF-C-1ΔN in this study and were even exacerbated when we tried to obtain the complex at high protein concentrations. Aggregation problems were also noticed during dialysis to decrease the salt concentration of samples, even though care was taken to maintain a salt concentration higher than 150 mM NaCl. In order to increase the stability of the protein, we tested different osmolytes to minimize aggregation of RF-C-1ΔN. Light scattering was used to detect aggregation in the sample (see under “Experimental Procedures”). Light scattering allows a rapid and accurate measurement of the hydrodynamic radius of the complex. The standard deviation of the calculated mean radius is represented by the polydispersity coefficient (Cp). A Cp value of <15% suggests a monodisperse solution with undetectable aggregation, whereas a Cp of >15% is indicative of aggregation. Thus, by comparing the polydispersity values, the optimal osmolyte can be evaluated.

The fraction of RF-C-1ΔN obtained after the PCNA-agarose column was either further passed over the MonoS column in the standard HEG buffer system, or various osmolytes were added and MonoS chromatography was carried out in their presence. The five osmolytes tested were 100 mM urea, 100 mM arginine (pH 7), 100 mM glycine (pH 7), 1 mM ATP (pH 7.5), and 0.05% ampholytes (pH 3.5–9.5). The purified proteins were filtered through an 0.1 μm filter and injected into the apparatus. The results of the analysis are shown in Fig. 2 and Table I. In the absence of any osmolytes, the Cp of RF-C-1ΔN solution was >30%, suggesting substantial aggregation. Addition of glycine had no effect, and only a minor improvement was observed with 1 mM ATP present (Fig. 2B). Polydispersity was reduced to 20% when the buffer contained 100 mM of arginine or urea (Table I). The most dramatic stabilization was observed with broad-range ampholytes (pH 3.5–9) at 0.05 or 0.2%, which maintained RF-C-1ΔN essentially as a monodisperse solution

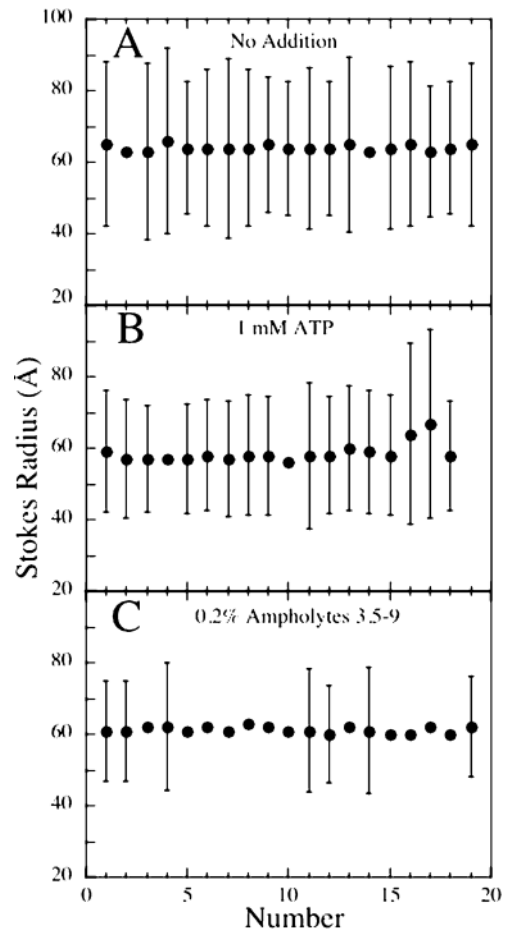


FIG. 2. **Light scattering measurements of RF-C.** The Stokes radius is determined in 19 consecutive determinations. The error bars indicate the degree of polydispersity in each determination. Additions to the basic RF-C buffer were none (A), 1 mM ATP (B), or 0.2% ampholytes, pH 3.5–9 (C).

TABLE I

Effect of osmolytes and ligands on the aggregation state of RF-C-1ΔN

Light scattering was carried out as described under “Experimental Procedures.” The mean solution radius and degree of polydispersity (Cp) are given.

Additive	Radius (Å)	Cp (Å)
None	66	25
1 mM ATP	59	16
0.05% ampholyte	63	7
0.2% ampholyte	62	6
100 mM glycine	65	25
100 mM arginine	60	14
100 mM urea	60	12

with a Cp value of about 10% (Fig. 2C and Table I). The measured Stokes radius of 62 Å is consistent with that of a globular complex with a molecular mass of 240 kDa, close to the predicted value of 221 kDa.

DNA Binding Activity of RF-C-1ΔN—Surface plasmon resonance was used to assess the binding of the full-length and truncated RF-C complex to SS DNA. The chip contained a primed 71-mer SS oligonucleotide that was attached via a biotin-streptavidin linkage (Fig. 3). The response signal when RF-C was flowed across the surface was 4-fold higher than the response signal obtained with RF-C-1ΔN, indicating a major contribution of the ligase homology domain of Rfc1p to binding. These data were corrected for the difference in molecular weight between RF-C and RF-C-1ΔN. The binding of RF-C to DNA is increased by ATP, and even more so by the nonhydro-

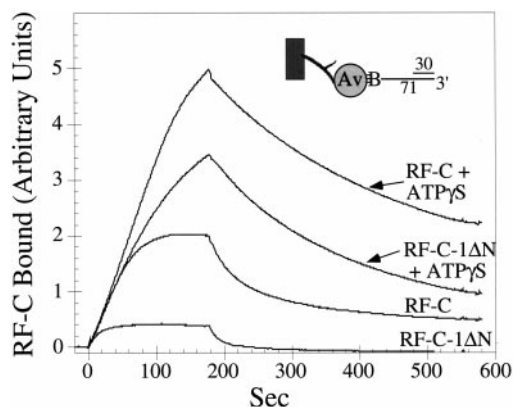


FIG. 3. The ligase homology domain of RF-C enhances nonspecific DNA binding. Surface plasmon resonance measurements were carried out as described under “Experimental Procedures.” A schematic of the sensor chip is shown (B, biotin; Av, streptavidin). Protein flow was started at $t = 0$ and stopped at $t = 175$ s. ATP γ S (10 μ M) was added where indicated. Measured response units were divided by the molecular weight of RF-C or RF-C-1 Δ N as appropriate to obtain molar responses (in arbitrary units). The maximal signal for RF-C + ATP γ S at 175 s was 1500 response units.

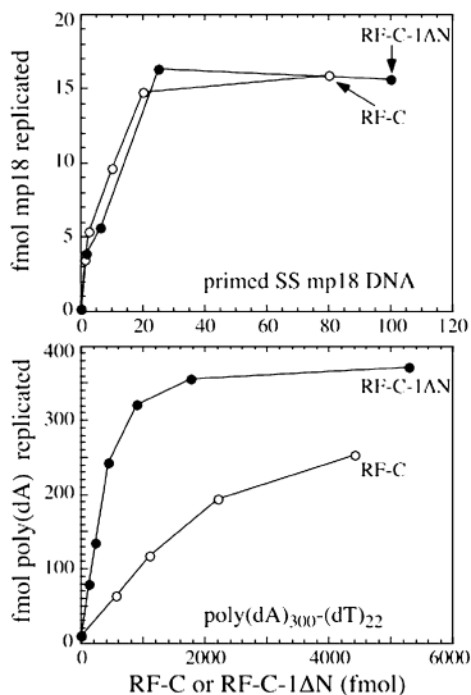


FIG. 4. Replication properties of RF-C and RF-C-1 Δ N on singly primed SS mp18 DNA (A) or poly(dA)₃₀₀-(dT)₂₂ (B). For details, see under “Experimental Procedures.”

lyzable analog ATP γ S (11, 12). In agreement with those results, strong binding to the chip was observed with either RF-C or RF-C-1 Δ N when ATP γ S was included in the buffer. The small difference in binding between the two complexes may reflect the residual contribution of the ligase homology domain (Fig. 3).

DNA Replication Activity of RF-C-1 Δ N—We compared the replication activity of wild-type RF-C with that of RF-C-1 Δ N using singly primed mp18 DNA. In this assay PCNA is loaded onto the primed circular template by RF-C, and processive replication is carried out by the PCNA-Pol δ complex. Increasing amounts of RF-C or RF-C-1 Δ N were preincubated with PCNA and DNA, and replication was started by the addition of Pol δ . In this assay, no significant differences were observed between the molar activities of RF-C and RF-C-1 Δ N (Fig. 4A).

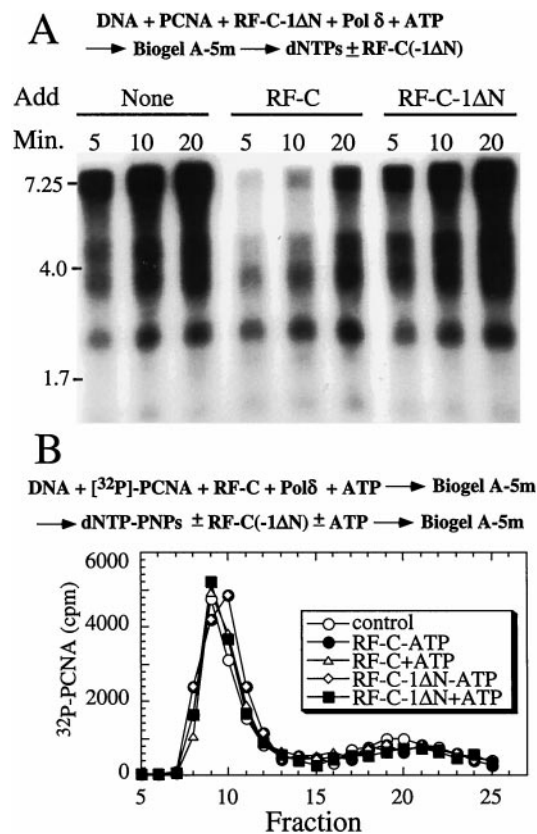


FIG. 5. Excess RF-C inhibits DNA replication without unloading PCNA. A, replication at 14 °C of isolated complexes without additional RF-C or with a 15-fold molar excess of RF-C or RF-C-1 Δ N as indicated. A schematic of the assay is indicated at the top. B, Biogel A-5m elution profiles of isolated replication complexes challenged with RF-C or RF-C-1 Δ N with or without added ATP. A diagram of the assay is given at the top. For details, see under “Experimental Procedures.”

This was the case regardless whether *E. coli* SSB or the yeast single-stranded binding protein replication protein A was used to coat the SS DNA (results not shown). Turnover of RF-C to load PCNA at additional primer termini is negligible in the mp18 replication assay. Therefore, we assessed loading on the homopolymeric template-primer system poly(dA)-oligo(dT) template, in which the DNA ends may promote complex dissociation (40). Indeed, on this DNA substrate, RF-C-1 Δ N shows about 5-fold more replication activity than RF-C (Fig. 4B), indicative of nonspecific binding of RF-C or a failure to dissociate after complete replication of the template.

Nonspecific binding of RF-C to SS DNA might also be inhibitory to replication if the bound RF-C could not be displaced by the polymerase. This inhibition by excess RF-C was actually observed, in particular when DNA replication was carried out at 14 °C. In the experiment shown in Fig. 5A, PCNA and Pol δ were loaded onto primed mp18 DNA by RF-C-1 Δ N, and the DNA-protein complex was separated from unbound proteins by filtration through a Biogel A-5m column. Replication was then started at 14 °C by addition of dNTPs, and after 1 min, a 15-fold molar excess of RF-C or RF-C-1 Δ N was added to the reaction, and time aliquots were analyzed by alkaline agarose gel electrophoresis (Fig. 5A). The data clearly show a much stronger inhibition of replication by RF-C (80%) than by RF-C-1 Δ N (20% inhibition).

One possible explanation of the observed results could be that excess RF-C unloads PCNA, thereby terminating replication, and that RF-C is more efficient at unloading than RF-C-1 Δ N. To investigate that possibility, the above assay was expanded with two modifications. First, a ³²P-labeled form of

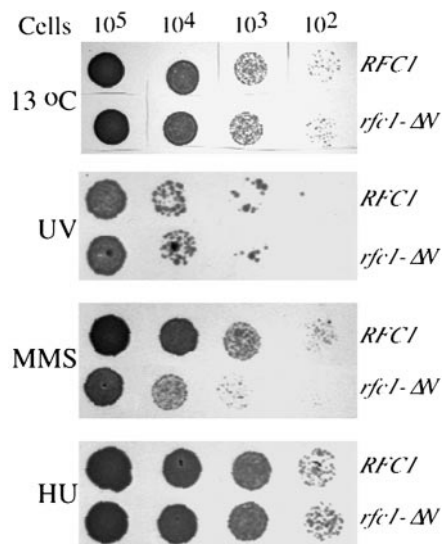


FIG. 6. Sensitivity of a *rfc1-ΔN* strain to DNA damaging agents. Serial 10-fold dilutions of the mutant and the isogenic wild-type strains were plated as drops (containing 10^2 , 10^3 , 10^4 , or 10^5 cells) on YPDA medium and grown at 13 °C for 7 days, irradiated with 100 J/m^2 UV light, and either grown at 30 °C for 3 days, grown on YPDA in the presence of 0.005% MMS for 2 days, or grown on YPDA in the presence of 100 mM hydroxyurea (HU) for 4 days. The experiment was carried out twice without significant variation, except for the MMS data. The *rfc1-ΔN* mutant was consistently between 2- and 10-fold more sensitive to MMS than the isogenic wild-type strain (the experiment showing the most dramatic difference is shown in the figure). There was no clear correlation in sensitivity to MMS with the concentration of MMS used (0.005–0.015%), the number of generations that the mutant had been propagated (30 or 70 generations), or the growth temperature (13, 30, or 37 °C). However, in each MMS experiment, several independent isolates of the mutant strain showed identical sensitivity.

PCNA replaced wild-type PCNA, which allowed us to monitor the fate of PCNA by scintillation counting. Previously, we have shown that this PCNA variant is catalytically indistinguishable from wild-type (41). Secondly, in order to investigate whether ATP or dATP, both of which are proficient in the loading reaction, affected inhibition of replication or the DNA-association status of PCNA, dAMP-PNP replaced dATP during DNA synthesis. This analog is active for incorporation by the polymerase but inactive for loading (7). With these modifications, a strong inhibition of replication by excess RF-C compared with excess RF-C-1ΔN was observed, similar to that shown in Fig. 5A (results not shown). Isolated replication complexes were incubated at 14 °C with dNTP-PNPs, and after 1 min, a 15-fold molar excess of RF-C or RF-C-1ΔN with or without 0.5 mM ATP was added to the reaction and incubation continued for an additional 19 min at 14 °C. The reaction was then chilled on ice and subjected to a second gel filtration column. Radioactivity, indicative of ^{32}P -PCNA, was then determined. The elution profiles in Fig. 5B clearly show that PCNA remains associated with the DNA under all conditions. Therefore, inhibition by excess RF-C is not due to unloading of PCNA.

Analysis of a Yeast Mutant Lacking the Ligase Homology Domain—The mutant *rfc1-ΔN* gene was introduced into haploid yeast cells as the sole source of *RFC1* as described under “Experimental Procedures.” Not only was the mutant viable, it also showed no growth defect at the three temperatures tested, *i.e.* 13, 30, and 37 °C (Fig. 6, data not shown). In particular, growth at 13 °C was tested because all conditional *RFC1* mutants isolated to date have been cold-sensitive alleles (42, 43). Microscopic examination of cells grown at 13 °C showed no increase in the percentage of large budded cells, which would have been indicative of a defect at G_2/M at the low temperature

(data not shown). This indicates that the N terminus of *RFC1* is also dispensable for RF-C activity *in vivo*.

To assess whether the ligase homology domain functions in DNA repair, the mutant was tested for sensitivity to different DNA damaging agents. Strains were grown in the presence of 110 mM hydroxyurea or 0.005–0.015% MMS or exposed to ultraviolet light. Again, these experiments were performed at 13, 30, and 37 °C. The mutant strain did not show any increased sensitivity to hydroxyurea or ultraviolet light at the three temperatures tested. However, a slight sensitivity to MMS was observed in the *rfc1-ΔN* mutant compared with wild-type (Fig. 6). The sensitivity varied between experiments, the mutant strain being between 2- and 10-fold more sensitive to MMS (see legend to Fig. 6).

The effect of deletion of the ligase homology on recombination was tested in a strain containing a direct repeat of the 2.2-kb *LEU2* gene, separated by 4.4 kb of DNA containing the *URA3* gene (plus 3 kb of vector sequences). Intragenic recombination between the repeats proceeds with deletion of the *URA3* gene and can be scored by plating on 5-fluoroorotic acid-containing plates (see under “Experimental Procedures” for details). Recombination frequencies were $1.4 \pm 0.4 \times 10^{-5}$ for the wild-type strain and $1.6 \pm 0.4 \times 10^{-5}$ for the *rfc1-ΔN* mutant. The virtual identity of the two frequencies indicates that there is no defect in homologous recombination in the *rfc1-ΔN* mutant.

RF-C is required for proper telomere maintenance (35). A *RFC1* deletion strain containing two plasmids, one with the wild-type *RFC1* on an *URA3* plasmid and one with the truncation gene *rfc1-ΔN* on a *TRP1* plasmid, was grown on 5-fluoroorotic acid medium to allow only growth of cells with *rfc1-ΔN* as the sole source for Rfc1p (see under “Experimental Procedures”). The cells were propagated in rich medium for up to 120 generations, and DNA prepared from cells after 30, 70, and 120 generations. The chromosomal DNA was digested with *XhoI*. *XhoI* cuts in the subtelomeric Y'-sequence and in wild-type strains produces a fragment of 1.1–1.4 kb that includes 0.2–0.4 kb of telomeric repeat DNA ($G_{1-3}T$) (35). A Southern blot analysis with a telomeric probe showed that the length of telomeric *XhoI* fragments was identical between the *rfc1-ΔN* strain and the wild-type control and that this length was maintained over 120 generations of growth. Therefore, strains deficient for the ligase homology domain of Rfc1p show no defect in telomere maintenance and telomere length regulation (Fig. 7).

DISCUSSION

The studies reported here show that heteropentameric RF-C can be overproduced in *E. coli* in sufficient quantities for biophysical studies. The success of the project hinged on the dispensability of the N-terminal domain of Rfc1p for function and on the concomitant overproduction of the argU tRNA for the rare arginine codons AGA and AGG. This strategy still produced the small Rfc proteins in an approximately 5-fold excess over Rfc1-ΔN, so that after purification about 1 mg of RF-C and 5 mg of Rfc2–5 were obtained per liter of cells. The Rfc2–5 complex will be of value in biophysical studies and in reconstitution studies with Rfc-like proteins that may take the place of Rfc1p to form alternative complexes. One of these potential *RFC1* homologues is *CTF18* (*CHL12*), which may function to link the replication apparatus to the chromosome segregation apparatus, and another is *RAD24*, which is involved in checkpoint function and interacts with the small RF-C subunits (44, 45).

A comparison of the replication properties of RF-C-1ΔN with wild-type RF-C isolated from a yeast overproduction strain showed no difference when primed circular mp18 DNA was the substrate (Fig. 4A). As no appreciable turnover of RF-C occurs

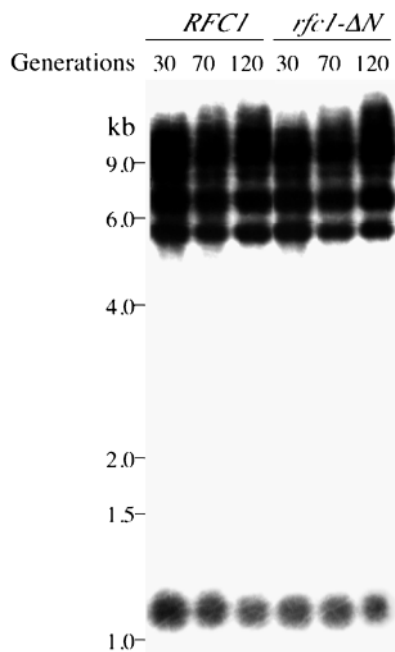


FIG. 7. The *rfc1-ΔN* strain is not defective for telomere length maintenance. The strains were grown at 30 °C in YPDA medium for the indicated number of generations. Southern analysis of telomere ends, migrating at ~1.2 kb, was performed. See under "Experimental Procedures" for details.

in this assay system, it allows us to quantitate the molar activity of the RF-C preparations: both are about 70–80% active. For both RF-C preparations, obtaining such an active enzyme preparation is critically dependent on stabilization of the enzyme and prevention of aggregation by including broad range ampholytes during purification and storage (Fig. 2). The results with the truncation enzyme differ substantially from analogous experiments with human RF-C, in which the presence of the N-terminal domain of Rfc1 was found to be very inhibitory for replication of circular DNA substrates (23, 25). In the yeast system, a lower replication activity of RF-C compared with RF-C-1ΔN was only observed on poly(dA)-oligo(dT) (Fig. 4B). In contrast to the circular mp18 system, replication of a linear template-primer may require multiple PCNA loading events by the clamp loader in order to achieve the formation of a productive complex between PCNA and Pol δ because the loaded PCNA can rapidly slide off the end of the linear DNA. Consequently, replication of linear DNA is less efficient than replication of circular DNA (40, 46). In addition, as complete replication of the linear template is expected to promote complex dissociation, recycling of the complex to a new template-primer is promoted. Therefore, it is likely that the decreased activity of RF-C in the linear DNA replication assay indicates slower dissociation of the wild-type enzyme in comparison to RF-C-1ΔN.

In a study to determine whether inhibition of replication could also occur through nonspecific binding of RF-C to SS DNA or at sites of secondary structure, we measured DNA replication by Pol δ holoenzyme in the presence of a large excess of RF-C or RF-C-1ΔN. At 30 °C, a moderate inhibition was observed by RF-C (data not shown), but this inhibition was accentuated at 14 °C (Fig. 5A). Through isolation of complex intermediates, we showed that this inhibition was not a result of unloading of PCNA at high concentrations of RF-C (Fig. 5B). Most likely, the inhibition occurs through the strong nonspecific binding of RF-C to SS DNA, as shown in Fig. 3. Inhibition may be accentuated at sites of secondary structures that would be stabilized at 14 °C. In agreement with this conclusion is the

observation that in the presence of excess RF-C, replication intermediates accumulate at pause sites (Fig. 5A).

Biochemical studies with the isolated human ligase homology domain show that DNA binding is strongly stimulated by the presence of a recessed or blunt end 5'-phosphate in partial duplex structures (26). In addition, the domain shows a binding preference for telomeric repeat sequences (27). The substrate binding specificity of this domain suggests a possible function in Okazaki fragment maturation or telomere maintenance. However, the preference for recessed 5'-phosphates would also be consistent with a function for the ligase homology domain in repair pathways such as base excision repair or nucleotide excision repair, which proceed via filling in of repair gaps.

Previously, Holm and co-workers (17) had shown that N-terminal truncations of the yeast *RFC1* gene up to amino acid ~150 could complement a cold-sensitive *rfc1-1* mutation (17). Similarly, these N-terminal truncations complemented an allele of *RFC1* inactivated by insertional mutagenesis in the middle of the gene. However, one N-terminal truncation that deleted into the ligase homology domain showed only partial complementation. In addition, a cold-sensitive allele of *RFC1*, *cdc44-10*, with two mutations in the ligase homology domain, G185E and P234L, showed cold sensitivity for growth and sensitivity to MMS (42). Most recently, Alani and co-workers (43) isolated another *RFC1* allele by insertional mutagenesis of transposon Tn3. Most likely expressed from a promoter inside the transposon, this mutant allele may make a truncated protein starting at amino acid 318, the first methionine after the insertion point, at a position very close to domain II. As with the double point mutant isolated by Holm and co-workers (17), the insertional mutant also shows cold sensitivity for growth, sensitivity to DNA damaging agents, and an increased rate of spontaneous mutations, suggesting that the N-terminal domain of Rfc1p may be important for DNA replication and DNA repair (42, 43).

In order to study the *in vivo* effect of the *RFC1* truncation that we used in our expression studies, we created a mutant strain with a complete deletion of the *RFC1* gene to avoid possible complications due to interallelic complementation. The complementing wild-type *RFC1* gene or the truncation *rfc1-ΔN* ($\Delta 3-273$) allele was carried on a centromere based plasmid under control of the native promoter. The mutant showed no defect in growth at any temperature in the presence or absence of the replication inhibitor hydroxyurea or in telomere maintenance (Figs. 5 and 6), nor could we detect a defect in homologous recombination in a intrachromosomal recombination assay. However, a slight sensitivity to MMS, but not to UV-irradiation, was observed in the *rfc1-ΔN* mutant, indicative of a minor repair defect. Perhaps the mutant is partially defective for base excision repair, which in yeast also uses the PCNA-Pol δ/ϵ replication system (47, 48).

The difference in observed phenotype between mutant strains with the *cdc44-10* or *rfc1::Tn3* alleles on one hand and the *rfc1-ΔN* allele on the other hand cannot be easily rationalized. Perhaps the mutations in *cdc44-10*, which allele was identified in a cold sensitivity screen, cause misfolding or destabilization of the entire Rfc1p subunit at the restrictive temperature. For the *rfc1::Tn3* allele, the observed defects may also be caused by the close proximity of the internal methionine start site to domain II or inappropriate expression of the truncated protein from the cryptic promoter inside the Tn3 cassette.

In conclusion, the successful overexpression of RF-C in bacteria and stabilization of the complex opens the way for more thorough biophysical and biochemical studies of the eukaryotic clamp loader. It also makes it possible to study mutants of RF-C that, because of their lethality, cannot be overproduced in

yeast. We have already used this overproduction system to isolate mutant RF-C complexes with mutations in the ATP-binding domains of several subunits.

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