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 RFCl–RFCl5. The RFCl gene was cloned under control of the strong inducible bacteriophage T7 promoter, yet induction did not yield detectable Rfc1p. However, a truncated form of RFCl 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 introduced into the vector. 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 RFCl 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 polymersases (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). Alternatively, RF-C function in vitro. In fact, deletion of the N-terminal domain of the protein (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-
Plasmid pBL72 was digested with HinIII and Furul and treated with DNA polymerase I, Klenow fragment plus dNTPs, and the resulting fragment containing the four small RFC genes was ligated into plasmid pBL840 digested with Smal. The resulting plasmid, pBL841, contains all five RFC genes in a counterclockwise orientation, each under the control of the T7 gene 10 promoter followed by a single copy of the gene 10 promoter. The proper sequences of all plasmids obtained by ligation purposes was DH5α, whereas BL21(DE3) was used for overexpression studies. Diploid yeast strain W303 (MATa/MATa ade2-1 ade2-1 ura3-1/ura3-1 his3-11/htis3-11 trpl-1/1trpl-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 KanMX 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. Haplod progeny carrying the disruption of the RFC1 gene and complementing plasmid pBL641 were identified by standard genetic techniques and PCR analysis. Strain PY171 (MATa rfc1-1::KanMX6 ade2-1 ura3-1 his3-11 trpl-1 leu2-3,112 can1-100 pBL641 (pRS316, RFC1, URA3)) was transformed with the plasmid encoding RFC1 and restored to growth on 5-fluoroorotic acid medium to give strain PY173-X (MATa rfc1-1::KanMX6 ade2-1 ura3-1 his3-11 trpl-1 leu2-3,112 can1-100 pBL642-x (pRS314, rfc1-1, TRP1)). Standard media were used (33). YPDA is YPD plus 20 μg/ml of adenine. DNA damage sensitivity measurements were carried out on YPDA plates to which was added, after autoclaving, 0.05% of methylmethane sulfonate (MMS) or 110 mM hydroxyurea. Strains PY173 and PY173-X were transformed with Hpal-cut pRS305-URA3 (Bluescript LEU2 URA3) to target integration in the LEU2 gene (34). To determine URA3 pop-out frequencies, a fluctuation analysis was carried out. 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 × 10⁵ 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.
yield of Rfc2–5 was 10–ml linear gradient from 150 to 600 mM NaCl in HEG buffer run at a constant voltage of 260–300 V. The dye front had entered the resolving gel by 1–2 cm. At this time, the gel was fraction was diluted with HEG buffer to a conductance of HEG 200 and RF-C N elutes at D concentrations corresponding to approximately 0.1 pmol of DNA complexes, were assayed in a 65–μl reaction containing 30 mM Tris-HCl, pH 7.8; 8 mM magnesium acetate; 100 μg/ml bovine serum albumin; 1 mM dithiothreitol, 1.65 mM [3H]dTTP, 100 μM each of dCTP, dGTP, 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 was added to the reaction or RF-C storage buffer was added in a control assay. At various time intervals, 20–ml aliquots were removed into 5 μl of 50 mM EDTA, 50% glycerol, and 2% SDS. The products were separated on an 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 and isolated by Biogel A-5m filtration as described above, except that 300 pmol of 32P-phPCNA replaced PCNA. The void volume fractions (fractions 8–10) from the Biogel column were pooled, and 55 μl of complex, corresponding to approximately 0.1 pmol of DNA complexes, were assayed in a 65–μl reaction containing 30 mM Tris-HCl, pH 7.8; 8 mM magnesium acetate; 100 μg/ml bovine serum albumin; 100 μM each of dCTP, dGTP, and dATP; and 12.5 μM [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 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 column. Three-drop fractions were collected and counted in a scintillation counter.

**Light Scattering Experiments**—Measurements were performed using a Surfotrack instrument (Anton Paar, Graz, Austria). Approximately 5 μg/ml of RF-C or RF-C-1 in HEG buffer was filtered through a Whatman Anotop 10 filter (0.1 μm) and measured at 20 °C in the absence or presence of various oligonucleotides. 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 mM solution of either wild-type RF-C or RF-C-1LON over the immobilized DNA substrate at a flow rate of 30 μl/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% amphotericin B 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 (15). Although this strategy yielded sufficient protein for many biochemical studies, about 2 mg of RF-C per g of cells, CFU was subject to several advantages and restrictions. First, overproduction of RF-C was 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
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 pBL414 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 RFC 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 an 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 reduced to 20% when the buffer contained 100 mM of arginine or urea (Table I). 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).

TABLE I

| 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-
lyzable analog ATPγS (11, 12). In agreement with those results, strong binding to the chip was observed with either RF-C or RF-C-1ΔN when ATPγS 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-1ΔN—We compared the replication activity of wild-type RF-C with that of RF-C-1ΔN 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-1ΔN 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-1ΔN (Fig. 4A).

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-1ΔN 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-1ΔN with or without added ATP. A diagram of the assay is given at the top. For details, see under "Experimental Procedures."

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-1ΔN. To investigate that possibility, the above assay was expanded with two modifications. First, a 32P-labeled form of

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. ATPγS (10 μM) was added where indicated. Measured response units were divided by the molecular weight of RF-C or RF-C-1ΔN as appropriate to obtain molar responses (in arbitrary units). The maximal signal for RF-C + ATPγS at 175 s was 1500 response units.

Fig. 4. Replication properties of RF-C and RF-C-1ΔN on singly primed SS mp18 DNA (A) or poly(dA)-(dT)22 (B). For details, see under "Experimental Procedures."

Fig. 5. DNA Replication Activity of RF-C-1ΔN—We compared the replication activity of wild-type RF-C with that of RF-C-1ΔN 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-1ΔN 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-1ΔN (Fig. 4A).
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 complexes was incubated at 14 °C with dNTP-PNPs, and after 1 min, a 15-fold molar excess of RF-C or RF-C-1 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 [32P]-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 G2/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 ± 0.4 × 10^{-6} for the wild-type strain and 1.6 ± 0.4 × 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 RF2–5 were obtained per liter of cells. The RF2–5 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 CTP18 (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-ΔN 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
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 rfc1-1ΔN (Δ3–273) 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 rfc1-1ΔN 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/e 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 rfc1-1ΔN 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

![FIG. 7. The rfc1-1ΔN 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.](http://www.jbc.org/content/early/2018/07/21/jbc.D118009203/F1.large.jpg)
Overproduction of RF-C

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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