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In vitro reconstitution of human replication factor C from its five subunits

(eukaryotic DNA replication/*in vitro* translation/proliferating cell nuclear antigen/Activator I)

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ABSTRACT **Replication factor C (RFC, also called Activator I) is part of the processive eukaryotic DNA polymerase holoenzymes. The processive elongation of DNA chains requires that DNA polymerases are tethered to template DNA at primer ends. In eukaryotes the ring-shaped homotrimeric protein, proliferating cell nuclear antigen (PCNA), ensures tight template–polymerase interaction by encircling the DNA strand. Proliferating cell nuclear antigen is loaded onto DNA through the action of RFC in an ATP-dependent reaction. Human RFC is a protein complex consisting of five distinct subunits that migrate through SDS/polyacrylamide gels as protein bands of 140, 40, 38, 37, and 36 kDa. All five genes encoding the RFC subunits have been cloned and sequenced. A functionally identical RFC complex has been isolated from *Saccharomyces cerevisiae* and the deduced amino acid sequences among the corresponding human and yeast subunits are homologous. Here we report the expression of the five cloned human genes using an *in vitro* coupled transcription/translation system and show that the gene products form a complex resembling native RFC that is active in supporting an RFC-dependent replication reaction. Studies on the interactions between the five subunits suggest a cooperative mechanism in the assembly of the RFC complex. A three-subunit core complex, consisting of p36, p37, and p40, was identified and evidence is presented that p38 is essential for the interaction between this core complex and the large p140 subunit.**

DNA replication in eukaryotes is dependent on three distinct DNA polymerases (pol), α, δ, and ε. DNA pol α, through its association with DNA primase, is responsible for the initiation of DNA synthesis and the production of pre-Okazaki DNA fragments. The function of the other two DNA pols remains to be completely elucidated but it is clear that they are involved in maturation of pre-Okazaki fragments on the lagging strand and synthesis of leading strand DNA. Both pol δ and pol ε are dependent on two auxiliary protein factors, proliferating cell nuclear antigen (PCNA) and replication factor C (RFC) (also called Activator I), for their processive function in strand elongation (1–7). PCNA is a ring-shaped homotrimeric protein that encircles DNA, acting as a sliding clamp that tethers DNA pol to template DNA (8–10). RFC is responsible for the loading of PCNA onto the DNA. RFC recognizes primer ends and contains DNA-dependent ATPase activity that is stimulated by PCNA (4, 5). Complexed with a primed template, RFC recruits PCNA and assembles it onto DNA in the presence of ATP. Subsequent ATP hydrolysis is required for the pol to enter the complex and to initiate chain elongation (2, 3). How RFC performs this topological task of loading PCNA onto the template is largely unknown. The unloading of PCNA from DNA, a step necessary to recycle PCNA during replication, is also achieved by RFC in an ATP-dependent reaction (11).

Human RFC was isolated from HeLa cells as a protein complex consisting of five different subunits (4, 12). The cDNAs for these five polypeptides have been cloned and sequenced. The calculated mass of the encoded subunits, deduced from their amino acid sequence, is 128 kDa, 42 kDa, 41 kDa, 40 kDa, and 39 kDa. Their migration on SDS/PAGE is p140, p40, p38, p37, and p36, respectively. Based on our previous description of these subunits, we refer to these proteins according to their SDS/PAGE migration (13–16). The large subunit (p140) contains a region homologous to prokaryotic DNA ligases (15, 17). All of the subunits contain conserved sequences characteristic of nucleotide binding proteins and also show significant homology to each other, with the highest similarities in several defined regions referred to as RFC boxes (13–16, 18). This apparent redundancy among the subunits has raised the question whether all five different subunits are necessary for RFC function. Furthermore, it is also uncertain whether the identified five subunits are sufficient for RFC activity.

In *Saccharomyces cerevisiae*, a functionally homologous five subunit protein complex (ScRFC) has been identified (18–22). The genes for ScRFC have been cloned and sequenced (18, 22–26) and all five of these genes are essential in *S. cerevisiae* suggesting that each gene has a unique function. Recently, it was shown that overexpression of the five ScRFC genes in *S. cerevisiae* leads to an increase of RFC activity in extracts prepared from these cells (22).

The RFC subunits in *S. cerevisiae* and humans are highly homologous. Their correspondence has been designated based on their sequence homology as follows: hRFC p140 corresponds to ScRFC1, hRFC p40 corresponds to ScRFC4, hRFC p38 corresponds to ScRFC5, hRFC p37 corresponds to ScRFC2, and hRFC p36 corresponds to ScRFC3.

In this report we have focused on the reconstitution of hRFC activity from its individual subunits. We demonstrate that the RFC complex can be assembled following coupled *in vitro* transcription and translation of the five cDNAs coding for the hRFC subunits. The five subunit complex can be immunoprecipitated with antibodies specific for one of the protein subunits. Furthermore, after purification, the five subunit RFC product formed *in vitro* supported DNA replication in a pol δ catalyzed primer elongation reaction dependent on PCNA and the human single-stranded DNA binding protein (HSSB, also called hRPA). We have also examined the interaction between the subunits of hRFC. We have detected a core complex

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Abbreviations: RFC, replication factor C, also called Activator I; HSSB, human single-stranded DNA binding protein, also called hRPA; PCNA, proliferating cell nuclear antigen; h, human; pol, polymerase; GST, glutathione-*S*-transferase.

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involving p36, p37, and p40, and we find that the interaction of the p140 subunit with this complex is dependent on the presence of the p38 subunit.

MATERIALS AND METHODS

DNA Templates for *in Vitro* Transcription/Translation. cDNAs encoding the five distinct subunits of hRFC (13, 14, 16) were cloned for expression in pET-derived vectors under the control of the phage T7 promoter. At the N-terminal end of the cDNAs coding for p36 and p38, a hexahistidine coding sequence was added. The cDNAs for p37 and p40 were not modified. The cDNA coding for p140 was expressed both in the unmodified form and fused at the N terminus to the DNA sequence coding for glutathione-*S*-transferase (GST). The resulting expression vectors were pET19bHisp36, pET3cp37, pET19bHisp38, pET5ap40, pET16ap140, and pET19bGSTp140.

***In Vitro* Transcription/Translation of RFC Subunits.** The *in vitro* transcription and translation of the cDNAs encoding the five subunits of hRFC were carried out with the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocols. In most cases reactions (25 μl) containing 0.5 μg of template DNA, reaction buffer, T7 RNA pol, 20 μM of each amino acid except methionine, 20 μCi of L-[^{35}S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq, Amersham; *in vivo* cell labeling grade), 20 units of RNase Block (Stratagene) and 12.5 μl of rabbit reticulocyte lysate were incubated at 30°C for 90 min.

In reactions in which the transcription/translation of more than one subunit was performed, up to 1.2 μg of template DNA was used; the concentrations of the different templates were adjusted to obtain stoichiometric expression. In experiments in which RFC was isolated from reactions coexpressing all five subunits, 20 μM of methionine was added, the reaction volume was increased to 250 μl, and the incubation time was reduced to 60 min.

Translation products were characterized by SDS/PAGE. After separation, gels were fixed in 25% 2-propanol/10% acetic acid, soaked in luminographic enhancer (Amplify, Amersham), dried, and exposed for autoradiography or for quantitation using the phosphorimager (Fuji).

Immunoprecipitation of Translation Products. One microliter of polyclonal antisera raised against one of the RFC subunits or 1 μg of affinity-purified antibodiy against GST was added to 5–10 μl of a transcription/translation reaction mixture after incubation as described above. After incubation on ice for 1 h, immunocomplexes were bound to 5 μl of protein A Sepharose beads (Pharmacia), equilibrated in RIPA/BSA buffer (25 mM Tris·HCl, pH 8.0/5 mM EDTA/1 mM DTT/0.2 M NaCl/0.5% Nonidet P-40/1% BSA). Incubation was on ice for 1 h with frequent shaking. The beads were then washed four times with 0.3 ml of the RIPA/BSA buffer and twice in RIPA buffer lacking albumin. Bound complexes were boiled for 5 min in SDS/PAGE sample buffer (15–50 μl) and aliquots were subjected to SDS/PAGE and then exposed for autoradiography as described above.

Purification of RFC from *in Vitro* Transcription/Translation Reactions. Reactions expressing all five RFC subunits (0.25 ml) were diluted 5-fold with loading buffer (25 mM Tris·HCl, pH 7.5/0.1 mM EDTA/2 mM DTT/0.05% Nonidet P-40/0.2 M NaCl) and then passed twice through a phosphocellulose column (0.1 ml). The column was then washed with 0.5 ml of the loading buffer and RFC was eluted from the column with 0.5 ml of the loading buffer containing 1 M NaCl. Fractions containing RFC were pooled, diluted to 0.1 M NaCl, and then concentrated 50-fold by centrifugation using an Ultrafree cone (Millipore).

Preparation of Replication Proteins. The following proteins were prepared from HeLa cell extracts as described: HSSB (27), PCNA (28), pol δ and RFC (29).

DNA Replication Assay for RFC Activity. The assay of RFC was carried out in reaction mixtures (20 μl) (40 mM Tris·HCl, pH 7.5/0.5 mM DTT/0.01% BSA/7 mM magnesium acetate/2 mM ATP/100 μM dATP/100 μM dGTP/100 μM dTTP/20 μM [α^{32}P]dCTP (10,000–20,000 cpm/pmol)/8 fmol of singly primed circular M13 DNA/0.5 μg HSSB/0.1 μg PCNA/50 fmol pol δ and 0.6 to 15 fmol RFC or RFC preparations to be assayed). After incubation for 30 min at 37°C reactions were stopped with 10 mM EDTA. Loading dye was added and the mixture subjected to alkaline agarose gel (1.5%) electrophoresis. Gels were dried and exposed for

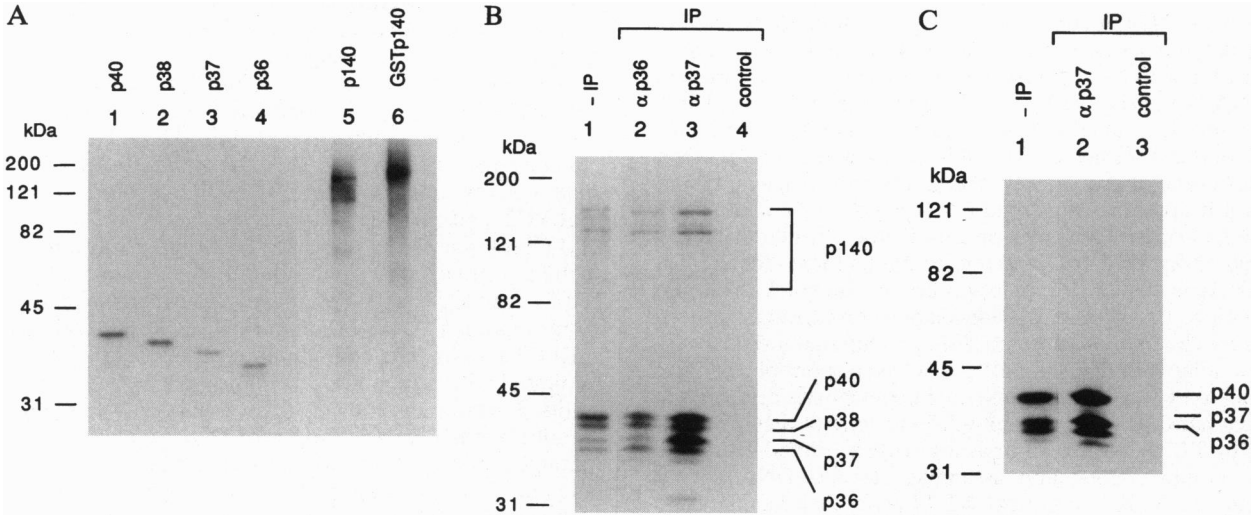

FIG. 1. (*A*) Transcription/translation products of the subunits of human RFC. Reactions (25 μl), as described, were incubated for 90 min; aliquots of each reaction (0.1 μl) were separated by SDS/10% PAGE. Lanes 1–6 contained products formed in the presence of the following cDNAs: p40, p38, p37, p36, p140, and GST-p140, respectively. (*B*) Complex formation after the simultaneous expression of the five subunits. Lane 1, an aliquot of the *in vitro* transcription/translation products formed in the presence of all five cDNAs was separated by SDS/10% PAGE. Lanes 2 and 3, aliquots of the material shown in lane 1 were immunoprecipitated with antisera against p36 and p37, respectively. Lane 4, a control immunoprecipitation using preimmune serum. (*C*) Formation of the core complex of p36, p37, and p40 subunits. The three subunits were coexpressed and an aliquot of the reaction was separated by SDS/PAGE (lane 1); immunoprecipitation with antiserum prepared against the p37 subunit (lane 2) and preimmune serum (lane 3).

autoradiography; quantitation was performed using the phosphorimager.

RESULTS

***In Vitro* Transcription/Translation of the Subunits of RFC.** The products obtained after *in vitro* transcription/translation of each cDNA coding for the hRFC subunits, as well as the fusion GST-p140 product, are shown in Fig. 1*A*. Each reaction yielded a labeled protein of the expected size, though smaller sized products were detected. Substantial numbers of smaller products were observed in reactions carried out with the large subunit (p140, lanes 5 and 6) and are most likely due to translational starts at internal methionine codons present in the *in vitro* transcribed RNA. Products formed with the p36 cDNA included the full-length p36 protein as well as smaller material (lane 4). The four small subunits are shown in Fig. 1*A* in near stoichiometric amounts; the yield of the p37 subunit (lane 3) appears to be lower than those of the other small subunits. However, this discrepancy is due to the lower number of methionine residues present in p37, resulting in reduced specific labeling of this subunit with [^{35}S]methionine compared with the other subunits.

Formation of the Five Subunit RFC Complex and a Three Subunit Core Complex. Coexpression of the five RFC subunits *in vitro* resulted in the production of all five products of expected size, visualized by autoradiography after SDS/PAGE analysis (Fig. 1*B*, lane 1). To determine whether coexpression of the five subunits led to formation of a complex containing each of the subunits, immunoprecipitation experiments were carried out using polyclonal antisera against either the p36, p37, p40, or the p140 subunit (Fig. 1*B*, lanes 2 and 3 show results obtained with α p36 and α p37, respectively). All five subunits coprecipitated indicating complex formation. In such reactions, 20–50% of the input labeled proteins coprecipitated. Glycerol gradient centrifugation of the five coexpressed RFC subunits yielded a complex containing all of the subunits with a sedimentation value similar to native RFC (data not shown). These observations indicated that the coexpressed subunits interacted to form a stable five subunit complex.

The only stable complex formed in reactions coexpressing fewer than all five subunits consisted of the three subunits p36, p37, and p40. Immunoprecipitation analysis showed that these three subunits interacted with one another (Fig. 1*C*). In these experiments, approximately 25% of the input proteins coprecipitated. In contrast to the *in vitro* assembled five subunit complex, the p36p37p40 complex was not stable during glycerol gradient centrifugation. A similar three subunit complex was assembled by mixing the individual proteins, purified after expression in either baculovirus infected Sf9 cells or *Escherichia coli* (data not shown).

Biological Activity of Reconstituted RFC. To investigate the biological activity of the five subunit RFC complex formed *in vitro*, the product was isolated from a transcription/translation reaction mixture. A phosphocellulose purification step, as described in *Materials and Methods*, removed the template DNAs and a substantial amount of the lysate proteins from the reaction mixture. The partially purified labeled RFC preparation contained near stoichiometric amounts of the five subunits (Fig. 2*A*). These preparations were examined for their ability to support the elongation of a singly primed M13 DNA in a replication reaction containing HSSB, pol δ, and PCNA. As shown in Fig. 2*B*, the *in vitro* synthesized RFC supported DNA synthesis in this assay (Fig. 2*B*, lanes 4 and 5). To rule out that endogenous RFC present in the reticulocyte lysate was responsible for the observed activity, transcription/translation reactions were carried out with cDNAs lacking the T7 promoter. After incubation, reaction mixtures were subjected to the same isolation procedure as those carried out with cDNAs containing the T7 promoter. Such preparation did not possess detectable RFC activity (Fig. 2*B*, lane 9). In keeping with the expected requirements for the elongation of a singly primed template, the RFC activity observed with the purified labeled product required PCNA, HSSB, and pol δ (Fig. 2*B*, lanes 6–8).

The level of RFC formed *in vitro* (described in Fig. 2*B*, lanes 4 and 5), was estimated (using Western blotting) to be 0.6 fmol/μl. The activity of the reconstituted RFC preparation in the replication assay corresponded to 0.2 fmol/μl, indicating that the reconstituted RFC was about 30% active.

Interactions Between the Individual Subunits of RFC. All possible permutations from two to four of the five subunits of RFC were coexpressed *in vitro*, and interactions between the different subunits were investigated by immunoprecipitation experiments. No stable complexes were detected in reactions

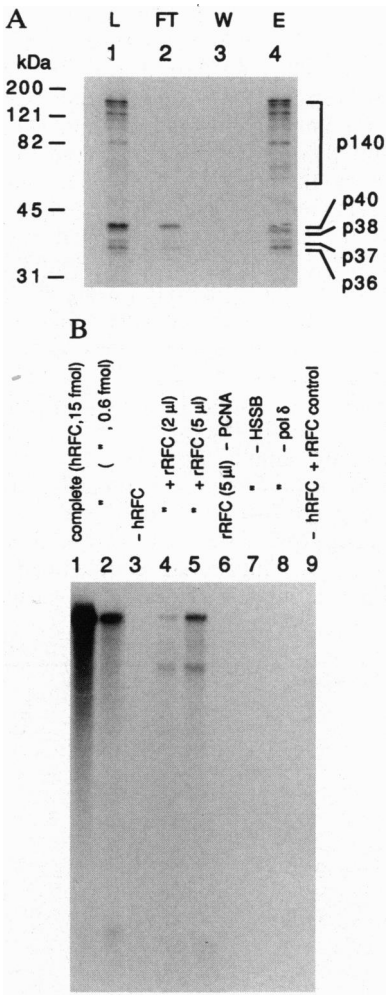

FIG. 2. (*A*) Phosphocellulose purification of *in vitro* reconstituted RFC. The cotranscription/translation products (lane 1) were subjected to phosphocellulose chromatography as described. SDS/PAGE analyses of the proteins that passed through the column (FT), eluted with 0.2 M NaCl (W) and eluted with 1 M NaCl (E) are shown in lanes 2–4, respectively. (*B*) *In vitro* synthesized RFC supports DNA synthesis. Reaction mixtures for the assay of RFC activity were as described. After alkaline agarose gel separation, dried gels were autoradiographed for 1 h. Reaction mixtures containing either 15 fmol, 0.6 fmol, or no RFC are shown in lanes 1–3, respectively; lanes 4 and 5 contained 2 and 5 μl of the *in vitro* synthesized RFC product, isolated after phosphocellulose chromatography as shown in *A*. Reaction mixtures containing 5 μl of the material used in lane 5, but lacking either PCNA, HSSB, or pol δ, are shown in lanes 6–8, respectively. A reaction mixture containing 5 μl of the products formed in a reaction containing cDNAs lacking the T7 RNA pol promoter, carried through the same phosphocellulose isolation procedure described in *A*, is shown in lane 9.

containing any two of the subunits; however, direct interactions between the following subunits were observed at a lower level of efficiency (1–2% of the input proteins coprecipitated): p36p40, p36p37, p37p40, and p140p38 (data not shown). Because the sensitivity of complex detection was relatively low, interactions between other subunit pairs cannot be excluded. The binding of p37 to p40 was shown previously using p40 bound to an Affi-Gel column (7) and an interaction between p36 and p40 was detected using the yeast two hybrid system (F.B.D. and J.H., unpublished results).

Upon addition of a third subunit, a stable complex was formed between p36, p37, and p40, as shown in Fig. 1*C*. A weak interaction between p37 and the p140p38 complex was also detected (data not shown).

Further information about complex formation was obtained from studies in which a single RFC subunit was omitted during *in vitro* synthesis RFC (Fig. 3). In the experiments shown in Fig. 3, the cDNA encoding GST-p140 was used instead of the cDNA expressing the native p140. Similar results were obtained using the unmodified p140 subunit. The resolution of p36 and p37 on SDS/PAGE was not completely reproducible. Where necessary, the presence of both p36 and p37 subunits was verified using antisera specific for each subunit.

The results obtained can be summarized as follows: in the absence of p140 a complex containing p36, p37, and p40 was formed; p38 was not coprecipitated using antibodies against p37 (Fig. 3, lanes 6 and 7). The coexpression of p140, p38, p37, and p36 (lacking p40) yielded a complex of the four subunits (lanes 9 and 10). However, only 10% of the complex was formed compared with the yield obtained with all five subunits (compare lanes 2 and 9). It appears that the p40 subunit may be dispensable for complex formation, but its presence may stabilize the five subunit complex, possibly through the formation of the p36p37p40 core. In the absence of the p38 subunit, the p36p37p40 complex was formed devoid of p140 (Fig. 3, lanes 11–13). This finding, coupled with the results of the experiment without p140 (lanes 6 and 7), suggests that p38 and p140 require each other for interaction with the core complex.

In the absence of the p37 or the p36 subunit, weak interactions were observed between p140 and p38, and p140, p38, and p37. These interactions, however, were not visible in Fig. 3 (lanes 14–19). The detection of GST-p140 in lane 16 with α p36 is due to a weak crossreactivity of the p36 antiserum with GST. The p140 subunit was not detected when the unmodified large subunit was used in place of the GST-p140 (data not shown).

DISCUSSION

By coexpressing the five subunits of human RFC in an *in vitro* transcription/translation system, the RFC complex was formed that possessed properties similar to RFC isolated from HeLa cells. The biological activity of the RFC product formed, measured by its ability to support the DNA elongation reaction, was lower than expected based on the level of methionine incorporated into the complex. A possible explanation for this discrepancy may be due to the synthesis of the large subunit of the RFC. The *in vitro* transcription/translation of the cDNA coding for this protein yielded several smaller sized products, most likely due to internal translational starts present in the RNA rather than degradation of the full-length protein as shown by kinetic analysis (data not shown). Thus, these translation products are presumably N-terminal deleted variants of p140. Such incomplete subunits were included in the reconstituted RFC, as well as full-length p140, suggesting that the N-terminal part of p140 may not be required for RFC complex formation. This notion was supported by the finding that the C-terminal half of p140 (spanning amino acids 555–1147) when expressed *in vitro* formed a complex with the four small subunits, whereas the N-terminal half (spanning amino acids 1–555) did not (data not shown). The C-terminal part of p140 contains the homology domains shared with the four small subunits (16, 18), and is probably sufficient to interact with these other subunits. However, the precise role of the

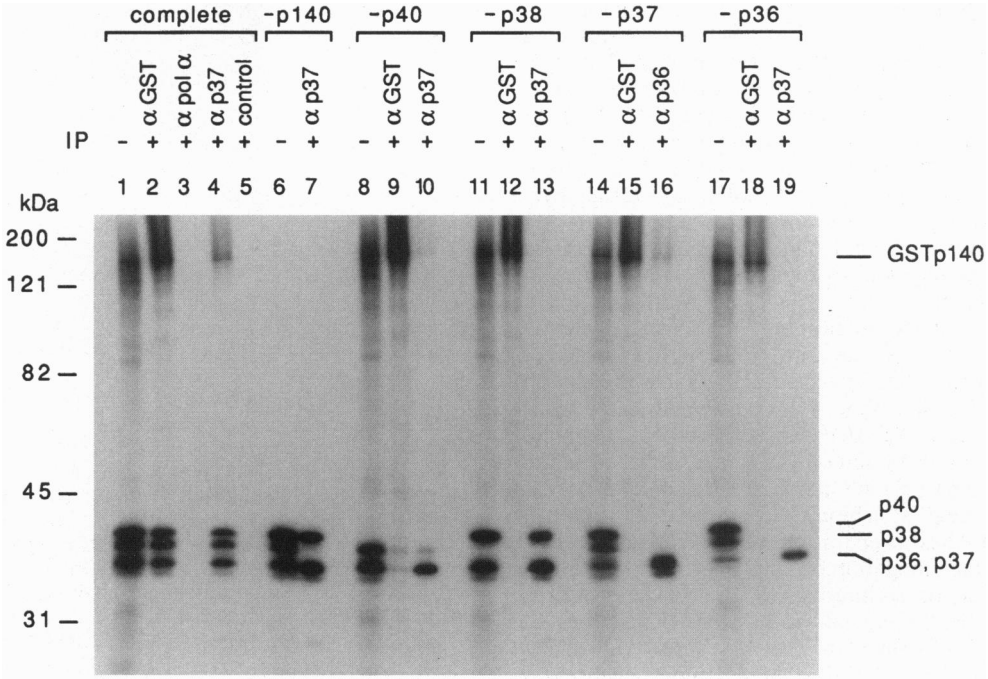

FIG. 3. Complexes formed upon omission of each RFC subunit. A complete reaction, expressing all five subunits (lane 1), and reactions in which the cDNA encoding either the p140, p40, p38, p37, or p36 subunit, was omitted (lanes 6, 8, 11, 14, and 17, respectively) are shown. Immunoprecipitations of reaction products were carried out using antibodies against GST (lanes 2, 9, 12, 15, and 18), p37 (lanes 4, 7, 10, 13, and 19), or p36 (lane 16). Controls are shown for the complete reaction immunoprecipitated with 1 μg of affinity-purified antibody against human pol α (lane 3) or preimmune serum (lane 5).

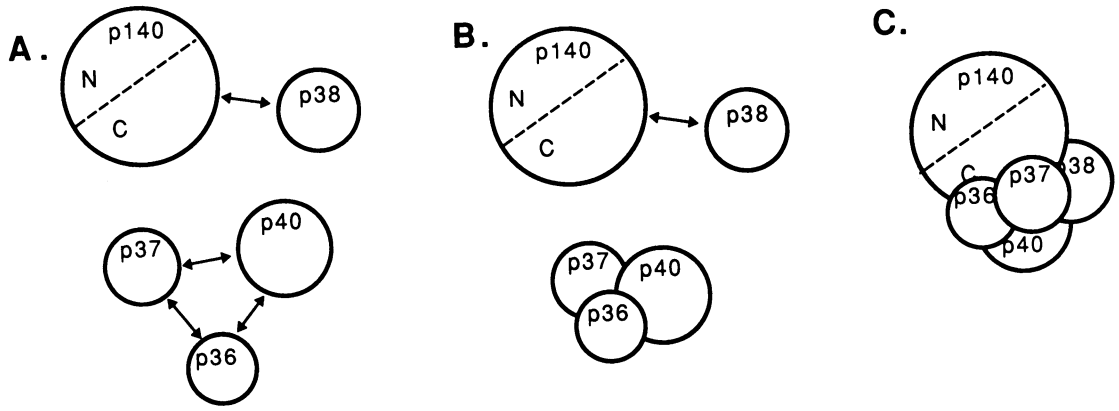

FIG. 4. A model summarizing the observed interactions between the human RFC subunits. (*A*) Weak interactions between individual subunits are indicated by arrows. (*B*) A stable core complex formed between p36, p37, and p40 subunits is presented. (*C*) The p38 and p140 subunits bind cooperatively to the core complex of p36, p37, and p40. The interaction between the C- terminal region of p140 and the other subunits is based on the observations presented in the *Discussion*.

N-terminal region in the biological activity of RFC remains to be elucidated.

The ratio of each of the subunits in the stable five subunit complex was quantitated using phosphorimager analysis of eight different preparations isolated either by immunoprecipitation, phosphocellulose chromatography, or after glycerol gradient centrifugation. The ratio of p36:p37:p38:p40:p140 was found to be 1:1.5:1.2:0.84:1.3, respectively. In some of these experiments, the resolution of the p36 and p37 subunits was inadequate and the sum of the radioactivity representing these two subunits was used. These findings suggest that the RFC complex most likely contains equimolar levels of each subunit.

The availability of highly labeled RFC subunits permitted us to examine the interactions between these proteins that lead to the assembly of the five subunit complex. Results presented here indicate that (*i*) no stable complex with two subunits was formed, though weak interactions were observed between the following: p36.p40; p36.p37; p37.p40, and p38.p140. (*ii*) A stable three subunit core complex was formed with p36, p37, and p40. (*iii*) The core complex interacted with p38 and p140 only when both subunits were present. These observations suggest a model for the highly cooperative interactions of the RFC subunit (Fig. 4).

The functions of the clamp loader and clamp are highly conserved between prokaryotes and eukaryotes (30). The functions of the clamp loader are carried out in *E. coli* by the five subunit γ complex and in T4 phage by the T4gp44gp62 complex (reviewed in ref. 30). The latter complex appears to be composed of four gp44 subunits and one gp62 subunit (31). Sequence homology was previously noted between the RFC subunits, T4 gp44 and the γ and δ subunits of the γ complex, particularly in the conserved regions found in the RFC boxes (16). These findings suggest that the 3-dimensional structure of the clamp loaders may also be conserved. Elegant crystallographic studies have shown that the structure of the clamps [PCNA (32), β (33), and T4 gp45 (J. Kuriyan, personal communication)] are conserved.

While the function of each RFC subunit remains to be elucidated, a number of the subunits have been shown to possess biological activities. The p140 subunit contains a DNA ligase domain, located within the N-terminal region, suggesting that this subunit can bind to DNA. DNA binding activity of the cloned and isolated N-terminal part of the subunit has been observed (H.F.-R. and J.H., unpublished results). Although all five subunits contain ATP-binding domains, only ScRFC2–34 ScRFC (26) and hRFC p40 (5, 13) have been shown to directly bind ATP, whereas ScRFC3 has been reported to contain ATPase activity that is stimulated by DNA (23). hRFC p40 binds to PCNA as detected by direct interaction studies (7). The role of the other subunits remains to be determined.

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