Deletion Analysis of the Large Subunit p140 in Human Replication Factor C Reveals Regions Required for Complex Formation and Replication Activities*

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Replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) are processivity factors for eukaryotic DNA polymerases δ and ε. RFC contains multiple activities, including its ability to recognize and bind to a DNA primer end and load the ring-shaped PCNA onto DNA in an ATP-dependent reaction. PCNA then tethers the polymerase to the template allowing processive DNA chain elongation. Human RFC consists of five distinct subunits (p140, p40, p38, p37, and p36), and RFC activity can be reconstituted from the first cloned gene products. To characterize the role of the large subunit p140 in the function of the RFC complex, deletion mutants were created that defined a region within the p140 C terminus required for complex formation with the four small subunits. Deletion of the p140 N-terminal half, including the DNA ligase homology domain, resulted in the formation of an RFC complex with enhanced activity in replication and PCNA loading. Deletion of additional N-terminal amino acids, including those constituting the RFC homology box II that is conserved among all five RFC subunits, disrupted RFC replication function. DNA primer end recognition and PCNA binding activities, located in the p140 C-terminal half, were unaffected in this mutant, but PCNA loading was abolished.

* These studies were supported by National Institutes of Health Grants GM 38559 (to J. H.) and GM 38839 (to M. O'D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Enrolled in the graduate program at the Physiologisch-chemisches Institut, Universität Tübingen, and supported by the German Aka demic Exchange Service (DAAD) through funds of the Zweites Hochschulsonderprogramm.
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1 The abbreviations used are: RFC, replication factor C, also called activator 1; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; HSSB, human single-stranded DNA-binding protein, also called RPA; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; RIPA, radioimmune precipitation buffer.

Received for publication, December 12, 1996

(Received for publication, December 12, 1996)
PCNA onto nicked circular duplex DNA, and to support RFC-dependent elongation of singly primed M13 DNA by pol δ.

MATERIALS AND METHODS

Templates for in vitro transcription/translation of the five subunits of hRFC were as described previously (17). Constructs for Expressing Deletion Mutants of p140—pET16p140C555 was derived from pET16p140 by excising a BspMI-BstBI fragment from the p140 coding sequence. The ends were filled in and religated. Expression from this vector results in a polypeptide spanning amino acids 1–555 of p140 plus three additional amino acids, TKK, at the C terminus. pET16p140N555 was obtained from pET16p140 by removing a Ncol-BspMI fragment, filling in the ends, and religation. This vector expressed amino acids 555–1148 of p140 with an additional methionine for translational start at the N terminus. pET16p140N604, −N667, −N776, −N822, and −N977 were cloned from pET16p140 by polymerase chain reaction (Expand High Fidelity, Boehringer Mannheim) using the following primers: p140N604 N terminus, 5′-CATGGCATGAGCAGACTTCGAACAAACTCC-3′; p140N822 N terminus, 5′-CATGCGATGACGAGTTGATGTGCTTTGCACT-3′; p140N776 N terminus, 5′-CATGCGATGACGAGTTGATGTGCTTTGCACT-3′; p140N822 N terminus, 5′-CATGCGATGACGAGTTGATGTGCTTTGCACT-3′; p140N977 N terminus, 5′-CAGTGGATGATCTAATGGTGAACACAACTCC-3′. The primer for the C terminus of all constructs was 5′-GGGGGTACCTACCATGGTATAAGCTGAGCCCCCTTCTTC-3′. Polymers of chain reaction products were cleaved with the restriction endonucleases Ncol and KpnI and ligated into the Ncol and KpnI sites of a modified pET19b vector (Novagen). This generated an artificial methionine in front of the p140 sequence starting at the respective amino acid indicated. Expression from these vectors ends at the C terminus of p140 (amino acid 1148). The validity of these constructs was confirmed by DNA sequencing. p140C1142 and p140C976 were generated by cutting pET16ap140 within the p140 coding sequence with the restriction endonucleases Xmal and AseNI, respectively. The linearized vector fragments were used directly in vitro transcription/translation reactions.

In Vitro Transcription/Translation—In vitro transcription/translation was performed as described (17). Linearized template DNA (0.4 µg) was added to a 10-µl transcription/translation reaction mixture. The translation products were quantitated by Western blotting of the p37 and p40 subunits and by comparing the level of [35S]methionine incorporated into these and other subunits.

Interaction of Translation Products—Interactions between subunits were studied by coexpressing the subunits in question in the in vitro transcription/translation system. When a linearized template was used, each subunit was expressed individually, then mixed immediately after an 1-h incubation of the in vitro translation reactions, followed by further incubation for 30 min at 30 °C. Polyclonal antisera against each of the subunits was prebound to a 5-µl suspension of protein A-Sepharose beads (Upstate Biotechnology Inc.) for 1 h on ice. Beads were washed with equilibration buffer (RIPA: 50 mM Tris/HCl, pH 8.0, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, and 1% BSA). Aliquots of the in vitro translation reaction products were added to the immunobeads and complexes were adsorbed for 1 h on ice with frequent shaking. The beads were then washed three times with 0.3 ml of RIPA and twice with 0.3 ml of RIPA without BSA and EDTA. Bound proteins were eluted with 25 µl of SDS-PAGE loading buffer and aliquots analyzed by 10% SDS-PAGE under reducing conditions. The amount of RFC bound to the beads was quantitated after SDS-PAGE analysis by phosphorimager (Fuji), using known amounts of the p37 and p40 subunits as standards.

Isolation of Replication Proteins—The following proteins were prepared from HeLa cell extracts as described: HSSB (23), PCNA (24), pol δ, and RFC (25).

Replication Activity of in Vitro Formed RFC Complexes—RFC complexes formed from 25 µl of in vitro translation reactions were isolated with immunobeads as described for immunoprecipitation. In each case, the amount of RFC adsorbed to the beads was quantitated as described above. After the beads were washed, 14.5 µl of the following reaction mixture was added directly to the washed beads: 40 mM Tris/HCl, pH 7.5, 7 mM magnesium acetate, 0.5 mM DTT, 0.01% BSA, 2 mM ATP, 100 µM each of dATP, dGTP, and dTTP, 20 µM [α-32P]dCTP (10,000 cpm/µmol), 10 fmol of singly primed circular M13 DNA, 320 ng of HSSB, 50 ng of PCNA, and 50 fmol of pol δ. Reactions were incubated for 30 min at 37 °C with frequent shaking and stopped with 10 mM EDTA. Aliquots (2 µl) were withdrawn and added to 0.1 ml of solution containing 0.67 mg/ml salmon sperm DNA and 67 µM sodium pyrophosphate, and the mixture was precipitated with 1 ml of 5% trichloroacetic acid. After 15 min on ice, the precipitate was filtered through glass fiber filters (Whatman) and acid-insoluble replication products were quantitated by liquid scintillation counting. To 10 µl of the remaining replication products, loading dye was added, and the mixture was subjected to alkaline agarose gel electrophoresis. Gels were dried and exposed for autoradiography. The amount of full-length replication products formed was quantitated by phosphorimager analysis.

Reactions in which RFC isolated from HeLa cells were used were carried out under the above conditions either in the absence of, or after binding to immunobeads as described above. Products formed were analyzed as described above.

DNA Binding Assay—The substrate used for DNA binding was a hair-pinned DNA structure, which was described previously and used to measure the binding of RFC to a DNA primer end (5, 26). The hairpin was formed from a synthetic 96-mer oligonucleotide after annealing in 20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl2. The DNA contained a biotin moiety at the 5′-end. Oligo(dA)20, also biotinylated at its 5′-end, was used as a single-stranded DNA substrate. Supercoiled pBlueBac II plasmid DNA was used as the double-stranded DNA competitor, where indicated. To a reaction mixture containing about 25 fmol of the in vitro translated complex, pure in vitro formed RFC complexes, or both of the DNA substrate was added and the mixture was incubated at 30 °C for 10 min. Avidinagarose beads (5 µl, Vector) in 10 µl of equilibration buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1 mM ATP, 2 mM DTT, 0.1% Nonidet P-40, and 1% BSA) was added to the reaction. Biotinylated DNA was bound to the avidin beads by incubating the mixture for 20 min on ice, with shaking. The beads were then washed three times with 0.3 ml of wash buffer (50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 2 mM MgCl2, 1 mM ATP, 2 mM DTT, 0.5% Nonidet P-40, and 1% BSA) and once with wash buffer without BSA. Bound protein was eluted with 25 µl of SDS-PAGE loading buffer. Elution could also be performed with 0.2 M NaOH or less efficiently by treatment with nuclease S1. Aliquots of the eluate were separated on SDS-PAGE and the gels processed as described under immunoprecipitation.

PCNA Binding of in Vitro Translated RFC—PCNA was overexpressed in Escherichia coli (24). Purified PCNA was covalently bound to activated Affi-Gel-15 (Bio-Rad) at a concentration of 10 mg/ml resin according to the manufacturer’s protocol. For control experiments, BSA was used to block in the resin in the same way. Aliquots of translation reactions expressing RFC complexes were added to 5 µl of the affinity beads in 5 mM Tris/HCl, pH 8.0, 200 mM NaCl, 1 mM MgCl2, 2 mM ATP, 2 mM DTT, 0.25% Nonidet P-40, 10% glycerol, and 2.5% BSA (binding buffer). Binding was carried out on ice for 4 h. The beads were then washed twice with 0.3 µl of equilibration buffer containing 1% BSA and once with equilibration buffer without BSA. Bound protein was eluted with 25 µl of SDS-PAGE loading buffer, and aliquots were separated on SDS-PAGE. The gels were processed as described under immunoprecipitation.

Loading of PCNA onto Nicked Circular DNA—RFC complexes formed from in vitro translation reactions were isolated with immunobeads as described for immunoprecipitation. After the beads were washed, the following reaction mixture was added to the washed beads: 0.5 µmol of singly nicked plBluescript DNA and 2.6 µmol of [3P]-labeled PCNA trimers (500–800 cpm/50 µl of incubation buffer (20 mM Tris/HCl, pH 7.5, 100 mM NaCl, 8 mM MgCl2, 0.5 mM ATP, 4% glycerol, 5 mM DTT, and 40 µg/ml BSA) (27, 28). The reaction was incubated for 15 min at 37 °C, stopped on ice and applied to a 5-ml gel filtration column (Bio-Gel A15m, Bio-Rad) equilibrated with incubation buffer. Fractions of 160 µl were collected at 4 °C, and [32P] was quantitated by Cerenkov counting.

RESULTS

Identification of a Region within p140 Required for Complex Formation—To identify regions in the subunit p140 that are required for complex formation, p140 deletion mutants were constructed, expressed in vitro, and assayed for their ability to assemble an RFC complex with the four small subunits. Fig. 1 shows the products obtained after in vitro transcription and translation of cDNAs encoding full-length p140 (lane 1) and deleted variants of this subunit (lanes 2–5). Each reaction mixture yielded a labeled protein of the expected size, although a substantial number of smaller products were formed. These
shown as a control (not the p38 subunit or the p140 variant (Fig. 1, lanes 1–5). Complex formation of p140 with the small subunits was detected by immunoprecipitation of a mixture of all five subunits (lane 6, 10% of the input material; lane 7, immunoprecipitate with antibodies against p37; lane 8, immunoprecipitate using preimmune serum). Requirements for complex formation were studied by replacing p140 with the respective deletion mutant as indicated (lanes 9, 11, 13, and 15, 10% of the input material; lanes 10, 12, 14, and 16, immunoprecipitate). An experiment omitting p140 is shown as a control (lanes 17 and 18). Under the conditions used, the p36 and p37 subunits were not resolved and are jointly labeled p36, p37.

An immunoprecipitation assay employing anti-p37 antibodies was used to analyze the formation of the five-subunit complex with the p140 deletion mutants described in Fig. 1. Deletion analysis of the p140 subunit revealed a region close to the C terminus that was required for complex formation with the small subunits of RFC. A C-terminal deletion of the p140 subunit that ended at amino acid 1142 (p140C1142; Fig. 1, lane 2) supported complex formation (lanes 9 and 10), as did full-length p140 (amino acids 1–1148, lanes 1, 6, and 7). However, p140C976, with another 166 C-terminal amino acids deleted, did not support RFC complex formation (Fig. 1, lanes 3, 11, and 12). Consistent with this, the N-terminal half of the p140 subunit (p140C555), spanning amino acids 1–555 that included the DNA ligase homology domain, was also unable to form the five-subunit RFC complex (Fig. 1, lanes 4, 13, and 14). These experiments were carried out with antibodies against the p37 subunit, so that immunoprecipitation of p140 variants incapable of supporting reconstitution of the five-subunit complex resulted in coprecipitation of the p36/p37/p40 core complex but not the p38 subunit or the p140 variant (Fig. 1, lanes 12 and 14; and control, omitting p140, lanes 17 and 18). p140C555 migrates through SDS-polyacrylamide gels as a band of 75 kDa although the calculated molecular mass of this fragment is 62 kDa. This aberrant migration is also observed with the full-length p140, a polypeptide of calculated molecular mass of 128 kDa that migrates through gels as a band of 140 kDa.

The C-terminal half of the p140 subunit (p140N555), spanning amino acids 555–1147, migrates at its calculated mass of 66 kDa. RFCp140N555 includes the RFC homology boxes II–VIII and was sufficient for complex formation with the small subunits (Fig. 1, lanes 5, 15, and 16).

To further delineate the region within the C terminus critical for complex formation, N-terminal deletions of the p140 subunit were examined starting at amino acids 604 after RFC box II, 687 after box IV, 776 after box VI, 822 after box VIII and 877 in the C terminus (p140N604, -N687, -N776, -N822, and -N877, respectively; Fig. 2, lanes 1–6). As shown, the large subunit derivative p140N822 supported formation of the five-subunit RFC complex, while p140N877 did not (Fig. 2, lanes 16–19). This indicated that a region between amino acids 822 and 1142, within the p140 C-terminal region that is not conserved among the RFC subunits, is necessary and sufficient for RFC complex formation.

Removal of the N-terminal Half of p140 Increases RFC Replication Activity—The replication activity of RFC complexes formed with the N-terminally deleted variants of p140 was examined. Complexes, assembled from in vitro translation reactions, were isolated on immunobeads, and their ability to support RFC-dependent DNA elongation was measured. The efficiency of the replication reaction with RFC bound to immunobeads was markedly reduced (approximately 6-fold) compared with reactions with soluble RFC (Fig. 3A, compare lanes 1 and 2), which was partly due to the limited efficiency of immunoprecipitation (25–35%). The elongation of singly primed single-stranded M13 DNA with immobilized RFC, catalyzed by pol δ, is dependent upon RFC, PCNA, and HSSB (Fig. 3A, lanes 1–8). The RFC complex formed with p140N555 (RFCp140N555) appeared to support replication more efficiently than the complex formed with full-length p140 (Fig. 3A, compare lanes 4 and 9). To quantitate this apparent difference, the RFC complexes formed with full-length p140 (RFCp140) or p140N555 were titrated by varying the amount of complex bound to immunobeads in the assay reaction (Fig. 3B). The efficiency of immunoprecipitation of RFCp140N555 was reproducibly twice the efficiency observed with RFCp140 (compare Fig. 1, lanes 7 and 16) and the input material for the reactions was adjusted accordingly. The activity of RFCp140N555 was approximately 2-fold greater than the activity of RFCp140 as measured by nucleotide incorporation, and 5-fold when formation of full-length replication products (7.2 kilobase pairs) in the singly primed M13 elongation reaction was measured (Fig. 3B, lanes 4–6 and 7–9). These differences were reproducibly observed in three additional experiments.

The measurement of RFC activity of complexes linked to immunobeads showed poor linearity as a function of RFC concentration in the M13 elongation assay (Fig. 3B). For this reason, these experiments were repeated using poly(dA)4000-oligo(dT)12–18 as the template in an RFC-dependent replication reaction. RFCp140N555 supported poly(dT)
synthesis 5 times more effectively than RFCp140 (data not shown). In this assay, the synthesis of poly(dT) showed a more linear response to RFC linked to immunobeads (10–40 fmol).

Similar observations were made with these complexes isolated from in vitro translation mixtures by phosphocellulose chromatography (data not shown). Thus assays using different DNA substrates and different isolation procedures indicated that the RFC complex containing the N-terminally deleted p140 was more active than the full-length p140 RFC complex.

A Region Including RFC Box II of p140 Is Indispensable for Replication Activity—To further identify regions within the p140 subunit required for replication, the N-terminally deleted variants of p140, p140N604 to p140N822, which lack some of the conserved RFC boxes but still form the five-subunit RFC complex, were examined for their ability to support DNA synthesis in the elongation reaction. As shown in Fig. 4, deletion of RFC box II from the p140 subunit (RFCp140N604) resulted in loss of replication activity, delineating a domain between amino acids 555 and 604 that is essential for replication activity.

Deletion of the last six amino acids of p140 (p140C1142) did not affect the activity of the RFC complex formed with this subunit (data not shown).

Binding of RFC Complexes Containing Deleted p140 to Primer Ends—For its function in replication, RFC must possess at least three activities: the ability to bind to DNA primer ends, to interact with PCNA, and to load PCNA onto DNA in an ATP-dependent reaction. To determine why the RFCp140N604 complex is inactive in DNA synthesis, we examined which of these functions is disrupted.

First, DNA binding properties of the RFC subunits and complexes were studied. p140, formed in the in vitro translation complex isolated on the beads in each case is indicated. An autoradiograph of the alkaline agarose gel in which the replication products were resolved is shown, and the quantitation of [α-32P]dCTP incorporated into replication products is given, as well as relative numbers for the amount of full-length (7.2 kilobase pairs) replication products formed (normalized against full-length products present in lane 6).
Deletion Analysis of hRFC p140

Fig. 5. DNA binding activity of RFC subunits and complexes. A, DNA binding properties of the single subunits. 5 μl of in vitro translation reactions, expressing about 25 pmol of the respective subunits or different regions of p140, were incubated with the indicated DNA substrates. The abbreviation hp refers to the addition of 10 pmol of biotinylated hair-pinned DNA containing a primer end, ss refers to addition of 10 pmol of biotinylated single-stranded oligo(dA-dC)_{18}, and ds refers to the addition of a 6-fold molar excess (in nucleotides) of double-stranded plasmid DNA (2 μg). Biotinylated substrates were bound to avidin beads, and bound protein was eluted and analyzed on SDS-PAGE. 10% of the material added to the binding reaction is shown. The elution of proteins bound to avidin beads and autoradiography were described under “Materials and Methods” under DNA binding assay.

reaction, bound to a DNA primer end formed by a hair-pinned DNA containing a recessed 3’-end (Fig. 5A, lanes 1 and 2), p140 specifically interacted with the primer end of this DNA substrate, since the binding was not competed by double-stranded DNA (lane 3), and p140 weakly bound to single-stranded DNA (lane 4). p140C555, containing the DNA ligase homology domain, bound to the primer end with the same efficiency as full-length p140 (lanes 6 and 7). p140N555, lacking this domain, still contained DNA binding activity, although the efficiency of binding was reduced (lanes 8 and 9). The four small subunits of RFC did not bind to DNA under the conditions used (lanes 10–17).

DNA binding by RFC complexes is shown in Fig. 5B. The complex containing full-length p140 bound specifically to the primer end (Fig. 5B, lanes 1–5). RFCp140N555 bound with reduced efficiency (lanes 6 and 7), as expected from the observed behavior of free p140N555 (see Fig. 5A). RFCp140N604, the complex inactive in replication, bound with equal efficiency as RFCp140N555 (lanes 8 and 9). DNA binding of the RFC complex was lost only after the p140 deletion extended to amino acid 776 (lanes 10–13). Likewise, p140N687 alone, rather than in the RFC complex, bound to primer ends, while p140N776 did not, suggesting that stable DNA binding of the RFC complex is mediated mainly by the large subunit. C-terminal deletion of p140N555 to amino acid 976 resulted in the loss of DNA binding by the subunit (data not shown). This localizes the region critical for DNA binding activity in the C-terminal half of p140 between amino acids 687 and the C terminus.

These results indicated that the inability of RFCp140N604 to support DNA synthesis was not due to its deficiency in binding DNA primer ends.

Binding of PCNA by RFCp140N604—We compared the abilities of RFC complexes containing full-length or N-terminally deleted p140 to bind PCNA. The RFCp140N604 complex interacted with PCNA but not with BSA covalently linked to a solid phase, as did the RFC complexes containing the full-length large subunit and p140N555 (Fig. 6). This suggests that the replication defect in RFCp140N604 is not solely due to its inability to interact with PCNA. However, it cannot be excluded that multiple contact sites between PCNA and RFC are required and that one of these sites may be absent in the RFCp140N604 complex.

RFCp140N604 Cannot Load PCNA onto DNA—We determined whether the RFC complex formed with the p140N604 subunit that was inactive in replication was able to load PCNA

Fig. 6. Interaction of RFC complexes containing full-length or N-terminally deleted p140 with PCNA. In vitro translation reactions expressing the small RFC subunits and either p140, p140N555, or p140N604 (RFC, N555, and N604 in the figure) were incubated with PCNA- or BSA-affinity beads as described under “Materials and Methods.” 10% of the translation reaction mixture (lanes 1, 4, and 7), the material that eluted from the PCNA beads (lanes 2, 5, and 8), and the material that eluted from the BSA beads (lanes 3, 6, and 9) were analyzed on SDS-PAGE.
onto DNA. The loading of \[^{32}P\]-labeled PCNA onto nicked circular DNA was assayed by separating the \[^{32}P\]PCNA-DNA complex from free \[^{32}P\]PCNA after the loading reaction by filtration through a sizing column. As shown in Fig. 7, RFCp140N604 was inactive in loading PCNA, whereas the RFCp140N555 complex catalytically loaded PCNA onto DNA. RFCp140N555 immobilized on beads (40 fmol) loaded 1.1 pmol of PCNA onto DNA, and the reaction was dependent on the addition of ATP (data not shown). A similar amount of RFCp140 loaded 0.2 pmol of PCNA. This indicated that the RFCp140N555 complex was 5 times more active in the PCNA loading reaction than RFC containing the full-length p140 subunit, consistent with the differences observed in the elongation reaction of primed DNA substrates (Fig. 3). Similarly, the unloading reaction, in which \[^{32}P\]PCNA is released from the \[^{32}P\]PCNA-DNA complex, was catalyzed more efficiently by RFC containing p140N555 than RFC containing p140N604 (data not shown).

These results suggest that the loss of replication activity observed with RFCp140N604, which possessed DNA and PCNA binding activities, was most likely due to its inability to load PCNA onto DNA.

**DISCUSSION**

Deletion analysis of the large subunit p140 of human RFC presented in this report provides a step toward the functional characterization of this polypeptide. A summary of the p140 variants constructed, as well as the activity of RFC complexes containing these variants, is presented in Fig. 8. A region was defined within amino acids 822–1142 that is required for the formation of the RFC complex. All of the small subunits also require sequences close to their C termini for complex formation (29), suggesting that the unique sequences in these regions govern the interactions between the five subunits. This might explain why all five subunits are required to form the RFC complex despite the redundancy in their conserved regions. The only other stable complex formed by the RFC subunits consists of the three subunits p36, p37, and p40. This core complex is inactive in replication, although it contains some of the enzymatic properties of RFC.\(^2\)

In the yeast *Saccharomyces cerevisiae*, the genes coding for the homologous RFC complex have been identified (3, 19, 20, 31–36). The genes for the corresponding subunits of yeast and human include highly homologous RFC boxes as well as several conserved C-terminal regions. These homologous C-terminal sequences may include sites critical for subunit interactions that have been conserved through evolution.

The p140 subunit was shown to contain two independent DNA binding domains. Apart from a DNA binding activity that has been mapped to the ligase homology domain (15, 21, 22), a separate region located in the C-terminal half of the subunit was found to recognize primer ends. This confirms previous observations that different nonoverlapping parts of this protein can bind DNA (37). Both the p37 subunit and the p36-p37-p40 complex have been shown to bind specifically to DNA primer ends under conditions less stringent than those used to bind p140 (7).\(^2\) However, further studies will be necessary to establish the significance of these DNA binding activities.

When RFC complexes were formed with N-terminally deleted variants of the p140 subunit, it was found that the N-
terminal half (amino acids 1–555) of p140 was not required for RFC to load PCNA onto DNA and to support elongation of primed DNA templates. The resulting complex (RFCp140N555) was 2–5 times more active than wild-type RFC in catalyzing PCNA loading and the elongation reaction. Two hypothetical explanations for this effect should be mentioned. The N-terminal half of p140 contains a strong DNA binding activity, whereas the C-terminal half binds DNA less effectively, but sufficiently to support RFC activity. The catalytic turnover of RFC may be enhanced by the absence of the strong but dispensable N-terminal DNA binding activity. The p140 N-terminal half also contains two potential CDC2 kinase sites (15) whose phosphorylation status may regulate RFC activity. RFCp140N555 lacks these sites and thus might escape this regulation. A similar mechanism has been described for mammalian DNA ligase I (38), where the enzymatic activity, located in the C terminus, is blocked by interaction with the N terminus. This blockage was released after phosphorylation or alternatively by deletion of the N terminus. Further studies with RFCp140N555 purified from baculovirus-infected insect cells will be necessary to examine this effect in more detail.

The in vivo function of the p140 N-terminal half remains speculative. Although the DNA binding activity of this region may not be essential for RFC function in replication, it might direct RFC to sites involved in other DNA transactions, such as DNA repair or recombination. Furthermore, while the studies presented here show that the p140 N-terminal half is dispensable for the elongation reaction, more elaborate interactions between the replication proteins probably take place at a replication fork, which might involve the N terminus of the p140 subunit.

In S. cerevisiae several mutations in the gene encoding the RFC large subunit (cdc44) have been identified that cause cold sensitivity, elevated levels of spontaneous mutations and increased sensitivity to DNA damaging agents. One such mutation was located in the DNA ligase homology domain (30, 39). The function of the p140 N-terminal half remains speculative for the elongation reaction, more elaborate interactions between the replication proteins probably take place at a replication fork, which might involve the N terminus of the p140 subunit.

Deletion Analysis of hRFC p140

In RFCp140N604 is that the absence of amino acids 555–604 of p140 leads to a structural distortion in the RFC complex that renders it inactive. However, deletion of a similar fragment, containing RFC box II, from any of the small subunits (p36, p37, or p40) did not lead to a comparable inactivation of the RFC complex (29).

In summary, we have defined a region around RFC box II in the large subunit, which when deleted inactivated RFC in loading PCNA. However, the reasons for this loss of function and a general understanding of how the RFC complex works as a molecular machine assembling the PCNA ring around DNA await elucidation of its three-dimensional structure.

Acknowledgments—We thank Charles E. Stebbins for a preparation of PCNA used in this study and Dr. Z.-Q. Pan for continuous helpful discussions.

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Frank Uhlmann, Jinsong Cai, Emma Gibbs,
Mike O'Donnell and Jerard Hurwitz
J. Biol. Chem. 1997, 272:10058-10064.
doi: 10.1074/jbc.272.15.10058

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