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Identification of Replication Factor C from *Saccharomyces cerevisiae*: A Component of the Leading-Strand DNA Replication Complex

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A number of proteins have been isolated from human cells on the basis of their ability to support DNA replication in vitro of the simian virus 40 (SV40) origin of DNA replication. One such protein, replication factor C (RFC), functions with the proliferating cell nuclear antigen (PCNA), replication protein A (RPA), and DNA polymerase δ to synthesize the leading strand at a replication fork. To determine whether these proteins perform similar roles during replication of DNA from origins in cellular chromosomes, we have begun to characterize functionally homologous proteins from the yeast *Saccharomyces cerevisiae*. RFC from *S. cerevisiae* was purified by its ability to stimulate yeast DNA polymerase δ on a primed single-stranded DNA template in the presence of yeast PCNA and RPA. Like its human-cell counterpart, RFC from *S. cerevisiae* (scRFC) has an associated DNA-activated ATPase activity as well as a primer-template, structure-specific DNA binding activity. By analogy with the phage T4 and SV40 DNA replication in vitro systems, the yeast RFC, PCNA, RPA, and DNA polymerase δ activities function together as a leading-strand DNA replication complex. Now that RFC from *S. cerevisiae* has been purified, all seven cellular factors previously shown to be required for SV40 DNA replication in vitro have been identified in *S. cerevisiae*.

The study of eukaryotic DNA replication has been dependent, in part, on the isolation of enzymes involved in DNA synthesis. In the absence of well-characterized mammalian origins of DNA replication, the mammalian DNA viruses, particularly simian virus 40 (SV40), have served as good model systems for understanding some aspects of eukaryotic DNA replication. The SV40 system has been used to identify proteins from human cells that are required for DNA replication from the virus origin sequences. While origins of DNA replication have been characterized in *Saccharomyces cerevisiae*, a primary hindrance in the study of yeast DNA replication has been the inability to reconstitute initiation of DNA replication with cellular extracts.

The SV40 DNA replication system is based on the observation that protein extracts from primate cells, when supplemented with purified SV40 large T antigen (TAg), efficiently replicate plasmid DNAs containing the SV40 origin of DNA replication (24, 25, 41, 52). Fractionation of these extracts has allowed the reconstitution of a core replication reaction by using seven highly purified cellular components in addition to viral TAg (45, 50, 51; for reviews, see references 4, 9, and 40). Kinetic analysis of SV40 DNA replication has identified at least three steps: presynthesis, initiation, and elongation (4, 5, 11, 14, 22, 35, 44, 45, 50, 51, 54). A characteristic time lag of 8 to 10 min is due to presynthesis events, in which TAg forms an ATP-dependent multimeric complex by binding to the replication origin and locally unwinding the DNA at the origin (5, 12, 13, 26, 30, 35, 54). Further unwinding requires the action of the TAg DNA helicase activity, a topoisomerase, and a single-stranded DNA binding protein, replication protein A (RPA) (formerly called RF-A [16, 53, 54]). In the absence of other DNA replication factors, TAg continues to unwind the template DNA, producing a highly unwound DNA molecule known as form U (11, 14, 44, 54). In the presence of human RPA and other DNA replication proteins, unwinding of DNA at the replication origin is coupled with initiation of DNA synthesis. At initiation, DNA polymerase α(polα)-DNA primase complex interacts with TAg and is stimulated by RPA (10, 27). Short RNA primers, synthesized by primase, are extended by the polα activity to make Okazaki fragments. The DNA polymerase polδ and the proliferating cell nuclear antigen (PCNA), a processivity factor for polδ (34, 43), are known to be essential for reconstitution of SV40 DNA replication in vitro (22, 28, 32–34, 50, 51). This observation is supported by genetic and biochemical studies with *S. cerevisiae* which show that polδ and PCNA are required for DNA replication (3, 6).

Replication factor C (RFC) is an essential factor for elongation of DNA synthesis during SV40 DNA replication in vitro. In human cells, RFC is a multisubunit enzyme with polypeptides with molecular masses of 140, 42 (doublet), and 37 kDa (23, 47). RFC binds in a structure-specific manner to primer-template DNA junctions, is a DNA-dependent ATPase, and stimulates both polα and polδ activities under certain conditions (23, 46, 48–50). The ATPase activity of RFC is stimulated by the polδ processivity factor, PCNA. RFC, in combination with RPA and PCNA, will cooperatively stimulate DNA synthesis by polδ on a primed, single-stranded DNA template (23, 46). These three accessory factors do not cooperate to stimulate polα, although RPA and RFC in the absence of PCNA will stimulate this polymerase under certain conditions. Analysis of the replication products of reactions in the absence of polδ or polα is consistent with the proposal that polδ synthesizes the continuously replicated leading strands while polα synthesizes shorter lagging strands. Direct experimental evidence dem-

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onstrates that polδ, PCNA, and RFC are responsible for leading-strand synthesis during SV40 DNA replication in vitro. Thus, when either polδ, PCNA, or RFC is left out of the SV40 reaction mixture, only short lagging-strand products are formed (45, 50). Furthermore, since these experiments are performed with highly purified enzymes, they demonstrate that polα can synthesize short lagging strands.

Significant information has been acquired from the study of SV40 DNA replication in vitro, yet these studies will not reveal whether these factors are required for DNA replication in vivo. For example, SV40 DNA replication in vitro can be reconstituted with polα (53) or both polα and polδ (22, 45, 50, 51). However, in *S. cerevisiae*, three DNA polymerases, polα (18), polδ (6, 39), and polε (29), have been shown to be essential for viability. To bridge the gap between in vitro and in vivo studies, we have searched for functional homologs in *S. cerevisiae* so that ultimately, a combined biochemical and genetic analysis can be pursued.

The conservation of structure and function in the replication apparatus of organisms as diverse as bacteriophage T4, *Escherichia coli*, humans, and yeasts has recently become apparent. For example, RFC and PCNA are functionally related to the products of bacteriophage T4 genes 44/62 and gene 45, respectively (48). In addition, functional homologs of polα-DNA primase (31, 38), polδ (3), and PCNA (1) have been purified in *S. cerevisiae*. Recently, an RPA homolog was purified from *S. cerevisiae* as a factor that functions in unwinding the SV40 origin of DNA replication. Yeast RPA is a multisubunit phosphoprotein which exhibits chromatographic behavior, subunit structure, and DNA binding activity similar to those of its human homolog (8).

The conservation of the DNA replication apparatus in diverse organisms prompted us to search for an RFC-like activity in *S. cerevisiae*. Because of the inability to reconstitute the whole yeast DNA replication reaction, we have devised a subassay to purify RFC. Here we report the purification of yeast RFC and demonstrate that yeast RFC shares many properties with its human and phage T4 homologs. These studies also indicate that RFC is a component of the leading-strand DNA replication complex in *S. cerevisiae*.

MATERIALS AND METHODS

Yeast strain. DNA polδ, RPA, and RFC were purified from the diploid protease-deficient strain BJ926 (*MATa/α TRP1/trp1 his1/HIS1 pep4-3/pep4-3 prb1-1122/prb1-1122 prc1-126/prc1-126 can1/can1 gal2/gal2*) (19).

Template DNAs. Single-stranded pUC118 DNA was prepared from *E. coli* cells infected with phage as described previously (37). This template was annealed at a unique site to a 17-base sequencing primer (New England Biolabs no. 1211) in a primer-to-template ratio of 3:1 in a solution containing 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA (TE) and 50 mM NaCl. Poly(A) (10,000 nucleotides; Boehringer Mannheim), poly(dA) (250 nucleotides; Pharmacia), and oligo(dT) (12 to 18 nucleotides; Pharmacia) were each annealed in TE with 50 mM NaCl at the ratio indicated above.

Purification of polδ and RPA from *S. cerevisiae*. Polδ was purified from 10 liters of the protease-deficient strain BJ926 by five steps (phosphocellulose column [2.5 by 8 cm], Mono Q HR10/10, heparin-agarose column [1.2 by 8 cm], phenyl superose HR5/5, and hydroxylapatite column [0.5 by 3 cm] chromatography). This purification scheme is a slight modification of the original method (3). The second step, DEAE–high-pressure liquid chromatography (HPLC), was substituted for by a Mono Q column run in DE buffer (3), pH 7.0. Polδ was eluted from the column by a linear gradient of KCl from 0 to 0.4 M, with the peak fractions centered at 200 mM KCl, whereas polα activity peaked at 320 mM KCl. The gradient was not run to a high enough salt concentration to elute polε from the column. The fourth step, propyl HPLC, was substituted for by a phenyl superose column. The sample was loaded at 1.6 M $(NH_4)_2SO_4$ in PR buffer (3), and polδ was eluted by a linear reverse gradient of $(NH_4)_2SO_4$ from 1.2 to 0 M. Polδ eluted in fractions centered at 0.5 M $(NH_4)_2SO_4$. The final step used conventional hydroxylapatite column chromatography. The purified polδ had a specific activity of 10^4 U/mg of protein [1 U is equivalent to 1 pmol of dAMP incorporated into poly(dA) · oligo(dT) in 30 min]. This polymerase was stimulated threefold by PCNA with poly(dA) · oligo(dT) as a template-primer at pH 8.0 and has subunits with the same molecular masses on a sodium dodecyl sulfate (SDS)-polyacrylamide gel as those previously published (one subunit at 125 kDa and four proteins between 55 and 66 kDa) (3).

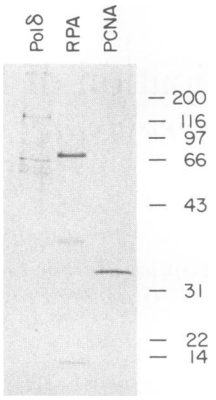

FIG. 1. Purified DNA replication proteins. SDS-PAGE of purified polδ, RPA, and PCNA from *S. cerevisiae*. A total of 50 ng, 200 ng, and 10 U of PCNA, RPA, and polδ, respectively, were loaded onto each lane. The gel was silver stained. The positions of the molecular weight markers (in thousands) are shown on the right.

RPA was purified from BJ926 as described in the original method (Affi-Gel blue, single-stranded DNA cellulose, and Mono Q chromatography) (8). The final concentration of RPA was 0.2 mg/ml. The preparations of polδ and RPA used for all figures in this paper are described in the legend to Fig. 1.

pT7/PCNA expression construct. Overexpression of PCNA in *E. coli* followed a modified version of a procedure for expression of *S. cerevisiae BCY1* protein (17). A unique *Nde*I site was engineered into the PCNA gene (2) at the initiator methionine, and a unique *Bam*HI site was engineered into the 3′ untranslated region. The 25-mers 5′CA AAA GAGAG AACATATGTT AGAAGC and 5′TATTT TTAGG ATCCA ACTAT ATAGAT were used to construct the *Nde*I site and the *Bam*HI site (both underlined), respectively. These two oligonucleotides were used to prime a polymerase chain reaction containing 2 μg of *S. cerevisiae* genomic DNA and 1 U of *Taq* polymerase (Perkin-Elmer Cetus). The resulting 827-bp polymerase chain reaction product coding for PCNA was isolated by agarose gel electrophoresis. The T7 vector, pAR3038 2xT, used to express PCNA is a derivative of pAR3038 (15), which has two transcriptional terminators cloned into the *Pvu*II site to prevent read-through transcription into the phage T7 gene 10

promoter (16a). The expression plasmid pT7/PCNA was constructed by digesting the PCNA polymerase chain reaction fragment and pAR3038 2xT with *Nde*I and *Bam*HI and ligating the two cleaved products. The PCNA ATG is 9 nucleotides downstream from the Shine-Dalgarno sequence in this construct. The plasmid pT7/PCNA was transformed into *E. coli* BL21(DE3), which contains the phage DE3 as a lysogen (42). This phage contains the T7 gene 1 (RNA polymerase) under *lac* UV5 control.

PCNA expression and purification. An overnight culture (7.5 ml) of BL21(DE3)(pT7/PCNA) grown in Luria broth plus ampicillin (50 μg/ml) was used to inoculate 2 liters of the same medium and was grown at 37°C. When the culture reached an A_{600} of 0.35, isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM and growth was continued for 3 h. Cells were harvested by centrifugation and lysed with a French press (1,000 lb/in^2) in 40 ml of buffer L (25 mM Tris-HCl [pH 7.4], 1 mM EDTA, 25 mM NaCl, 0.01% Nonidet P-40 [NP-40], 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT]). The suspension was adjusted to 0.1 M NaCl and centrifuged for 1 h at 31,000 rpm in a 60 Ti rotor at 4°C. This supernatant was adjusted to 0.2 M NaCl and loaded onto a Q-Sepharose column (1.2 by 16 cm), equilibrated with 0.2 M NaCl in buffer A (25 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.01% NP-40, 10% glycerol, 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM PMSF, 5 mM DTT). The column was washed with 40 ml of the same buffer, and the protein eluted with a 200 ml gradient from 0.2 to 0.7 M NaCl in buffer A. PCNA eluted in fractions centered at 0.4 M NaCl. In this step, as in all subsequent steps (except S-Sepharose), an SDS–12.5% polyacrylamide gel was run and stained with Coomassie blue to determine which fractions contained PCNA. Pooled fractions were dialyzed in HAP buffer (25 mM KPO_4 [pH 7.0], 0.01% NP-40, 10% glycerol, 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM PMSF, 5 mM DTT). The dialysate was loaded onto an S-Sepharose column (1.2 by 16 cm) that was equilibrated with HAP buffer. PCNA flowed through this column. The protein concentration in the flowthrough was determined (7). This step removes a potent single-stranded DNA nuclease and a few low-molecular-weight contaminating proteins. The third step, hydroxylapatite chromatography, removed any residual nuclease activity. The pooled protein peak from the S-Sepharose flowthrough was loaded onto a hydroxylapatite column (2.5 by 11 cm) equilibrated with HAP buffer and was washed with 100 ml of the same buffer. PCNA was eluted with a 500-ml gradient of 0.025 to 0.5 M KPO_4 in HAP buffer. PCNA-containing fractions, eluting as fractions centered at 0.3 M KPO_4, were pooled and dialyzed against 1.2 M NaCl in buffer A (without glycerol). A phenyl-Sepharose column (1.2 by 11 cm) was used as a last step to remove any remaining contaminants. The protein was loaded onto the column equilibrated with 1.2 M NaCl in buffer A (without glycerol), and PCNA eluted with a reverse linear gradient from 1.2 to 0 M NaCl in buffer A (without glycerol). PCNA-containing fractions were pooled and dialyzed against 20% sucrose–25 mM NaCl in buffer A. This preparation yielded 11 mg of purified PCNA. The protein preparation used in all experiments reported in this paper is described in the legend to Fig. 1.

DNA synthesis and product analysis. Fifteen-microliter replication reaction mixtures were as described previously (48) except that 60 ng of primed-pUC118 single-stranded DNA was used as a template and the NaCl concentration was raised to 100 mM. Unless indicated otherwise, 0.06 to 0.12 U of polδ, 190 ng of PCNA, 225 ng of RPA, and various amounts of RFC (either crude or purified as indicated) were incubated at 37°C for 30 min. To assay for incorporated nucleotides, reaction mixtures were spotted onto DEAE-paper (DE-81; Whatman), washed three times with 0.5 M Na_2PO_4 and once with distilled water, and counted. *E. coli* SSB (340 ng) can be substituted for RPA in this reaction and was used instead of RPA during purification of RFC. A 10-μl aliquot was removed from the reaction mixture for product analysis, and the reaction was terminated with a 10-μl proteinase K solution as previously described (46). Samples were run on a 2% alkaline agarose gel (37) and then fixed in a 10% methanol–10% acetic acid solution, dried onto DEAE-paper, and autoradiographed.

RFC purification. All steps were performed at 0 to 4°C. Ten liters of *S. cerevisiae* BJ926 was grown in a fermentor at 30°C to an A_{600} of 4.0 and glass bead broken, the crude lysate was spun, and the supernatant was precipitated with $(NH_4)_2SO_4$ as previously described for the purification of polδ (3). The resulting $(NH_4)_2SO_4$ pellet was resuspended by Dounce homogenization in 35 ml of buffer A containing 25 mM NaCl and was dialyzed overnight against 1 liter of the same buffer. We used *E. coli* SSB (65 μg/ml) instead of RPA in the assay to purify RFC. The chromatographic behaviors of yeast RFC and human RFC proved to be very similar and, therefore, the chromatographic scheme used to purify RFC from *S. cerevisiae* (phosphocellulose column [4 by 12 cm], hydroxylapatite column [2 by 12 cm], phosphocellulose column [0.9 by 9 cm], single-stranded DNA cellulose column [0.9 by 7 cm], Mono Q HR5/5, and a 15 to 35% glycerol gradient) was a slight modification of that described for human RFC (47). Elution points for human RFC and yeast RFC are the same at all steps. Unless otherwise indicated, all buffers used were as described previously (47), except that the DTT concentration was raised to 5 mM and the following protease inhibitors were used at all steps: 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, and 1 mM PMSF. The third step, the second phosphocellulose column, was loaded at 0.1 M KPO_4, and a linear gradient was run from 0.1 to 0.5 M KPO_4. Two peaks of activity were identified by the DNA synthesis assay in the presence of polδ, *E. coli* SSB (Pharmacia), and PCNA. One peak centered at 0.25 M KPO_4, and another centered at 0.4 M KPO_4. The peak eluting at 0.25 M KPO_4 was used to further purify RFC. Upon further purification, no RFC activity was found in the 0.4 M KPO_4 peak. The salt step elutions used during the single-stranded DNA cellulose column were replaced with a linear gradient from 0.1 to 0.7 M NaCl. RFC eluted in a peak centered at 0.45 M NaCl. This is the first stage during the purification at which DNA synthesis was absolutely dependent on polδ because of previous comigration of contaminating polymerase(s) with RFC. The Mono Q gradient was performed as described previously (47) except that the buffer was adjusted to pH 8.0. Purified glycerol gradient fractions of RFC fractions were stored at −70°C.

ATPase assay. The assay for *S. cerevisiae* RFC ATPase was essentially as described previously for human RFC ATPase (48).

DNA binding studies. Either ^{32}P-labeled poly(dA)$_{250}$ (50,000 cpm [2 ng] per reaction mixture) or ^{32}P-labeled oligo(dT)$_{12-18}$ was incubated with the indicated amount of RFC at 0°C in a 10-μl reaction mixture for 30 min. The buffer used was as described for the polδ synthesis assay but contained 60 mM NaCl and no nucleotides except γ-^{35}S-ATP

(10 μM). Reaction mixtures were filtered through alkali-washed nitrocellulose, and bound radioactivity was counted.

Protein analysis. SDS-PAGE of purified proteins was done as described previously (20), and proteins were silver stained by the method of Wray et al. (55).

RESULTS

Identification and purification of RFC. RFC from human cells was originally identified as an essential factor required for SV40 DNA replication in vitro (47). Subsequent biochemical analysis of RFC demonstrated that it was a DNA-dependent ATPase and a primer-template DNA-binding protein. Moreover, RFC cooperatively stimulated polδ activity in the presence of RPA and PCNA (46). In this previously described reaction, PCNA, RPA, and RFC act cooperatively to stimulate polδ DNA synthesis on a uniquely primed, single-stranded M13 template. The addition of RFC to the assay results in a 25-fold increase in DNA synthesis and production of long DNA strands. Therefore, we devised a similar assay using polδ, RPA, and PCNA from *S. cerevisiae* to identify yeast RFC.

A necessary first step in developing this assay was the purification of yeast polδ, PCNA, and RPA. Polδ was purified from *S. cerevisiae* by a modified version of a published method (3) and was shown by SDS-PAGE to have a similar polypeptide structure (125, 60, 55, and 53 kDa). The gene for *S. cerevisiae* PCNA (2) was cloned into a phage T7 expression vector, and PCNA was purified from *E. coli*. This PCNA migrated as a 33-kDa polypeptide on SDS-PAGE and stimulated DNA synthesis by purified polδ threefold, with poly(dA) · oligo(dT) as a primer-template. The products of this reaction were long DNA strands consistent with PCNA functioning as a processivity factor for yeast polδ (1). In contrast, PCNA did not affect polδ activity when natural DNA templates were used. RPA, which contains subunits of 69, 36, and 13 kDa, was purified by a published method (8). An SDS-polyacrylamide gel of the proteins used in these experiments is shown in Fig. 1. Studies of the replication properties of these purified proteins show that these yeast activities behave similarly to their human homologs. PCNA did not stimulate DNA synthesis, nor did it lengthen the products formed by polδ on a uniquely primed, circular, single-stranded DNA (primed pUC118) in the presence or absence of RPA. By analogy with the human system, it seemed likely that a yeast homolog of RFC might be required for DNA synthesis by polδ. We therefore used this system to search for activities in yeast extracts that would stimulate DNA synthesis by polδ.

Two fractions derived from an *S. cerevisiae* extract were added to reaction mixtures containing primed pUC118 single-stranded DNA, polδ, PCNA, and RPA (or *E. coli* SSB) to identify an RFC activity. These two fractions were obtained by fractionating a crude enzyme extract by phosphocellulose column chromatography and eluting proteins with either 0.33 or 0.66 M NaCl. The 0.33 M NaCl fraction, known to contain the majority of DNA polymerase activity (data not shown), did not stimulate DNA synthesis. Upon addition of a 0.66 M NaCl fraction, DNA synthesis was stimulated 10-fold, and therefore this fraction was used for further purification.

The chromatographic scheme used to purify the stimulatory factor consisted of fractionation on hydroxylapatite, a second phosphocellulose column, single-stranded DNA cellulose and Mono Q column chromatography, and, finally, glycerol gradient sedimentation (Table 1). The specific activities recorded in Table 1 for fractions 1 and 2 are high compared with the specific activities of later fractions because of the assay measuring RFC activity as well as a cofractionating but minor DNA polymerase activity. This DNA polymerase activity separated from RFC during chromatography on the second phosphocellulose column. There were two peaks of stimulatory activity eluting from this column, one eluting with 0.25 M KPO_4 and another with 0.4 M KPO_4. We discriminated between RFC activity and that of the contaminating DNA polymerase by observing whether the two activities were dependent on the addition of exogenous polδ. Thus, while RFC activity (0.25 M PO_4) required the addition of polδ to stimulate DNA synthesis, the contaminating DNA polymerase activity (0.4 M PO_4) did not. Only the first peak of activity exhibited polδ dependence and was further fractionated. No RFC activity could be found in the second peak, even after further fractionation.

TABLE 1. Purification of scRFC

Step and fraction	Total protein (mg)	Activity	
		Total (U)	Specific (U/mg)
1. Phosphocellulose	336.00	369,231	1,099[a]
2. Hydroxylapatite	93.00	47,320	509[a]
3. Phosphocellulose	33.60	13,200	393
4. ssDNA[b] cellulose	1.00	4,824	4,824
5. Mono Q	0.08	1,430	17,875
6. Glycerol gradient	0.04	1,080	27,000

[a] Specific activity calculations reflect RFC as well as a contaminating polymerase activity (see Materials and Methods).
[b] Single-stranded DNA.

Glycerol gradient analysis of the peak Mono Q fraction showed that a single peak of DNA synthesis activity (Fig. 2A) sedimented with an apparent molecular mass of 180,000 Da. Examination of the glycerol gradient fractions by SDS-PAGE showed that fractions containing this activity had four polypeptides with apparent relative mobilities of 110, 42, 41, and 36 kDa (Fig. 2B). Since these four polypeptides cofractionated on a number of different chromatographic resins and cosedimented in a glycerol gradient with RFC activity, it is likely that yeast RFC exists as a multiprotein complex. Furthermore, the sizes of the yeast RFC subunits are similar to those of human RFC, which is a complex of polypeptides with molecular masses of 140, 42 (doublet), and 37 kDa. We refer henceforth to this protein complex as scRFC to distinguish it from the analogous activity from human cells, termed hRFC. Similarly, we refer to the other yeast activities involved in scRFC function as scRPA, scpolδ, and scPCNA when it is necessary to distinguish them from their human-cell counterparts.

DNA binding activity of scRFC. scRFC was demonstrated to stimulate DNA synthesis by polδ in the presence of scPCNA and scRPA in a manner similar to that of hRFC in the presence of the analogous human activities (Fig. 2A). Previous studies have demonstrated that hRFC bound in a structure-specific manner to single-stranded DNA (a template for DNA synthesis) annealed to an oligonucleotide primer. To test whether scRFC had a similar binding specificity, a nitrocellulose filter binding assay was used with ^{32}P-labeled poly(dA) and specified amounts of oligo(dT) (Fig. 2A and 3A). Analysis across the glycerol gradient showed that a primer-template DNA binding activity cosedimented with scRFC activity (Fig. 2A). Furthermore, in the absence of oligo(dT), scRFC failed to bind poly(dA) (Fig. 3A). In contrast, the addition of increasing amounts of

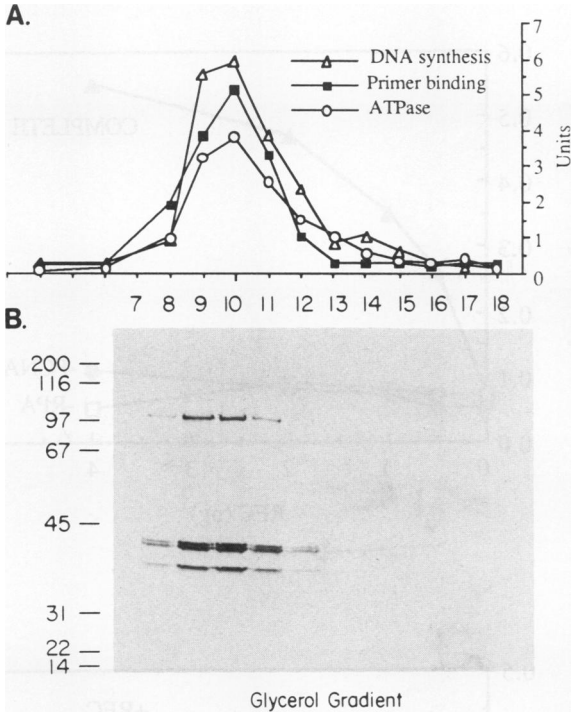

FIG. 2. Glycerol gradient sedimentation of RFC. (A) A Mono Q fraction was sedimented in a 15 to 35% glycerol gradient. RFC was assayed by stimulation of polδ on primed pUC118 single-stranded DNA in the presence of RPA and PCNA (units are in tens). In addition to this assay, the fractions were also assayed for a DNA-stimulated ATPase activity (picomoles of P_i released divided by 50) and a DNA-primer binding activity (DNA bound, percent input divided by 10). Sedimentation was from right to left. (B) A sample from every fraction of the gradient was subjected to SDS-PAGE analysis, and fractions 6 through 18 are shown. Lane numbers correspond to numbers of the fractions. Molecular mass markers (in kilodaltons) for the SDS-polyacrylamide gel: myosin (200), β-galactosidase (116), phosphorylase *b* (92), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (31), trypsin inhibitor (21), lysozyme (14).

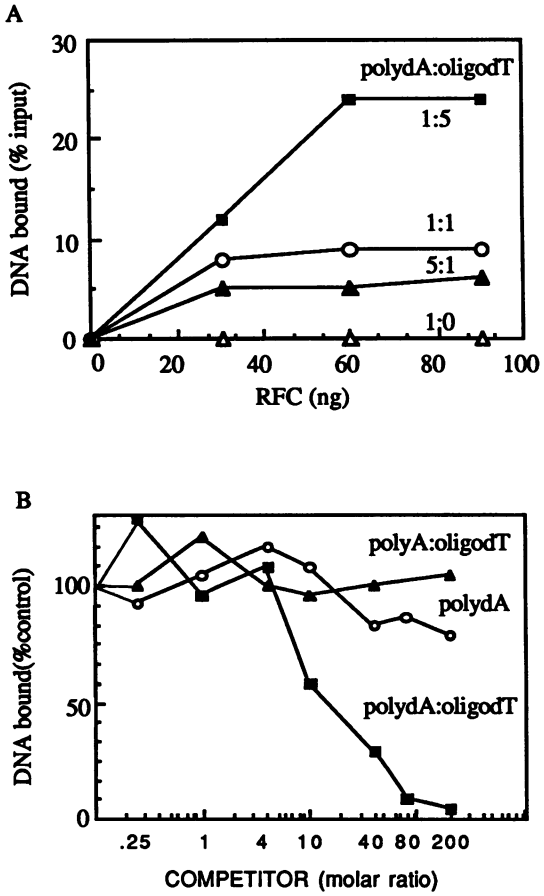

FIG. 3. DNA binding activity of RFC. (A) Ability of RFC to bind [^{32}P]poly(dA)$_{250}$ with increasing amounts of oligo(dT)$_{12-18}$ annealed. The ratios reflect moles of poly(dA) to moles of oligo(dT). Nitrocellulose filter binding reaction mixtures were incubated for 30 min at 0°C and then filtered through alkali-washed nitrocellulose. The 100% value of input poly(dA) was 50,000 cpm/2 ng of nucleotide. (B) Ability of different DNAs or RNAs to compete for RFC binding to DNA primer ends. Reaction mixtures contained 30 ng of RFC and poly(dA) · [^{32}P]oligo(dT) with the indicated molar amounts of competitor. The 100% value corresponds to 15% binding of the input label.

oligo(dT), which increased the primer-to-template ratio, resulted in increased retention of the labeled poly(dA). The DNA binding specificity of scRFC was further analyzed by measuring the ability of different DNAs to compete with scRFC for binding to poly(dA) · oligo(dT). Neither poly(dA) nor poly(dA) · oligo(dT) competed for scRFC binding; however, oligo(dT)-primed poly(dA) effectively competed for binding (Fig. 3B).

Characterization of scRFC ATPase. A second well-characterized function of the T4 phage 44/62 and hRFC proteins is their DNA-dependent ATPase activity. Consistent with scRFC being the yeast homolog of hRFC, a DNA-activated ATPase activity cosedimented with the complex of four scRFC subunits in a glycerol gradient (Fig. 2A). We demonstrated that, like hRFC (23, 48), this ATPase was stimulated by poly(dA) · oligo(dT) (data not shown) and PCNA (Fig. 4). There appears to be a very weak, intrinsic ATPase activity in the PCNA preparation. This contaminating ATPase can be removed from purified PCNA (data not shown).

Cooperative stimulation of polδ. Experiments were performed with scRPA, scRFC, scPCNA, and scpolδ to test for cooperative stimulation of DNA synthesis. Previous experiments have shown that hRPA, hPCNA, and hRFC function cooperatively to stimulate the activity of polδ isolated from calf thymus or human cells (21, 46). These four DNA replication factors combined to synthesize long DNA strands, consistent with their role as parts of a leading-strand DNA polymerase holoenzyme. To test for a similar cooperative interaction with yeast replication factors, a single-stranded DNA template 3 kb in length and annealed to a unique oligonucleotide primer was used. In this reconstituted system, containing all three accessory factors plus scpolδ, an eightfold stimulation of scpolδ activity was observed with optimal amounts of scRFC (Fig. 5A). This synthesis was absolutely dependent upon scPCNA. In the absence of scRPA, a small amount of incorporation was observed. Figure 5B demonstrates that scRFC is required for DNA synthesis by scpolδ reaction with various concentrations of bacterially expressed PCNA. Addition of 400 pg of scPCNA, an amount calculated to be a 3-to-1 molar ratio of scPCNA to template, saturated the reaction mixture. This finding suggests that stoichiometric amounts of scPCNA efficiently stimulate the reaction.

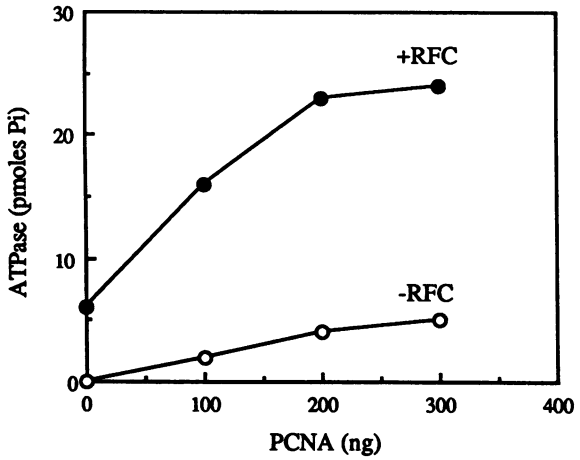

FIG. 4. ATPase activity of RFC. Titrations of PCNA with (+) and without (−) 2 ng of RFC. Reaction mixtures contained 12 μM poly(dA) · oligo(dT) (as nucleotide) and [γ-^{32}P]ATP, and the amount of P_i released was measured.

The products of these and other reactions were also examined by alkaline agarose gel electrophoresis. scpolδ in combination with either scRPA or scPCNA or both showed minimal DNA synthesis (Fig. 6, lanes 1, 2, and 4), and no products could be detected by autoradiography. scpolδ in the presence of scRFC yielded a small amount of DNA synthesis, and short products could be seen (approximately 100 bases in length) (Fig. 6, lane 3). Addition of scRPA inhibited this small amount of DNA synthesis and the short DNA products (Fig. 6, lane 5). In contrast, DNA synthesis by scpolδ was stimulated by scPCNA with scRFC, and the distribution of DNA products extended to full length (Fig. 6, lane 6). Addition of scRPA to this reaction mixture stimulated DNA synthesis fourfold more but did not alter the size distribution of DNA products (Fig. 6, lane 7).

We found that hRFC could not substitute for scRFC in cooperatively stimulating scpolδ in the presence of scRPA and scPCNA (Fig. 7). Equal-mass amounts were added to the reaction mixture as determined by silver staining of an SDS-polyacrylamide gel. As much as 30 ng of hRFC did not stimulate DNA synthesis in this reaction (data not shown). This result demonstrated that the interaction of scRFC with either scpolδ or scPCNA has species-specific requirements.

DISCUSSION

RFC activity from *S. cerevisiae* was purified and characterized and shown to share many activities with its human-cell and phage T4 counterparts. Importantly, one of these activities includes its stimulation of DNA synthesis by scpolδ in the presence of scPCNA and scRPA. With the identification of scRFC, it will now be possible to pursue a genetic analysis to obtain information about its in vivo function.

The yeast factor was purified to homogeneity by six steps and shown to consist of four polypeptides. The comigration of these polypeptides on a number of different chromatographic resins argues that they form a complex constituting the scRFC activity. Assuming a 1:1:1:1 stoichiometry of these polypeptides, the complex has a molecular mass of 230 kDa. This molecular mass is consistent with its glycerol gradient sedimentation behavior, further indicating that these four polypeptides exist as a complex.

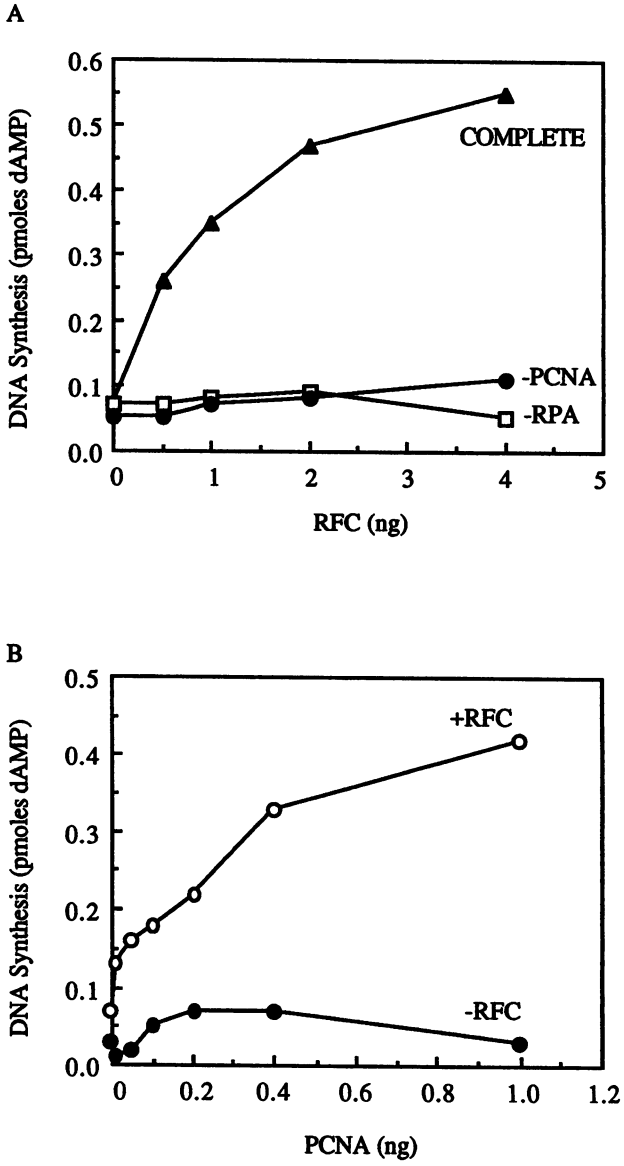

FIG. 5. Stimulation of polδ DNA synthesis activity by RFC, RPA, and PCNA. Titrations of RFC (A) and PCNA (B) in polδ DNA synthesis reactions. A typical reaction mixture (10 μl) contained 40 ng of primed, single-stranded pUC118 DNA, 200 ng of RPA, and 0.1 U of polδ. Titrations of the indicated amounts of PCNA and RFC were performed. In reactions with RFC and PCNA, 4 and 1.6 ng were used, respectively. Reaction mixtures were incubated for 30 min at 37°C, and incorporated radioactivity was measured. + and −, presence and absence of a component, respectively.

scRFC is structurally and functionally related to hRFC by a number of criteria. scRFC behaves similarly to hRFC on several chromatographic resins and exists as a multisubunit protein with polypeptide molecular weights remarkably similar to those of hRFC. The apparent number of hRFC polypeptides can vary from three to five, depending on gel electrophoresis conditions, suggesting that at least one of the doublets is posttranslationally modified. scRFC exhibits three activities previously found to be associated with hRFC: primer-template DNA binding, DNA-activated ATPase, and polδ stimulation (21, 23, 46, 48–50). It has been shown that the 140-kDa subunit of hRFC binds primer-template DNA and

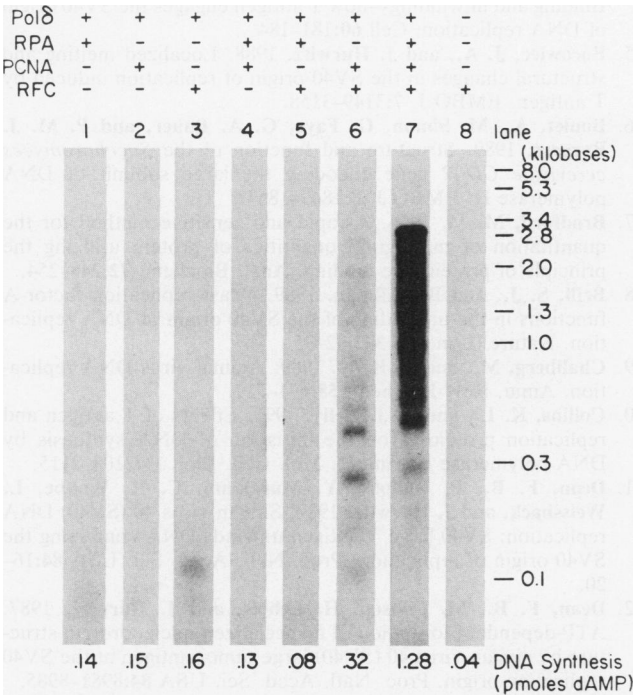

FIG. 6. Product analysis of DNA synthesis by polδ on primed single-stranded pUC118 DNA in the presence or absence of RPA, RFC, and PCNA. Reaction mixtures contained 40 ng of primed, single-stranded pUC118 DNA, 0.1 U of polδ (lanes 1 through 7), 150 ng of RPA (lanes 1, 4, 5, and 7), 120 ng of PCNA (lanes 2, 4, 6, and 7), and 30 ng of RFC (lanes 3, 5, 6, 7, and 8). The presence and absence of components are indicated by + and −, respectively. Reaction mixtures were incubated for 37°C for 30 min and then subjected to electrophoresis in a 1% alkaline agarose gel. Markers were derived from *Hin*dIII-digested adenovirus type 2 DNA. All samples were run on the same gel, and reactions 2, 4, and 6 were done in an experiment separate from that of reactions 1, 3, 5, 7, and 8.

that the 41-kDa subunit binds ATP (49). By analogy, we suggest that the scRFC 110-kDa subunit functions in primer-template binding and that the 42- and/or 41-kDa subunit(s) functions as an ATPase.

The scRFC doublet at 41 and/or 42 kDa might be due to phosphorylation. scRPA is an example of a posttranslationally modified replication protein, in which the p36 subunit exists as a doublet because of phosphorylation in the S and G_2 phases of the cell cycle (36). Phosphorylation of scRFC could trigger the activity of the enzyme, change its location within the cell, and/or allow it to associate with or dissociate from other proteins of the DNA replication apparatus. Experiments are under way to address these questions directly.

It is interesting that either *E. coli* SSB or scRPA can function to stimulate scpolδ in the presence of scPCNA and scRFC. This lack of species specificity also occurs in the human leading-strand complex, in which *E. coli* SSB, adenovirus DNA-binding protein, or the plage T4 gene 32-encoded protein, all single-stranded DNA-binding proteins, can substitute for human SSB (21). These data, along with DNase I footprinting experiments using hRFC in the presence of hPCNA and hRPA (49, 50), indicate that the function of an SSB in the cooperative stimulation of polδ may be to facilitate the recognition of primer-template junctions by RFC-PCNA by removing secondary structures from DNA. Alternatively, the coating of single-stranded DNA by RPA might exclude RFC-PCNA from binding nonspecifically to single-stranded DNA. These models are supported by our finding that the addition of scRPA to reaction mixtures containing scRFC and scPCNA results in a fourfold stimulation of DNA synthesis by scpolδ but that the distribution of DNA products is unchanged (Fig. 6, lanes 6 and 7). Thus, RPA allows polδ to initiate synthesis more frequently, but its processivity is unchanged. It might be that the effective concentration of RFC and PCNA is higher in the presence of RPA, allowing more efficient recognition of primer ends by polδ.

There is an apparent discrepancy in the ATPase and DNA synthesis assays. Full stimulation of the ATPase activity of 2 ng of RFC required 200 ng of PCNA. For DNA synthesis, however, 0.8 ng of PCNA was required to fully stimulate the replication activity of 4 ng of RFC. The difference in the protein ratios required for the two assays is 500-fold. It is possible that the PCNA-stimulated ATPase activity of RFC is not required for replication. Alternatively, PCNA might efficiently activate the RFC ATPase activity when both proteins are part of a multiprotein complex at the replication fork.

There is a species-specific requirement for scRFC in the scpolδ replication complex, since hRFC cannot substitute for scRFC. It is likely, therefore, that RFC will have interacting domains with PCNA, polδ, or both. The cooperative manner in which these factors interact supports this idea. Other proteins likely to interact include PCNA and polδ (PCNA is the polδ processivity factor) and PCNA and RFC (PCNA and RFC are required for ATPase stimulation). Further study of the species-specific interactions among these different proteins may help to identify interacting domains.

Polδ, PCNA, RFC, and RPA have been shown to be required for leading-strand synthesis during SV40 DNA replication. In the bacteriophage T4 system, the functional homologs of these activities are the products of gene 43 (polymerase), genes 44/62 (RFC), and gene 45 (PCNA).

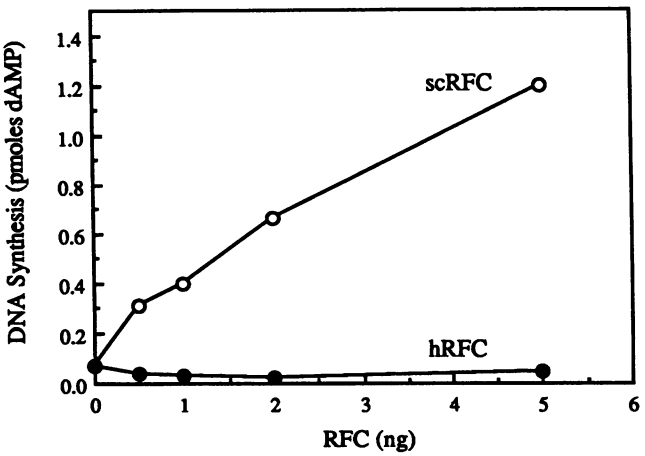

FIG. 7. Titrations of scRFC and hRFC in DNA synthesis reactions containing scpolδ, scPCNA, and scRPA. Reaction mixtures contained 40 ng of primed single-stranded pUC118 DNA, 0.1 U of scpolδ, 120 ng of scPCNA, and 150 ng of scRPA. The indicated amounts of scRFC and hRFC were added to the reaction mixtures and incubated for 30 min at 37°C. Incorporated radioactive nucleotides were measured.

These T4 proteins constitute the holoenzyme complex and are required for both leading- and lagging-strand synthesis of phage T4 DNA replication in vitro. In fact, the prokaryotic T4 DNA polymerase holoenzyme complex can substitute for hpolδ, hPCNA, and hRFC during replication of SV40 DNA (27, 45). Thus, in both eukaryotes and prokaryotes there are two polymerase complexes required at a replication fork, one for leading- and the other for lagging-strand synthesis. However, the eukaryotic system is more complicated, requiring distinct multisubunit polymerases.

Primase activity, which is uniquely associated with polα, is required to synthesize the primers for leading and lagging strands. DNA polδ has been shown to function for leading-strand synthesis during SV40 DNA replication and is required for S-phase transition in *S. cerevisiae* (6, 39). Recently, a third essential DNA polymerase, polε, in *S. cerevisiae* has been described (29). Therefore, it will be necessary to address the exact roles of polδ and polε in replication of cellular DNA. Biochemically, however, DNA replication in *S. cerevisiae* is proving to be very similar to that in SV40, during which polδ, RFC, PCNA, and RPA constitute a processive replication complex. The yeast proteins are conserved in function and subunit structure compared with the human enzymes, suggesting that these yeast factors might constitute the leading-strand DNA replication complex in *S. cerevisiae*.

The purification of scRFC completes the identification and purification of all seven yeast homologs of human proteins required for SV40 DNA replication in vitro. These proteins include RPA, PCNA, polδ, polα, topoisomerases I and II, and now RFC. The cellular equivalent(s) of TAg has not been identified, however, in yeast or human cells. This protein(s) would likely have an origin recognition function and helicase activity. In *E. coli*, these functions are fulfilled by the three different products of the genes: DnaA, DnaB, and DnaC. The yeast system now offers a biochemical approach to identifying a eukaryotic initiation protein. The use of scRFC in the presence of DNA polymerases, their accessory factors, topoisomerases, and a yeast origin of replication may be of use in identifying such an initiation protein(s). Alternatively, the yeast genetic system can now be exploited. The identification of scRFC genes will allow the investigation of the in vivo function of scRFC, with the possibility of identifying interacting genes through mutagenesis and a search for suppressors. Such an approach may help to reveal the function of numerous genes identified as DNA synthesis mutants that have yet to be assigned roles in DNA replication.

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