Reconstitution of Recombinant Human Replication Factor C (RFC) and Identification of an RFC Subcomplex Possessing DNA-dependent ATPase Activity*

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Replication factor C (RFC) is a five-subunit protein complex required for coordinate leading and lagging strand DNA synthesis during S phase and DNA repair in eukaryotic cells. It functions to load the proliferating cell nuclear antigen (PCNA), a processivity factor for polymerases δ and ε, onto primed DNA templates. This process, which is ATP-dependent, is carried out by 1) recognition of the primer terminus by RFC (2) binding to and disruption of the PCNA trimer, and then 3) topologically linking the PCNA to the DNA. In this report, we describe the purification and properties of recombinant human RFC expressed in Sf9 cells from baculovirus expression vectors. Like native RFC derived from 293 cells, recombinant RFC was found to support SV40 DNA synthesis and polymerase δ DNA synthesis *in vitro* and to possess an ATPase activity that was highly stimulated by DNA and further augmented by PCNA. Assembly of RFC was observed to involve distinct subunit interactions in which both the 36- and 38-kDa subunits interacted with the 37-kDa subunit, and the 40-kDa subunit interacted with the 36-kDa subunit-37-kDa subunit subcomplex. The 140-kDa subunit was found to require interactions primarily with the 38- and 40-kDa subunits for incorporation into the complex. In addition, a stable subcomplex lacking the 140-kDa subunit, although defective for DNA replication, was found to possess DNA-dependent ATPase activity that was not responsive to the addition of PCNA.

Knowledge of the mechanism of mammalian DNA replication is important not only for understanding how this process is regulated in normal cells, but also for the design of therapies against cancers in which this vital function is perturbed. Replication of the mammalian genome has been investigated *in vitro* using the SV40 DNA virus as a model system, because all of the factors required for SV40 DNA replication, except for large T antigen, the initiator protein and helicase, are host cell-encoded. Recently, all of the factors required for SV40 DNA replication *in vitro* have been identified, and data from numerous biochemical studies suggest the following model for how these factors function (Refs. 1 and 2, and references therein): 1) following template preparation (origin recognition and local unwinding, more extensive DNA unwinding by T antigen, and coating of the single strands by replication protein A (RPA)[1]),

primer synthesis and limited primer extension is carried out by the polymerase α primase complex to yield an initiator DNA; 2) a switch from usage of polymerase α to polymerase δ (pol δ) as the primary replicative polymerase is accomplished by recognition of the initiator DNA primer terminus by RFC; 3) next, PCNA, the processivity factor for pol δ, is loaded onto the DNA by RFC; 4) leading and lagging strand DNA synthesis is catalyzed by pol δ; and 5) finally, Okazaki fragments are processed by the coordinate activities of FEN-1, ribonuclease H, and DNA ligase I. By this model, a potential rate-limiting step for efficient DNA synthesis is the activity of RFC, which facilitates DNA synthesis by the highly processive pol δ. As implicated by *in vitro* studies, the importance of RFC for DNA replication has been confirmed by *in vivo* studies that have demonstrated that the *Saccharomyces cerevisiae* homologue of human RFC is essential for viability and functions in DNA replication and repair (3–8).

In contrast to the function of RFC in DNA replication, its role in DNA repair is less clear. Nevertheless, both biochemical and genetic studies have suggested a requirement for RFC in DNA repair (9–13). Mutations in the *S. cerevisiae* gene encoding the large subunit of RFC (*cdc44*) have been shown to render the cell sensitive to exposure to the alkylating agent methylmethane sulfonate and UV radiation, but not γ irradiation, suggesting a role for RFC in the base excision and nucleotide excision repair pathways (8). Consistent with this hypothesis, mutations in the *pol30* gene encoding PCNA have been found to suppress the DNA repair defect in *cdc44* mutants (8, 13). In addition, *in vitro* reconstitution studies have demonstrated a requirement for RFC for the pol δ (or ε)-catalyzed DNA synthesis step during nucleotide excision repair (14, 15).

RFC has been shown to consist of five subunits, one large subunit of approximately 100–140 kDa (140 kDa for human, 103 kDa for yeast) and four small subunits ranging from 40 to 36 kDa (16–20), which have been found to share considerable sequence identity and similarity. In addition, a significant degree of sequence identity has been found to exist between homologous subunits of not only yeast and human RFC but also between the eukaryotic RFCs and the *Escherichia coli* γ and δ′ subunits of the γ complex, the functional equivalent of RFC in prokaryotes (21, 22).

All five subunits of RFC have been shown to contain seven conserved regions that include the nucleotide binding motif found in all known ATPases, and the "DEAD box" motif found in RNA and DNA helicases (6). One additional region, conserved only among the large subunits, has been noted to share a significant degree of similarity with prokaryotic DNA ligases

[1] The abbreviations used are: RPA, replication protein A; RFC, replication factor C; PCNA, proliferating cell nuclear antigen; pol δ, polymerase δ; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; AcMNPV, *Autographa californica* nuclear polyhedrosis virus.

and has been termed therefore the "ligase homology box" (6). Biochemical studies have shown that RFC possesses a structure-specific DNA binding activity, displaying a preference for substrates with a 5′-overhang (23), and an ATPase activity that is stimulated by DNA and further augmented in the presence of PCNA (24, 17). To investigate the many interesting structure-function relationships in human RFC, we have reconstituted in active form a recombinant human RFC in Sf9 cells and characterized the subunit requirements for complex assembly and activity.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses—Recombinant baculoviruses expressing each of the subunits of replication factor C were created using the Baculogold baculovirus expression system developed by PharMingen. Baculovirus transfer vectors for each gene were constructed as follows. pVL1393–140kDa was constructed by removing the 140-kDa coding sequence from pRFC+ and cloning it into pVL1393. pVL1393–36kDa was made by removing the 36-kDa subunit coding sequence from p36NL with *Nde*I and *Eco*RI and placing it into the *Sma*I and *Eco*RI sites of pVL1393. pAcSG2–37kDa was generated by removing the gene for the 37-kDa subunit from pET-37kDa with *Nde*I and *Bam*HI and cloning it into the *Stu*I and *Bgl*II sites of pAcSG2. pVL1393–38kDa was created by removing the 38-kDa coding sequence from pSK-38kDa and placing it into the *Bam*HI and *Kpn*I sites of pVL1393.

Baculoviruses expressing NH_2-terminal HA epitope tagged 140- and 37-kDa subunits were generated by cloning each of these subunits in a CITE vector containing the HA epitope coding sequence (N-CITE, kindly provided by Drs. Bill Tansey and Winship Herr, Cold Spring Harbor Laboratory (CSHL)). The 140-kDa subunit sequence was cloned into N-CITE vector by digesting the plasmid pRFC+ with *Hae*II, ligating on the oligonucleotide *Hae*II (5′-AATTGCGC-3′; Nucleic Acid Chemistries facility, CSHL), purifying the DNA by Sephadex G-25 spin column chromatography, and then incubating the DNA with exonuclease VII. Afterward, the DNA was extracted with phenol-chloroform and digested with *Xba*I, and the 140-kDa gene fragment was gel-purified and ligated to N-CITE cleaved with *Sma*I and *Xba*I. The resulting derivative of N-CITE contained the 140-kDa subunit gene 3′ to the HA epitope coding sequence. The HA epitope-140-kDa subunit cassette was excised using *Nco*I and *Pst*I and cloned into the *Sma*I and *Pst*I sites of pVL1393.

For construction of an amino-terminal HA epitope 37-kDa subunit gene, the 37-kDa subunit coding sequence was cloned into the *Eag*I and *Pst*I of N-CITE by removing from pET-37kDa the gene plus an additional 1024 base pairs (746 base pairs 5′ and 278 base pairs 3′ of the 37-kDa subunit gene) with *Eag*I and *Nhe*I. The resulting plasmid was then digested with *Nde*I and *Sma*I and recircularized to remove the additional sequence at the 5′-end of the gene and to juxtapose the HA epitope and 37-kDa subunit coding sequences. The HA epitope-37-kDa subunit cassette was then removed with *Nco*I and *Bam*HI and cloned into the *Sma*I and *Bgl*II sites of pVL1393. The N-CITE derivatives were sequenced with the CITE-1 primer (5′-GGGGACGTGGTTTTCCTTT-3′; Nucleic Acid Chemistries facility, CSHL) to determine if in frame fusions were obtained, and the pVL1392 and pAcSG2 derivatives were sequenced with the BVPolH primer (5′-TCGTAACAGTTTTGTAATAA-3′; Nucleic Acid Chemistries facility, CSHL). The transfer vectors were used to create recombinant baculoviruses by homologous recombination in Sf9 cells. Baculovirus clones were isolated by plaque purification, amplified once, and then screened for expression of human RFC subunits by SDS-PAGE (Anderson's modified Laemmli procedure (25)) and immunoblotting. Viruses expressing RFC subunits were then further amplified until a titer of $>1 \times 10^8$ plaque-forming units/ml was achieved.

Expression and Purification of Human RFC in Sf9 Cells—Maintenance and infections of Sf9 cells were performed in Grace's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Hyclone). The optimal multiplicity of infection was empirically determined for each recombinant virus. Each virus was used at a multiplicity of infection of 10, and expression of more than one protein at a time was accomplished by the infection of multiple viruses simultaneously. For purification of the five-subunit RFC complex, cells in 5 T-175-cm² flask (2×10^7 cells/flask) were infected with each RFC subunit virus (bvHA-140kDa, bv40kDa, bv38kDa, bv37kDa, and bv36kDa), harvested by removal with a cell scraper 48 h postinfection, washed once with phosphate-buffered saline, and then resuspended in buffer PC (50 mM KPO_4,

pH 7.4, 7 mM CHAPS, 1 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin) plus 300 mM NaCl (10 ml of buffer/2×10^7 cells) and incubated on ice for 30 min. Next, the lysate (1.2 mg/ml) was disrupted by Dounce homogenization (10 strokes with a "B" pestle) and centrifuged at $16,000 \times g$ for 30 min at 4 °C, and the supernatant was incubated for 8 h at 4 °C with 200 μl/50 ml of clarified whole cell lysate of 12CA5-protein A-Sepharose (50% slurry, 500 μg to 1 mg of antibody/ml of bead) prepared as described by Harlow and Lane (26). After binding, the resin was washed three times for 15 min at 4 °C with 20 ml/wash of buffer PC plus 300 mM NaCl, and RFC was then eluted by incubating the beads at 37 °C for 15 min in 1 ml of buffer PC plus 300 mM NaCl plus 0.5 mg/ml HA peptide (YPYDVPDYA, Protein Chemistries Facility, CSHL). The elution was repeated two more times, and the first and second eluates were pooled (total of 90–100 μg of protein), concentrated 4-fold on a Centricon 30 (Amicon), and then 200 μl of the concentrated material was further purified by glycerol gradient sedimentation (4.8 ml of 15–40% glycerol gradient in buffer PC plus 300 mM NaCl centrifuged in a SW55Ti rotor at 50,000 rpm for 24 h at 4 °C). The gradients (two total) were fractionated from the top (200 μl/fraction) and assayed for DNA synthesis and ATPase activity. Active fractions were pooled and stored at −80 °C (total amount of protein was 50 μg). A subcomplex of RFC lacking the 140-kDa subunit was purified as described above, except the glycerol gradient was centrifuged for 19 instead of 24 h.

Immunoprecipitation of RFC Subunits—Infections and lysate preparation were performed as described for the RFC purification except that 1×10^7 cells were lysed with 1 ml of buffer PC plus 300 mM NaCl, and the lysate was not homogenized but repeatedly pipetted (10 times) before centrifugation. The clarified lysate was then incubated with 40 μl of 12CA5-protein A-Sepharose (50% slurry) for 4 h at 4 °C with rocking, and then the beads were collected by centrifugation at $1000 \times g$ in an Eppendorf microcentrifuge, washed for 15 min three times with buffer PC plus 300 mM NaCl, and finally resuspended in $2 \times$ SDS-PAGE sample buffer. The immunoprecipitates were analyzed by SDS-PAGE followed by silver staining and/or immunoblotting.

Purification of Other Replication Proteins—Human RFC was purified from 293 nuclei as described previously (16). DNA pol δ and topoisomerases I and II were purified from calf thymus as indicated in Refs. 16 and 27. SV40 large T antigen was purified from baculovirus-infected Sf9 cells as delineated in Ref. 28. PCNA and RPA were purified from *E. coli* as described previously (28, 29). Fraction IIA, a 0.2 M NaCl, 0.33 M NaCl phosphocellulose fraction, which supports SV40 DNA replication when supplemented with topoisomerases I and II, RPA, PCNA, and RFC, was prepared as described previously (16).

DNA Replication Assays—The in vitro assay for SV40 DNA replication was performed as described previously (16) using fraction IIA and purified SV40 large T antigen, topoisomerases I and II (1 unit of topoisomerase activity is the amount of enzyme that converts 50% of the substrate DNA into relaxed forms in 30 min at 37 °C in a 25-μl reaction containing 500 ng of DNA), RPA, PCNA, and RFC. 50-μl reactions were performed with 3000 units of topoisomerase I (1000 units/μl), 64 units of topoisomerase II (64 units/μl), 640 ng of RPA, 1.54 μg of PCNA, 10 μg of SV40 large T antigen, the indicated amount of RFC (and 0.56 mM CHAPS contributed by baculovirus-derived RFC), 6.7 μg of fraction IIA, and 500 ng of SV40 origin containing plasmid pSV011. The final NaCl concentration was 25 mM in the experiment described in Fig. 3*A* and 35 mM NaCl in that described in Fig. 9.

M13 DNA synthesis reactions were carried out as described previously (30) in 50 mM NaCl using 4 μg/ml single primed M13mp18 DNA (M13mp18 primer 4995; maps to nucleotide 4995), 10 ng of pol δ, 20 μg/ml PCNA, 10 μg/ml RPA, and the indicated amount of RFC (0.7 mM CHAPS contributed by baculovirus-derived RFC) in a 30-μl reaction. All reactions for both SV40 and M13 DNA synthesis were incubated for 1 h at 37 °C and stopped by the addition of 10 mM EDTA, and the dAMP incorporation was determined by spotting a fraction of the reaction onto DE81 paper (Whatman) that was then washed in 0.5 M Na_2HPO_4 as described previously (31). In addition, the replication products from SV40 DNA replication reactions were purified by proteinase K (200 μg/ml) treatment at 37 °C in 1% SDS, phenol/chloroform extraction, and ethanol precipitation and then analyzed by neutral agarose gel electrophoresis and autoradiography.

ATPase Assays—20-μl reactions were performed in 30 mM HEPES, pH 7.5, 30 mM NaCl, 1 mM dithiothreitol, 7 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 100 μM ATP, 3 pmol of [α-³²P]ATP (ICN Pharmaceuticals, Inc.; specific activity, 800 Ci/mmol), and 0.25 pmol of recombinant RFC (plus 0.7 mM CHAPS contributed by the protein) in the presence or absence of either 25 μM poly(dT)·oligo(dA) (Pharmacia Biotech, Inc.; 1:4 molar ratio of poly(dT) (average length of 250 bases) to oligo(dA) (length

of 20 bases)) or 25 μM poly(dT) only. For historical reasons, the five-subunit RFC complex glycerol gradient fractions were assayed in 50 mM Tris, pH 7.5, 2 mM MgCl$_2$, 30 mM NaCl, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 100 μM ATP, and 3 pmol of [α-^{32}P]ATP. The conditions for these reactions were subsequently discovered to be suboptimal; therefore, the specific activity of the recombinant RFC and stimulation by PCNA appears lower than in other experiments. Reactions were incubated for 1 h (or the indicated time) at 37 °C and stopped by the addition of EDTA to a final concentration of 10 mM, and the amount of ADP produced was determined by TLC. A small fraction of each reaction (2 μl) was spotted onto a polyethyleneimine-cellulose plate (pre-developed in 1 M formic acid), and the plate was then developed in a buffer of 1 M formic acid and 0.5 M LiCl$_2$ for 40 min. The amount of ADP produced was determined by scanning the plates using a FUJI BAS-100 phosphor imager.

Protein Quantitation—Protein preparations were quantitated by Bradford assay, using the Bio-Rad Bradford reagent, and by Coomassie-staining SDS-PAGE gels. Immunoblots were developed using the SuperSignal Substrate detection system (Pierce).

Other Reagents and Protocols—The 12CA5 monoclonal antibody against the HA epitope was obtained from the Monoclonal Antibody Facility at CSHL. The antibody against the 140-kDa subunit of RFC, monoclonal antibody 6, was generated as described previously (21). Rabbit antibodies directed against the 40- and 37-kDa subunits of RFC were kindly provided by Dr. Jerard Hurwitz, Memorial Sloane-Kettering Cancer Center. Protein A-Sepharose and CHAPS were purchased from Pharmacia and Sigma, respectively. Enzymes that were not purified in the laboratory were purchased from New England Biolabs. All other protocols that were not outlined were performed as described by Sambrook *et al.* (32).

RESULTS

Reconstitution and Characterization of Baculovirus Human RFC—Seven recombinant baculoviruses, each encoding one of the five RFC subunits were constructed and used to infect Sf9 cells. As shown in Fig. 1*A* (*lanes 5, 7, 8,* and *9*), the 40-kDa (*lane 5*), 37-kDa (both native NH$_2$ terminus (*lane 7*) and an NH$_2$-terminal HA epitope variant (*lane 8*)), and 36-kDa (*lane 9*) subunits were expressed well. In contrast, the 140-kDa (both native NH$_2$ terminus (Fig. 1*A, lane 3*) and NH$_2$-terminal HA epitope variant (Fig. 1*A, lane 4*)) and 38-kDa (Fig. 1*A, lane 6*) subunits were consistently not expressed as well. Expression of the large subunit was confirmed by immunoblotting (data not shown), and expression of the 38-kDa subunit was confirmed by analysis of protein synthesis by [^{35}S]methionine labeling of the cells 42 h postinfection, when viral mRNAs were predominantly translated (Fig. 1*B, lane 5*).

Using the HA epitope variant of the 140-kDa subunit (HA-140kDa), we observed co-purification of the 40-, 38-, 37-, and 36-kDa subunits with the HA-140kDa subunit on a column consisting of the monoclonal antibody 12CA5 linked to protein A-Sepharose (Fig. 2*A, lanes* labeled E_1 and E_2), suggesting that the RFC complex was reconstituted in the infected cells. A functional RFC complex was formed as judged by co-sedimentation of both DNA synthesis and ATPase activities (Fig. 2, *C* and *D*) with all five subunits in a 15–40% glycerol gradient (Fig. 2*B, lanes 14–17*). In addition, recombinant RFC was capable of supporting SV40 DNA synthesis *in vitro* as shown in Fig. 3*A*, with a specific activity comparable with that of authentic RFC isolated from human 293 cells as shown in Fig. 3*B*.

Similar to native RFC, the recombinant RFC was observed to hydrolyze ATP in a DNA-dependent manner, displaying a preference for primer-template substrates compared with unprimed single-stranded DNA (Fig. 4*A*). The approximate rate of ATP hydrolysis as determined from a time course of the reaction (Fig. 4*B*) was 0.95 pmol/min, indicating a specific activity of 3.7 mol of ADP produced/mol of RFC/min. The ATPase activity of recombinant RFC in the presence of DNA was found to be stimulated 4-fold by PCNA (Fig. 4*C*), and no stimulation by PCNA was observed in the absence of DNA or in the absence of primer (data not shown).
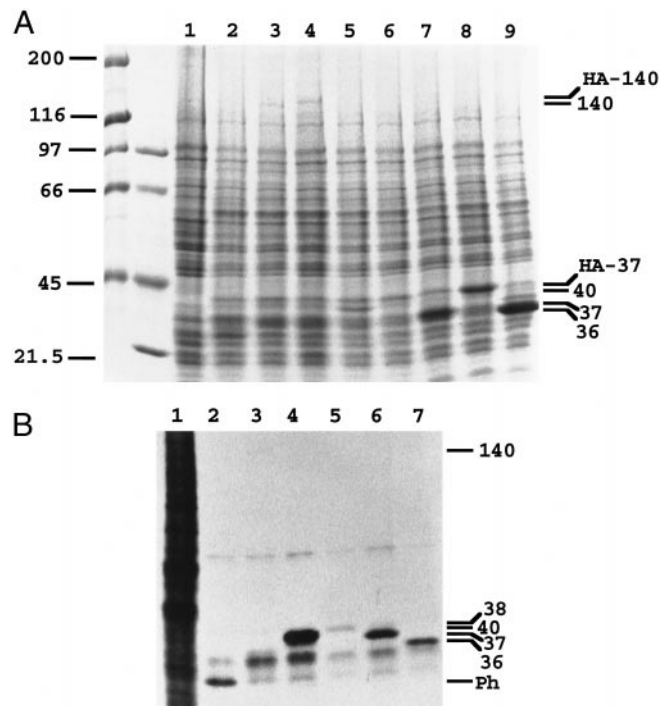

FIG. 1. **Expression of the subunits of human RFC in Sf9 insect cells.** Infections of Sf9 cells with recombinant baculoviruses each encoding a subunit of RFC were carried out as described under "Materials and Methods." *A*, 1 × 10^6 cells were infected with recombinant baculoviruses expressing the indicated RFC subunit. After 48 h, they were harvested and lysed in 1 × SDS-PAGE sample buffer and analyzed by SDS-PAGE. The resulting gel was stained with Coomassie. The *lanes* represent cells infected with wild-type AcMNPV (*lane 2*), bv140kDa (*lane 3*), bvHA-140kDa (*lane 4*), bv40kDa (*lane 5*), bv38kDa (*lane 6*), bv37kDa (*lane 7*), bvHA-37kDa (*lane 8*), and bv36kDa (*lane 9*). In *lane 1*, the cells were mock-infected with media. *B*, 1 × 10^6 cells were infected with the indicated baculoviruses. After 42 h, they were labeled for 20 min with [^{35}S]methionine. After harvesting, the cells were lysed in 1 × SDS-PAGE sample buffer and analyzed by SDS-PAGE and fluorography. Lysates from cells infected with the following viruses were loaded in the indicated *lanes*: wild-type AcMNPV (*lane 2*), bv140kDa (*lane 3*), bv40kDa (*lane 4*), bv38kDa (*lane 5*), bv37kDa (*lane 6*), bv36kDa (*lane 7*). In *lane 1*, the cells were mock-infected with media. The 140-kDa subunit typically was labeled poorly; therefore, expression of this subunit was confirmed by immunoblotting (data not shown).

Analysis of the Subunit Interactions Necessary for Complex Assembly—The arrangement within the RFC complex of the five subunits was investigated by performing immunoprecipitation experiments from extracts prepared from cells infected with subsets of viruses. Using an HA epitope-tagged variant of the 37-kDa subunit, we tested which of the other RFC subunits could associate with the 37-kDa subunit. When Sf9 cells were co-infected with all five RFC subunit viruses, bvHA-37kDa, bv140kDa, bv40kDa, bv38kDa, and bv36kDa, all of the untagged RFC subunits were observed to co-purify with the HA epitope-tagged 37-kDa subunit (Fig. 5*A*, silver-stained gels, *lane 5*; Fig. 5*B*, immunoblots of the samples in Fig. 5*A* probed with either an α-140kDa monoclonal antibody or α-40kDa rabbit antibody, *lane 5*). The samples in Fig. 5 were subjected to electrophoresis four separate times, and the silver-stained gels (Fig. 5*A*, *top* and *bottom*) were developed either completely (*top*) to visualize the 140-kDa subunit or less extensively (*bottom*) to view the small subunits, which, due to their similar molecular weights, run close together on the gel. Subsequent purification of the proteins shown in Fig. 5*A, lane 5*, by phosphocellulose chromatography, single-stranded DNA cellulose column chromatography, and glycerol gradient sedimentation confirmed the presence of a functional five-subunit RFC complex (data not shown). Thus, a functional five-subunit RFC complex could be
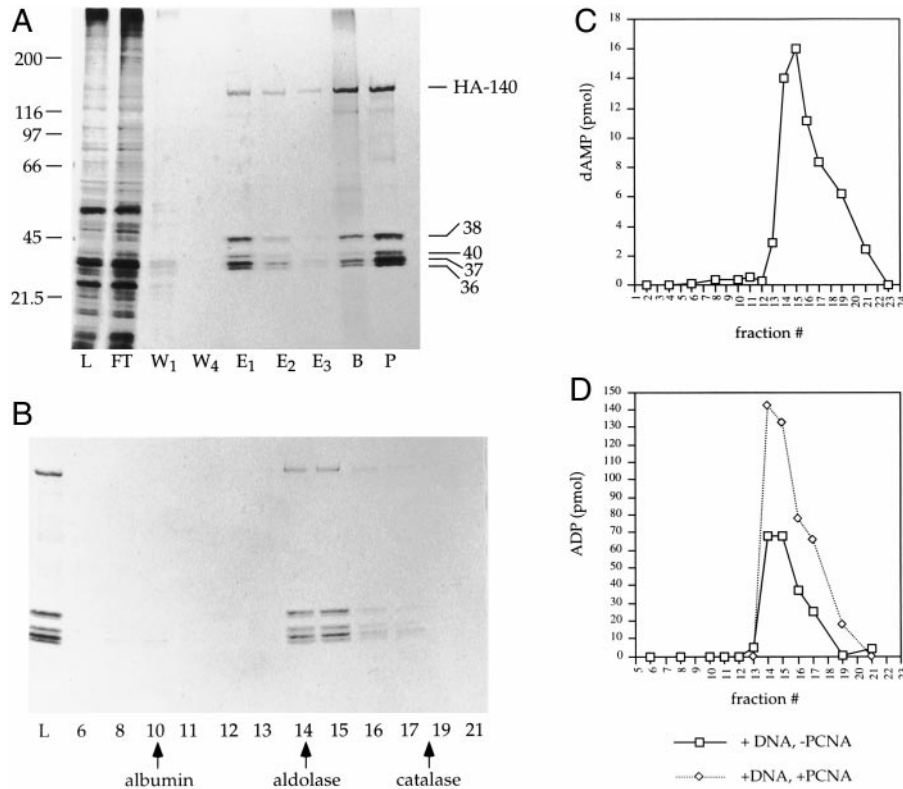
FIG. 2. **Purification of recombinant human RFC from Sf9 cells.** *Panel A*, infection and lysis of Sf9 cells was as described under "Materials and Methods." A whole cell lysate from 1×10^8 cells was incubated with 12CA5-protein A-Sepharose, after which the beads were washed and RFC was eluted with HA peptide (0.5 mg/ml) as described under "Materials and Methods." A fraction (5 µl) of each stage of the purification was analyzed by SDS-PAGE, and the resulting gel was silver-stained. *Lane L*, the load onto the beads; *lane FT*, the material not bound to the beads; *lane W₁*, the first wash from the beads; *lane W₄*, the fourth wash from the beads; *lanes E₁, E₂,* and *E₃*, the first, second, and third elutions, respectively, from the beads; *lane B*, the material that remained bound to the beads after the elutions; *lane P*, the concentrated pooled elutions. *Panel B*, glycerol gradient sedimentation profile of the concentrated pooled elutions from the 12CA5-agarose beads. The glycerol gradients (15–40%) were sedimented as described under "Materials and Methods," and 5 µl of each fraction was analyzed by SDS-PAGE and silver staining. *Lane numbers* correspond to fraction numbers (removed from the top of the gradient), and the *arrows below* denote the positions of the peaks of the protein standards albumin, aldolase, and catalase from an independent gradient simultaneously sedimented. *Panel C*, 1 µl of the glycerol gradient fractions in *panel B* were assayed for the ability to support pol δ DNA synthesis on singly primed M13 DNA as described under "Materials and Methods." *Panel D*, 2.5 µl of the glycerol gradient fractions in *panel B* were assayed for ATPase activity as described under "Materials and Methods." Each of the indicated fractions were tested in the absence (*squares*) and presence (*diamonds*) of 20 µg/ml PCNA. All reactions contained 25 µM poly(dT)·oligo(dA).

purified using two different epitope-tagged versions of the complex.

When the 140-kDa subunit was omitted from the co-infection, a quaternary complex consisting of the 40-, 38-, 37-, and 36-kDa subunits was observed (Fig. 5A, *lane 6*, and Fig. 5B, α40kDa immunoblot, *lane 6*), indicating no dependence on the 140-kDa subunit for the small subunits to assemble into a complex. However, assembly of the 140-kDa subunit into the complex was observed to be dependent upon the 38- and 40-kDa subunits, because omission of the 38-kDa subunit from the complex resulted in complete loss of the 140-kDa subunit from the complex (Fig. 5A, *lane 8*, and Fig. 5B, α140kDa immunoblot, *lane 8*), and omission of the 40-kDa subunit resulted in significantly reduced assembly of the 140-kDa subunit into the complex (Fig. 5A, *lane 7*, and Fig. 5B, α140kDa immunoblot, *lane 7*). The omission of the 36-kDa subunit was observed to diminish dramatically the incorporation of the 40-kDa subunit into the complex (Fig. 5A, *lane 9*, and α40kDa immunoblot in Fig. 5B, *lane 9*). In addition, a small reduction in the incorporation of the 140-kDa subunit (Fig. 5A, *lane 9*, and Fig. 5B α140kDa immunoblot, *lane 9*), presumably due to loss of the 40-kDa subunit, was detected. Neither the 38-kDa subunit nor the 36-kDa subunit was found to manifest a dependence on the other RFC subunits for assembly into a complex with the 37-kDa subunit, since omission of each of the other RFC subunits had no effect on the ability of the 36- and 38-kDa subunits to

co-immunoprecipitate with the 37-kDa subunit (Fig. 5A, *lanes 5, 6, 7,* and *9* (36-kDa subunit) and *lanes 5, 6, 7,* and *8* (38-kDa subunit)).

To further characterize the subunit interactions, we tested pairwise interactions between the untagged RFC subunits and either the HA-37kDa subunit (Fig. 6) or the HA-140kDa subunit (Fig. 7). The 36- and 38-kDa subunits were found to interact directly with the 37-kDa subunit (Fig. 6, *lanes 5* and *6*), whereas the 140- and 40-kDa subunits were not (Fig. 5, *lane 10*, and Fig. 6, *lane 4*). However, the 40-kDa subunit was discovered to interact with the 37-kDa subunit when the 36-kDa subunit was co-expressed with these two subunits (Fig. 6, *lane 9*). The interaction was found to be facilitated specifically by the 36-kDa subunit, since neither the 38-kDa subunit (Fig. 6, *lane 8*, and immunoblot analysis not shown) nor the 140-kDa subunit (data not shown) was capable of promoting this interaction. Thus, the 40-kDa subunit appears to interact either directly with the 36-kDa subunit, or with a unique interface created by the 36-kDa subunit-37-kDa subunit interaction.

Analyses of pairwise subunit interactions between untagged RFC subunits and the HA epitope-tagged 140-kDa subunit were difficult because of the substantial degradation of this subunit in the absence of more than one small subunit (Fig. 7A and data not shown). However, weak interactions between some of the small subunits and the HA-140kDa subunit were detectable when a single subunit was omitted from the co-

FIG. 3. **SV40 DNA synthesis *in vitro* by recombinant RFC and comparison of the pol δ-stimulatory activities of recombinant and authentic RFC.** *A*, reconstitution of SV40 DNA synthesis *in vitro* was performed as described under "Materials and Methods." The recombinant RFC was titrated in this assay, and the amount of DNA synthesis was quantitated and graphed. The DNA synthesis product from the reactions containing 0.03, 0.13, 0.26, and 0.51 pmol of RFC was analyzed by neutral agarose gel electrophoresis and autoradiography. The graph shows incorporation of dAMP in the reconstituted replication reaction. *B*, DNA synthesis by pol δ on singly primed M13 DNA was performed as described under "Materials and Methods." Both the recombinant RFC (*diamonds*) and authentic RFC derived from 293 cells (*squares*) were titrated in this assay, and the amount of DNA synthesis was graphed as a function of protein concentration.
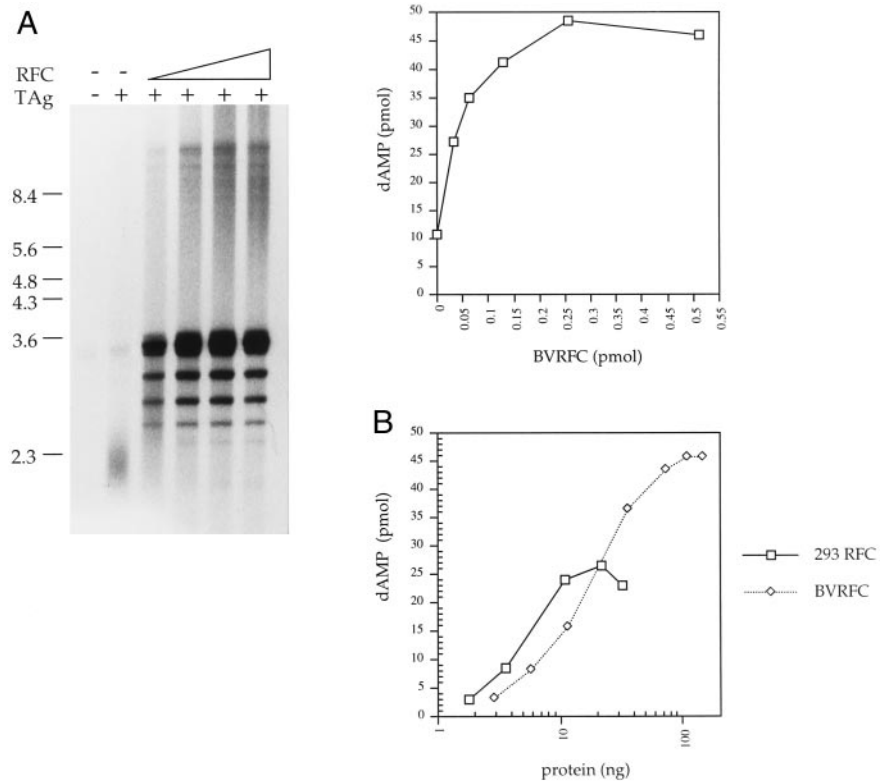
infections. When either the 40- or 38-kDa subunit was omitted, only the 36- and 37-kDa subunits were found to co-immunoprecipitate with the HA-140kDa subunit (Fig. 7*A*, silver-stained gel, *lanes 6* and *7*, and data not shown). Upon omission of the 37-kDa subunit, unexpectedly, only the 36-kDa subunit was found to interact with the 140-kDa subunit; neither the 40- nor the 38-kDa subunit was detected in the immunoprecipitate (Fig. 7*A*, *lane 8*; Fig. 7*B*, α40kDa immunoblot of the samples in Fig. 7*A*). When the 36-kDa subunit was excluded from the co-infection, an interaction between the HA-140kDa subunit and the 38-, 37-, and 40-kDa subunits was observed (Fig. 7*A*, *lane 9*; Fig. 7*B*, α40kDa immunoblot, *lane 9*). The detection of this complex is consistent with the results in Fig. 5 that show that a complex consisting of the 140-kDa, 40-kDa, 38-kDa, and HA-37kDa subunits can be formed in the absence of the 36-kDa subunit (Fig. 5, *lane 9*). The significance for RFC complex assembly of the interaction between the 140-kDa subunit and 36- and 37-kDa subunits is questionable for two reasons. 1) the 140-kDa subunit manifested no requirement for the 36-kDa subunit for incorporation into the RFC complex. As shown in Fig. 5, *lanes 7* and *9*, omission of the 40-kDa subunit (*lane 7*) had a greater effect on the assembly of the 140-kDa subunit into complex than omission of the 36-kDa subunit (*lane 9*), which instead hindered assembly of the 40-kDa subunit. 2) No direct interaction between the 140- and 37-kDa subunits was observed in the experiment in Fig. 5, *lane 10*. Therefore, the interaction between the 140-kDa subunit and 37-kDa subunit most likely is indirect and is mediated probably through the 36-kDa subunit, which was found to interact directly with the 37-kDa subunit (Fig. 6, *lane 6*). Thus, it appears that for RFC complex assembly (summarized in Fig. 7*D*), both the 38- and 40-kDa subunits are required for the 140-kDa subunit to assemble into the complex. These two subunits in turn interact with the 37- and 36-kDa subunits, the 38 kDa subunit directly with the 37-kDa subunit and the 40-kDa subunit either directly with the 36-kDa subunit or with a unique protein surface created by the interaction of the 36- and 37-kDa subunits.

A Subcomplex of RFC Lacking the 140-kDa Subunit Is Inactive for DNA Replication but Possesses ATPase Activity—Given the previous observations suggesting that a stable complex between the four small subunits of RFC can be formed in the absence of the large subunit, we next attempted to isolate the complex of four small subunits to analyze its biochemical properties. Similar to the entire RFC complex, the 40-, 38-, and 36-kDa subunits were observed to co-purify with the HA epitope-tagged 37-kDa subunits through 12CA5-protein A-Sepharose chromatography (Fig. 8*A*), and all four small subunits appeared to co-sediment on a 15–40% glycerol gradient (Fig. 8*B*, *lanes* corresponding to fractions 10 and 12). Fractions 10–12 from the glycerol gradient were pooled, and the purified small subunit complex was tested for the ability to support SV40 DNA replication, pol δ DNA synthesis on singly primed M13, and ATPase activity (Fig. 9 and Table I). Unlike the entire RFC complex, the small subunit complex appeared to be unable to support full SV40 DNA synthesis *in vitro*, with only aborted initiation events detected (Fig. 9 *lanes labeled BVRFC-140*) (16, 33). In the M13 DNA synthesis assay, no stimulation of pol δ DNA synthesis was observed with the amounts of small subunit complex used in the SV40 DNA synthesis experiment (data not shown). Collectively, these results indicate a requirement for the 140-kDa subunit for DNA synthesis.

When the small subunit complex was assayed for ATPase activity in the presence or absence of poly(dT)·oligo(A) (Table I), an ATPase activity that was highly stimulated (approximately 20-fold) by DNA was detected. Attempts to purify further the glycerol gradient pool by ion exchange chromatography on phosphocellulose, Affi-Gel blue Sepharose, and Q-Sepharose were futile, because under the conditions used, neither the RFC subunits nor this DNA-dependent ATPase activity interacted with any of these resins; both the activity and the RFC proteins were recovered in the flow-through (data not shown). Hence, this DNA-dependent ATPase activity appears to be intrinsic to the small subunits of RFC. In addition, the activity of the small subunit ATPase was examined in the presence of PCNA (Fig.
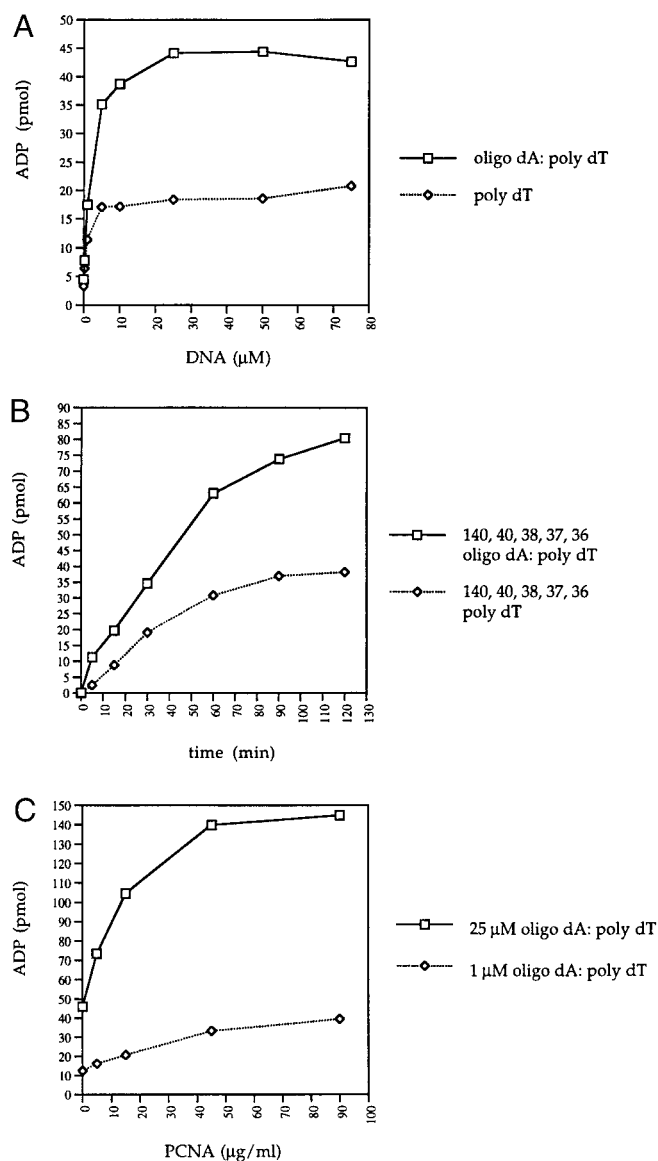
FIG. 4. **Characterization of the ATPase activity of recombinant human RFC.** ATPase assays were performed with 0.25 pmol of RFC and the indicated amount of either poly(dT)·oligo(dA) or poly(dT) only as described under "Materials and Methods." *A*, ATPase assays were performed in the presence of increasing concentrations (0.25, 1, 5, 10, 25, 50, and 75 μM) of either poly(dT)·oligo(dA) (*squares*) or poly(dT) only (*diamonds*). The amount of ADP produced by RFC in the absence of DNA in this experiment was 45 pmol. *B* shows the time course of ATP hydrolysis in the presence of either poly(dT)·oligo(dA) (*squares*) or poly(dT) only (*diamonds*). *C* shows an analysis of RFC ATP hydrolysis in the presence of increasing concentrations (5, 15, 45, and 90 μg/ml) of PCNA with either 25 μM poly(dT)·oligo(dA) (*squares*) or 1 μM poly(dT)·oligo(dA) (*diamonds*). The amount of ADP produced in the presence of 90 μg/ml PCNA with 25 μM poly(dT)·oligo(dA) but without RFC was 0.39 pmol.

10). In contrast to the entire five-subunit complex, this small subunit complex appeared to be refractory to PCNA, indicating a requirement for the 140-kDa subunit not only for DNA synthesis but also for PCNA-dependent ATPase activity.

DISCUSSION

Biochemical characterization of mammalian DNA replication has been hampered due to lack of sufficient quantities of the replication factors necessary for such studies. Here we report the reconstitution of human replication factor C in Sf9 cells, characterization of its activities *in vitro*, and analysis of the subunit requirements for complex assembly and activity.
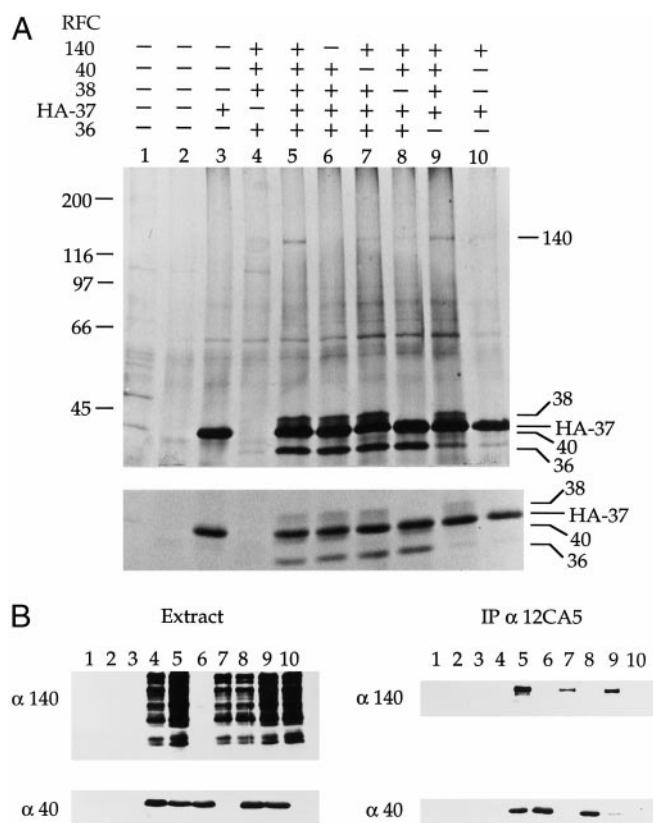

FIG. 5. **Immunoprecipitation of the 140-, 40-, 38-, and 36-kDa subunits with the HA-37kDa subunit.** Immunoprecipitation of the HA epitope variants of RFC using the α-HA monoclonal antibody 12CA5 was carried out as described under "Materials and Methods." The proteins bound to the beads were analyzed by SDS-PAGE, and the gels were subsequently silver-stained (*A*) or subjected to immunoblot analysis using antibodies against either the 140- or 40-kDa subunits (*B*). Lysates from cells infected with the pertinent viruses (denoted as *plus signs*) were analyzed and loaded in the numbered *lanes* as indicated. Loaded in *lane 1* is the immunoprecipitate from cells mock-infected with media, and in *lane 2* is the immunoprecipitate from cells infected with wild-type AcMNPV. *A*, two silver-stained gels, one developed thoroughly (*top*) and the other moderately (*bottom*), were prepared with the samples from the immunoprecipitation reactions described above to allow detection of the 140-kDa subunit (*top gel*) and 40-kDa subunit (*bottom gel*), respectively. *B* shows immunoblots of the lysates used for the immunoprecipitation reactions described above before the addition of 12CA5 (*left*) as well as the immunoprecipitation reactions (*IP*) (*right*) using antibodies against the 140-kDa subunit (*top*) and 40-kDa subunit (*bottom*).

Similar expression of RFC has been reported elsewhere (34–36). The five subunits of RFC share a significant degree of homology, which is manifested as seven conserved sequence motifs. These include the P-loop and DEAD-box motifs found in other ATP/GTPases. Despite the apparent functional similarity, *in vivo* the function of each subunit is not redundant. This raises the question of why so many similar subunits are necessary. The studies in this report suggest that all five RFC subunits are essential to form a stable complex competent for DNA synthesis. Central to the complex is the 37-kDa subunit, which functions as a scaffold on which the other subunits can assemble (Fig. 7*D*). The direct interaction of the 36- and 38-kDa subunits with the 37-kDa subunit are required for incorporation of the 40- and 140-kDa subunits, respectively, into the complex. The stability of the 140-kDa subunit in the complex depends on the presence of both the 38- and 40-kDa subunits. In the absence of the 140-kDa subunit, the 40-kDa subunit displays an absolute requirement for the 36-kDa subunit for incorporation into a subcomplex. However, a subcomplex con-
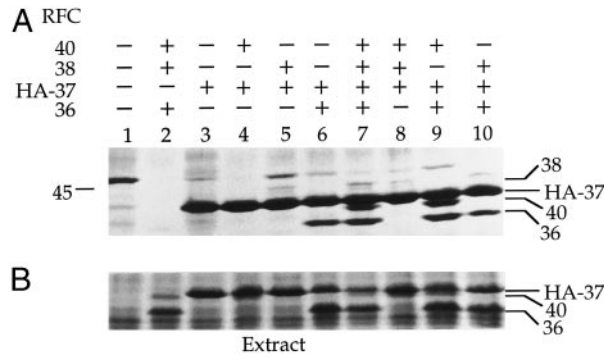
FIG. 6. **Immunoprecipitation of the 40-, 38-, and 36-kDa subunits with the HA-37kDa subunit of RFC.** The 40-, 38-, and 36-kDa subunits were examined for the ability to interact with the 37-kDa subunit. Infections with the indicated viruses, preparation of cell lysates, and immunoprecipitations were carried out as described under "Materials and Methods." 5-μl aliquots of the cell lysates were removed before the addition of 12CA5 and analyzed by SDS-PAGE and Coomassie staining (*B*). The immunoprecipitates were analyzed by SDS-PAGE and silver staining (*A*). The numbered *lanes* contain immunoprecipitates from the indicated infections, with viruses used for infections denoted as *plus signs*. The immunoprecipitate loaded in *lane 1* was from cells infected wild-type AcMNPV. The 38-kDa subunit was not visible in these whole cell lysates.

sisting of the 140-, 40-, 38-, and 37-kDa subunits can form, albeit inefficiently, in the absence of the 36-kDa subunit, implying that other protein-protein interactions, most notably with the 140-kDa subunit, may stabilize the 40-kDa subunit in the complex.

The subunit interactions required for the assembly of RFC have also been investigated by Uhlmann *et al.* (34) and Podust and Fanning (35). In each report (this one and the aforementioned ones), a stable interaction between the 40-, 37-, and 36-kDa subunits was reported. Moreover, this report and that of Podust and Fanning (35) have described the assembly of this subcomplex, which was found to involve a direct interaction between the 37- and 36-kDa subunits and the interaction of the 40-kDa subunit with the 37-kDa subunit-36-kDa subunit complex. A requirement for the 38-kDa subunit for incorporation of the 140-kDa subunit into the RFC complex has been shown here and in Uhlmann *et al.* (34). Unique findings regarding RFC assembly reported here are 1) a stable interaction of the 38-kDa subunit with the 37-kDa subunit that was not found to be dependent on any other subunit (Figs. 5*A* and 6), 2) the requirement for the 40-kDa subunit for efficient incorporation of the 140-kDa subunit (Fig. 5*A*), and 3) formation of a stable complex between the 140-, 40-, 38-, and 37-kDa subunits in the absence of the 36-kDa subunit (Figs. 5*A*, 7*A*, and 7*B*). Discordant with the results in Figs. 5*A* and 6*A* are those presented in Uhlmann *et al.* (34), which 1) implied that the 40-kDa subunit is dispensable for complex assembly and 2) did not address the dependence on the 36-kDa subunit for assembly of the 40-kDa subunit into the RFC complex but instead advocated a direct interaction between the 40- and 37-kDa subunits. The requirement for the 36-kDa subunit for a 37-kDa subunit-38-kDa subunit interaction described by Podust and Fanning (35) is contradictory to the results shown in Fig. 6, which demonstrated a direct interaction between the 38- and 37-kDa subunits in the absence of any other RFC subunit. In summary, we believe the RFC complex is probably organized as two "domains," one consisting of the 40-, 36-, and 37-kDa subunits and the other consisting of the 140- and 38-kDa subunits. These two subcomplexes are juxtaposed by the 40-kDa subunit-140-kDa subunit and 38-kDa subunit-37-kDa subunit interactions, which, if eliminated, are predicted to result in the loss of complex formation (see Fig. 7*D*). This model provides an expla-
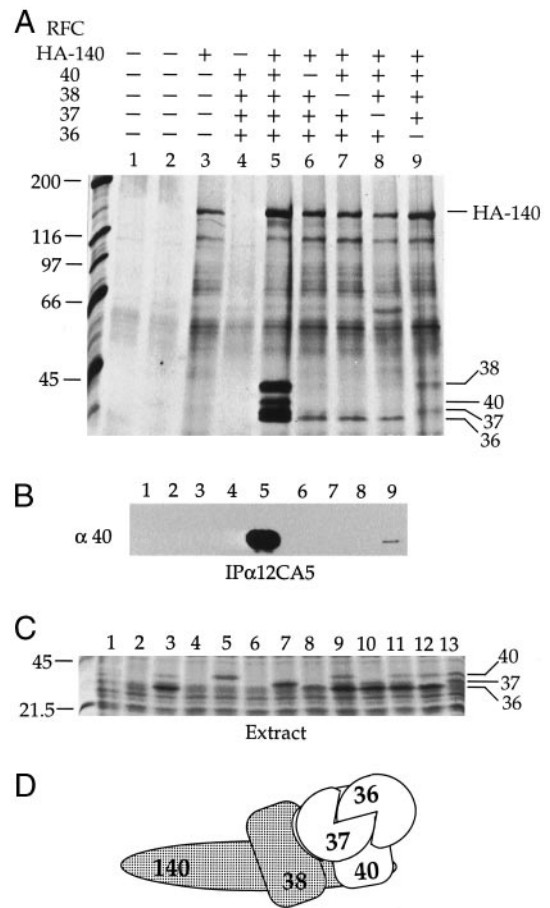

FIG. 7. **Immunoprecipitation of the small subunits of RFC with the HA-140kDa subunit.** The small subunits of RFC were tested for the ability to interact with the 140-kDa subunit. Lysates from cells co-infected with the viruses noted below were used in immunoprecipitation reactions (procedure described under "Materials and Methods"). The immunoprecipitates were analyzed by SDS-PAGE, and the gels were either silver-stained as shown in *panel A* or subjected to immunoblot analysis using an antibody against the 40-kDa subunit as shown in *panel B*. The numbered *lanes* were loaded with the immunoprecipitates from the indicated infections with the viruses used denoted by *plus signs*. In *lanes 1* and *2*, the cells were either mock-infected with media only (*lane 1*) or infected with wild-type AcMNPV (*lane 2*). In *panel C* is a Coomassie-stained gel of an aliquot (5 μl) of the lysates used for the immunoprecipitations in *panels A* and *B* plus lysates from additional control infections with single subunits, which were used for immunoprecipitation reactions but not loaded on the gels in *panels A* and *B* because they were redundant. The aliquots were removed before the addition of antibody. The numbered *lanes* correspond to cells infected with media only (*lane 1*); wild-type AcMNPV (*lane 2*); bv40kDa, bv38kDa, bv37kDa, and bv36kDa (*lane 3*); bvHA-140kDa (*lane 4*); bv40kDa (*lane 5*); bv38kDa (*lane 6*); bv37kDa (*lane 7*); bv36kDa (*lane 8*); bvHA-140kDa, bv40kDa, bv38kDa, bv37kDa, and bv36kDa (*lane 9*); bvHA-140kDa, bv38kDa, bv37kDa, and bv36kDa (*lane 10*); bvHA-140kDa, bv40kDa, bv37kDa, and bv36kDa (*lane 11*); bvHA-140kDa, bv40kDa, bv38kDa, and bv36kDa (*lane 12*); bvHA-140kDa, bv40kDa, bv38kDa, and bv37kDa (*lane 13*). In *panel D* is a model for the organization of the subunits within the RFC complex. The complex is illustrated as two "domains," one consisting of the 40-, 36-, and 37-kDa subunits and the other consisting of the 140- and 38-kDa subunits (depicted in *gray*). These two subcomplexes are connected by the 40-kDa subunit-140-kDa subunit and 38-kDa subunit-37-kDa subunit interactions, the abolition of which results in the loss of complex formation.

nation for why omission of the 140- and 36-kDa subunits from the complex permits the formation of stable quaternary complexes of the 140-, 40-, 38-, and 37-kDa subunits (Fig. 5*A*, *lane 9*; Fig. 7*A*, *lane 9*) and 40-, 38-, 37-, and 36-kDa subunits (Fig. 5*A*, *lane 6*; Fig. 6, *lane 7*), respectively.

The primary role of RFC during DNA synthesis is to load PCNA, the processivity factor for pol δ, onto the DNA, thus
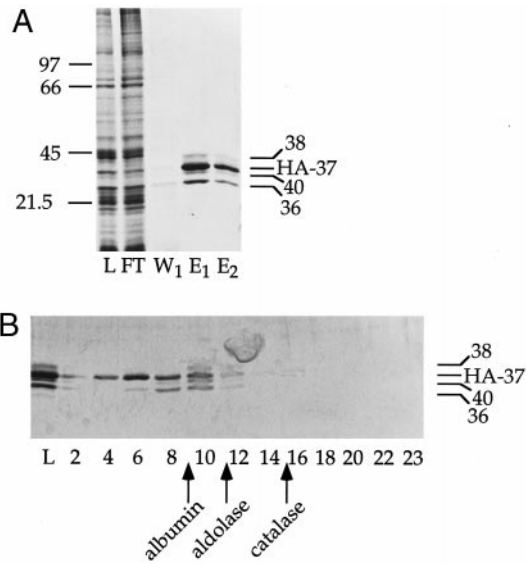
FIG. 8. **Purification of a subcomplex of RFC consisting of the four small subunits.** The infections, lysate preparation, immunoaffinity chromatography on 12CA5-protein A-Sepharose, and glycerol gradient sedimentation were accomplished as described under "Materials and Methods." 5-μl aliquots from each stage of the purification were removed and examined by SDS-PAGE and silver staining as shown in *panel A*. *Lane L*, the lysate before incubation with the beads; *lane FT*, the lysate after incubation with the beads; *lane* W_1, the first wash from the beads; *lanes* E_1 and E_2, the first and second elutions from the beads. *Panel B* shows fractions from the glycerol gradient (15–40%; fractionated from the top) that were analyzed by SDS-PAGE and silver staining. The *lane numbers* correspond to the fraction numbers, and the *arrows* denote the positions of the peaks of the protein standards from an independent gradient sedimented simultaneously.
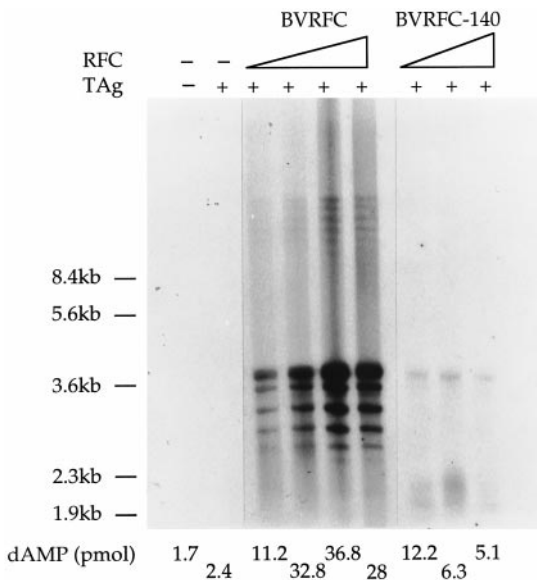

FIG. 9. **Impaired ability to support SV40 DNA replication by a subcomplex of RFC lacking the 140-kDa subunit.** *In vitro* SV40 DNA replication reactions were performed as described under "Materials and Methods" with increasing amounts of either the entire RFC complex (labeled *BVRFC*; 0.013, 0.03, 0.13, and 0.32 pmol used) or the 40-, 38-, 37-, and 36-kDa subunits (labeled *BVRFC-140*; 0.05, 0.13, and 1.3 pmol used). Graphed *below* is the amount of dAMP incorporated.

facilitating highly efficient leading and lagging strand DNA synthesis. This process entails recognition of the primer terminus by RFC, binding and disruption of the PCNA trimer, and topologically linking PCNA to the DNA. How these activities are distributed among the five subunits is unknown. *In vivo* experiments in yeast have shown a requirement for all five

TABLE I
ATPase activity of the four small subunit complex of RFC

Reactions were carried out as described under "Materials and Methods" in the presence or absence of 25 μM poly(dT) $\cdot$ oligo(dA) using 0.25 pmol of the entire RFC complex or 0.5 pmol of the four small subunit complex.

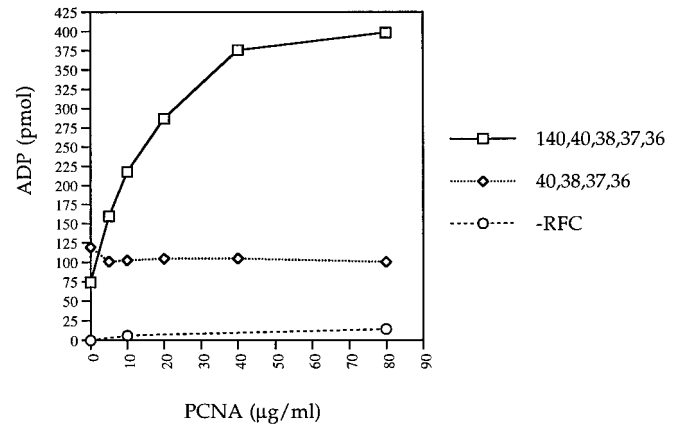| Protein complex		ADP
		pmol
140-, 40-, 38-, 37-, and 36-kDa subunits	Without DNA	3.5
	With DNA	31.2
40-, 38-, 37-, and 36-kDa subunits	Without DNA	1.1
	With DNA	29.9

FIG. 10. **The four small subunit complex ATPase activity is not responsive to PCNA.** ATPase reactions were performed in the presence of 25 μM poly(dT)·oligo(dA) as described under "Materials and Methods." The amount of ATP hydrolysis by 0.38 pmol of the entire RFC complex (*squares*) and the four small subunit complex (*diamonds*) were measured in the presence of increasing concentrations of PCNA. The amount of ADP formed in the absence of RFC is represented by the *circles*.

RFC subunits for viability (4–7, 37) and the involvement of RFC in DNA replication, DNA repair, and cell cycle checkpoint pathways (8, 14, 15, 38). However, the function(s) of each subunit in these cellular processes remain(s) unclear. *In vitro* experiments presented in this report and elsewhere have shown biochemically a requirement for the large subunit of RFC for DNA replication, because a subcomplex lacking the large subunit was found to be incompetent for DNA synthesis. RFC has been shown to be a structure-specific DNA-binding protein, displaying a preference for DNA molecules mimicking DNA replication substrates (23). Deletion analyses of the 140-kDa subunit have defined the presence of at least two "domains" involved in DNA binding, one at the NH_2 terminus that includes the ligase homology domain (39, 40) and one located near the COOH terminus (40). However, the specificity of these putative large subunit DNA binding domains for primer termini is inconclusive.

Present in all five RFC subunits are motifs termed RFC box III and RFC box V that are similar to the phosphate binding loop (P-loop) and DEAD box consensus sequences, respectively, found in proteins that hydrolyze ATP or other nucleotide triphosphates. The RFC complex possesses ATPase activity, and stable binding of RFC to DNA has been shown to require ATP binding and/or hydrolysis. Moreover, DNA appears to be a co-factor for the ATPase of RFC, stimulating this activity approximately 20-fold. The subunit(s) responsible for this activity is unknown. In this report, evidence is presented indicating that a complex of the small subunits, although inactive for DNA replication, is an efficient ATPase. Similar to the entire RFC complex, the ATPase activity of the small subunit complex was found to be highly stimulated by DNA, but, unlike the

entire RFC complex, its ATPase activity was not responsive to PCNA. This observation is consistent with the findings of McAlear *et al.* (8), which indicated that a direct interaction between the large subunit of yeast RFC (encoded by *cdc44*) and PCNA (encoded by *pol30*) is required for RFC function. The apparent inability of PCNA to stimulate the activity of the small subunit ATPase is contradictory to the observations recently reported by Cai *et al.* (41). Further investigation of the interaction between the small subunit complex and PCNA may shed some light on this issue.

As noted earlier, the 140-kDa subunit has been reported to contain two distinct DNA binding domains; but which, if either, of these regions is responsible for the DNA binding properties of RFC is unclear. Experiments presented in this report suggest that a complex of the small subunits of RFC is a DNA-stimulated ATPase, implying that at least one of these subunits has DNA binding activity. In the report of Uhlmann *et al.* (40), the four small subunits of RFC were tested individually for the ability to bind DNA, and none exhibited any DNA binding activity. Perhaps these subunits bind DNA cooperatively, and consequently, a complex of these subunits is required for stable DNA binding. Alternatively, a complex of the small subunits is necessary to form a functional ATPase that is competent to stably bind DNA. Indeed, although each subunit of RFC has the primary features of an ATPase, reconstitution experiments have revealed that a minimum of three subunits (the 40-, 37-, and 36-kDa subunits) are required for DNA-dependent ATPase activity *in vitro*.[2] How the DNA binding, ATP binding, and ATPase activities of RFC are distributed among the subunits remains to be determined.

In contrast to RFC, much more is known about the prokaryotic equivalent of RFC, the γ complex of *E. coli*. It consists of five subunits ($\gamma\delta\delta'\chi\varphi$) and like RFC possesses a DNA-dependent ATPase activity that is required for loading the β clamp, the *E. coli* equivalent of PCNA, onto DNA (42). Although all five subunits of the γ complex are necessary for optimal activity *in vitro*, the minimum complex active for DNA synthesis consists of γ, δ, and δ' (43). The γ and δ' subunits are homologous to the small subunits of RFC and contain nucleotide binding motifs (6, 22). However, the γ subunit has been demonstrated to be the sole functional ATPase in the complex, and it is optimally active only when present in a complex with the δ and δ' subunits (43, 44). The ATPase activity of the $\gamma\delta\delta'$ complex was found to be stimulated by a complex of the χ and φ subunits, as well as by the β clamp, which has been shown to interact with the δ subunit (45, 46). Given the similarity among all five RFC subunits and, consequently, the greater complexity of functional analyses, it will be interesting to see if a similar distribution of the functions of the complex among the five subunits is achieved in RFC. Further characterization of the conserved regions shared among the subunits will undoubtedly yield interesting insights into how multisubunit protein complexes coordinate multiple activities.

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