THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 13, Issue of March 30, pp. 10168–10177, 2001

Printed in U.S.A.

Human-*Saccharomyces cerevisiae* Proliferating Cell Nuclear Antigen Hybrids

OLIGOMERIC STRUCTURE AND FUNCTIONAL CHARACTERIZATION USING *IN VITRO* DNA REPLICATION*

Received for publication, September 29, 2000, and in revised form, November 20, 2000
Published, JBC Papers in Press, November 27, 2000, DOI 10.1074/jbc.M008929200

Ayodele Ola‡, Shou Waga§¶, Viola Ellison§, Bruce Stillman§, Mark McGurk‡, Irene M. Leigh‖, Naushin H. Waseem, and Ahmad Waseem‡ ‡‡**

*From the ‡Head and Neck Cancer Research Program, Guys, King's, and St. Thomas' Dental Institute and the **Division of Molecular and Medical Genetics, Pediatrics Research Unit, Guy's Campus, King's College London, London SE1 9RT, United Kingdom, the §Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724, and the ‖Imperial Cancer Research Fund Skin Tumor Laboratory, the Royal London Hospital, London E12AT, United Kingdom*

The proliferating cell nuclear antigen (PCNA) is a highly conserved protein required for the assembly of the DNA polymerase delta (pol δ) holoenzyme. Because PCNAs from *Saccharomyces cerevisiae* and human do not complement each other using *in vitro* or *in vivo* assays, hybrids of the two proteins would help identify region(s) involved in the assembly of the pol δ holoenzyme. Two mutants of human PCNA, HU1 (D21E) and HU3 (D120E), and six hybrids of human and *S. cerevisiae* PCNA, HC1, HC5, CH2, CH3, CH4, and CH5, were prepared by swapping corresponding regions between the two proteins. In solution, all PCNA assembled into trimers, albeit to different extents. These PCNA variants were tested for stimulation of pol δ and *in vitro* replication of M13 and SV40 DNA as well as to stimulate the ATPase activity of replication factor C (RF-C). Our data suggest that in addition to the interdomain connecting loop and C terminus, an additional site in the N terminus is required for pol δ interaction. PCNA mutants and hybrids that stimulated pol δ and RF-C were deficient in M13 and SV40 DNA replication assays, indicating that PCNA-induced pol δ stimulation and RF-C-mediated loading are not sufficient for coordinated DNA synthesis at a replication fork.

DNA replication is a fundamental biological process that is vital for cell proliferation. The mechanism of DNA synthesis and its regulation is highly complex requiring the interplay of many proteins that cooperate to duplicate the genetic information for the next generation rapidly and accurately (1–4). Factors required for DNA replication in eukaryotes have been identified using replication of simian virus 40 (SV40) as a model system because all of the proteins required for SV40, except the viral large T antigen, are host-provided (5–7). Systematic reconstitution of SV40 replication *in vitro* using highly purified proteins has provided important insights into their functions (8). Our current understanding of DNA replication in eukaryotes suggests that polymerase α (pol[1] α)−primase synthesizes the first RNA/DNA primer on the leading strand which is then followed by the binding of RF-C and loading of PCNA and a polymerase switch from pol α to pol δ. Thus pol δ holoenzyme performs processive leading strand synthesis, whereas pol α participates in RNA priming and lagging strand synthesis. However, complete synthesis of Okazaki fragments also requires participation of pol δ holoenzyme (9–15).

A vital element of the DNA replication machinery is the ability of DNA pol δ to execute processive DNA synthesis allowing the polymerase to move quickly along the template thousands of nucleotides without dissociation. This is accomplished in *Escherichia coli* by the β subunit of DNA pol III and in eukaryotes by PCNA, which is a processivity factor for pol δ (16). The crystal structure of the β subunit and PCNA showed a toroidal structure that can encircle the DNA (17, 18). PCNA is a homotrimer in which each subunit consists of two structurally related domains giving the molecule a 6-fold symmetry. The center of the ring is positively charged, and it is large enough to allow free passage of double-stranded DNA. PCNA is loaded onto DNA by the action of RF-C (11, 19), a complex of five different subunits (20, 21), in an ATP-dependent manner. The RF-C·PCNA complex then tethers pol δ onto the template to assemble a highly processive pol δ·PCNA·RF-C complex also known as the pol δ holoenzyme. However, in the absence of RF-C, PCNA can still load onto linear DNA at a double-stranded end, albeit at much lower efficiency (22). The orientation of the PCNA ring provides two surfaces, one facing the primer-template junction required for pol δ and RF-C binding and the other surface facing the double-stranded DNA for binding of other proteins (23, 24). The molecular interactions between different subunits of various proteins in the holoenzyme have only been partially studied.

Besides being required for coordinated leading and lagging strand DNA synthesis at a replication fork (25), PCNA has also been implicated in a variety of cellular processes such as cell cycle control (26), nucleotide excision repair (27, 28), postreplication mismatch repair (29, 30), base excision repair (31),

* This work was supported by the Special Trustees of Guy's Hospital, Guy's Dental Funds Committee, and the Wellcome Trust. 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.

¶ Present address: Dept. of Biochemistry and Molecular Biology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565 0871, Japan.

‡‡ To whom correspondence should be addressed: Dept. of Craniofacial Development, GKT Dental Institute, Floor 28, Guy's Hospital, King's College London, London SE1 9RT, United Kingdom. Tel.: 44-207-955-4992; E-mail: ahmed.waseem@kcl.ac.uk.

[1] The abbreviations used are: pol, polymerase; RF-C, replication factor C; PCNA, proliferating cell nuclear antigen; hPCNA, human PCNA; cPCNA, *S. cerevisiae* PCNA; PAGE, polyacrylamide gel electrophoresis; RPA, replication protein A; wt, wild type; DTT, dithiothreitol; interdomain connector.

TABLE I
Sequence of oligonucleotides used in site-directed mutagenesis of h- and cPCNA

Mutants	Restriction site created	Oligonucleotide used in mutagenesis 5′ → 3′[a]	Mutations[b]
HU1	Sac I	GGAGGCACTCAAGGAGCTCATCAACGAGG	D21E
HU2	*Spe* I	CGTGAACCTCACTAGTATGTCCAAA	c219 → t
HU3	*Sac* I	AGTTGATGGAGCTCGATGTTGAAC	D120E
HU4	*Sac* I	CAAGTGGAGAGCTCGGAAATGGAA	a522 → g t525 → c
CE1	*Sac* I	GTTTCAAAGAGCTCGTCCAGTTGG	D21E; C22L
CE2	*Spe* I	GGTATGGATCTAACTAGTCTAAGTAAAATCC	c219 → t t220 → a c221 → g a222 → t
CE3	*Sac* I	CTGAAATTGATGGAGCTCGATGCTGATTTC	I121L
CE4	*Sac* I	GTAGCTGACGGTGAGCTCGGATCAGGTTC	D174E; I175L
CE5	*Kpn* I	GACGTTCGGAGCTAGGTACCTATTGGACATCA	L210R t633 → c t634 → c

[a] Underlined nucleotides are those mutated from the wt sequence.
[b] Amino acid replacements are shown in upper case, and nucleotide changes are in lower case.

chromatin function (32–34), RNA transcription, and cytosine-5 methylation (35) (for reviews see Refs. 2, 4, 20, 36). PCNA therefore not only acts as a clamp for DNA polymerases, but it is also a multifunctional protein that may link multiple protein-protein interactions in replication, repair, recombination, and cell cycle regulation.

The primary structure of PCNA is highly conserved in the animal kingdom (31, 37, 38), and related proteins have been found in plant (39), yeast (40, 41), and virus (42). Recent mutation analyses of both human and yeast PCNA have shown that PCNA is remarkably resistant to amino acid substitution, but these studies have defined protein interaction sites on the surface of PCNA (23, 43–51). However, deletions in any part of the molecule including the N and C termini distort the tertiary structure, rendering the protein of limited use in functional assays. The *Saccharomyces cerevisiae* homolog of PCNA, pol30, shares 35% sequence identity with the human PCNA (hPCNA) and is able to enhance the processivity of the mammalian pol δ (52), but it is unable to complement hPCNA in SV40 DNA replication *in vitro* (53). This suggests that *S. cerevisiae* PCNA (cPCNA) has very low affinity for mammalian pol δ and RF-C, even though both yeast and mammalian PCNAs exist as trimers (54, 55). We therefore argued that by swapping different regions between h- and cPCNA, it should be possible to generate hybrids that were equivalent to large deletions in hPCNA while retaining a trimeric structure. Functional analyses of these hybrids would help to define region(s) of hPCNA involved in DNA replication.

We introduced mutations in the highly conserved region of PCNA and also generated a set of novel human-*S. cerevisiae* PCNA hybrids. These PCNAs were examined for their ability to stimulate pol δ and RF-C or to function in DNA replication assays. The stimulation of RF-C and pol δ involves associations on at least three sites located at the N and C termini, and at the interdomain connector (IDC) loop of PCNA. We have observed induction of RF-C-catalyzed ATPase activity by a PCNA hybrid that was predominantly dimeric, suggesting that PCNA trimers may not be necessary for stimulation of RF-C ATPase. Our data also suggest that assembly of the pol δ holoenzyme may require participation of other proteins at a replication fork.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA-modifying enzymes were obtained from Roche Molecular Biochemicals (Germany) and from Promega. Radioactive chemicals were bought from Amersham Pharmacia Biotech and ICN (U. K.). Synthetic oligonucleotides were supplied by Imperial Cancer Research Fund laboratories, U. K., and purified by urea-PAGE before use. $(dA)_{290-539}$ and oligo$(dT)_{12-18}$ were obtained from Amersham Pharmacia Biotech. The 69-nucleotide hairpin DNA used in the RF-C stimulation assay was constructed by ligating two oligonucleotides following the method described previously (9). Singly primed M13 DNA, M13mp18, was prepared according to the method described earlier (56). Anti-PCNA antibodies, PC-9 and PC-10, were obtained by growing respective hybridomas in Dulbecco's modified Eagle's medium + 10% fetal calf serum. Supernatants from the confluent cultures were stored in 0.2% (w/v) sodium azide until used. All other chemicals and reagents used in this study were molecular biology grade and were obtained from BDH/Merck and Sigma.

Plasmid pSV011 was constructed by ligating a *Hin*dIII-*Sph*I fragment containing the SV40 origin of replication into pUC18 as described earlier (25), and the construct was purified by Triton X-100 lysis of recombinant bacteria followed by CsCl gradient centrifugation. Construction of plasmids for the expression of the human, pThPCNA, and *S. cerevisiae*, pTyPCNA, PCNA has been described previously (41, 53).

Preparation of Replication Proteins—DNA pol δ from calf thymus, RPA from *E. coli*, and SV40 large T antigen from Sf9 insect cells infected with recombinant baculovirus were purified as described previously (15). Human RF-C was expressed and purified from baculovirus infected Sf9 cells as described earlier (57). The cytosolic replication extract (S100) from human 293 cells was prepared as described (7). Fractionation of S100 into fractions II and IA has been described previously (16). Fraction II contained multiple components including DNA polymerases α and δ, RF-C, topoisomerases I and II, DNA ligases, and other essential DNA replication proteins; RPA was the main component of fraction IA (9, 16, 58–60).

Site-directed Mutagenesis and Preparation of Human-S. cerevisiae PCNA Hybrids—Mutations were introduced into the respective cDNA for the wild type (wt) h- and cPCNA using the plasmid pThPCNA and pTyPCNA by polymerase chain reaction-mediated site-directed mutagenesis using the Quick Change mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used for generating mutants and the restriction sites created are shown in Table I. Four human and five *S. cerevisiae* PCNA mutants were prepared using this method. The mutations were confirmed by nucleotide sequencing.

The mutant cDNAs for h- and cPCNA were subjected to double restriction enzyme digestion using the restriction sites created during mutagenesis (see Table I) and the *Bam*HI site present at the 3′-end in all constructs. The insert and vector DNAs were ligated in different combinations such that the hPCNA inserts were swapped with the corresponding segment of cPCNA. This resulted in 10 different constructs containing part of the hPCNA fused with a part of cPCNA (see Table II), which were confirmed by nucleotide sequencing. The hybrids were named by two letters followed by a number in which the first letter was derived from the species contributing the N terminus followed by the species contributing the C terminus. For example, hybrids with the human sequence at the N terminus were named HC whereas those with *S. cerevisiae* sequence at their N terminus were named as CH. The contribution of the two PCNAs in each hybrid is shown in Table II.

Expression and Purification of Recombinant PCNA—Plasmids constructs encoding wt, mutant, and hybrid PCNA were transformed into BL21(DE3) cells for expression of protein. An overnight culture (0.75 ml) was used to inoculate 200 ml of LB + ampicillin and grown at 37 °C until A_{600} reached to 0.6. The protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and grown for 3–6 h at 37 °C.

TABLE II
Contribution of the h- and cPCNA in different hybrids used in this study along with the origin of IDC loop

PCNA hybrids	Contribution from hPCNA	Contribution from cPCNA	Origin of IDC loop[a]
	amino acids	*amino acids*	
HC1	1–20	21–258	*S. cerevisiae*
HC5	1–211	212–258	Human
CH2	73–261	1–72	Human
CH3	121–261	1–120	12% *S. cerevisiae* 88% Human
CH4	175–261	1–174	*S. cerevisiae*
CH5	212–261	1–211	*S. cerevisiae*

[a] IDC loop extends from residues 118 to 135.

The procedure for extraction and purification of PCNA from recombinant bacteria was carried out at 4 °C. Bacteria from 200-ml cultures were harvested at 3,000 rpm for 15 min, resuspended into 4 ml of lysis buffer (25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1 mM EDTA, 0.01% Nonidet P-40, 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT), and sonicated to disrupt cells and reduce viscosity. The mixture was adjusted to 0.2 M NaCl, cleared by centrifugation, and loaded onto a Q-Sepharose column (1.2 × 11 cm) equilibrated with 0.2 M NaCl and buffer A (50 mM Tris-HCl, pH 7.4, 20% glycerol, 2 mM EDTA, 0.02% Nonidet P-40, 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT). After washing the column, bound PCNA was eluted with a 0.2–0.7 M linear NaCl gradient in buffer A. The PCNA-containing fractions, identified by SDS-PAGE, were pooled and dialyzed against 25 mM KPO_4, pH 7.0, and buffer B (10 mM KPO_4, pH 7.0, 10% glycerol, 0.01% Nonidet P-40, 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) and loaded onto an S-Sepharose column (1.2 x 11 cm). The PCNA-containing flow-through fractions were pooled and loaded onto a hydroxyapatite column (2.5 × 11 cm) equilibrated with 25 mM KPO_4 and buffer B and eluted with a 0.025–0.5 M linear phosphate gradient in buffer B. The PCNA-containing fractions from the hydroxyapatite column were pooled, dialyzed against 1.2 M NaCl and buffer C (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.02% Nonidet P-40, 2 mM benzamidine, 2 μM pepstatin A, 10 mM $NaHSO_3$, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT), and 4 ml of the sample was loaded onto a phenyl-agarose column (1.2 × 11 cm). The protein was eluted with a decreasing linear salt gradient from 1.2 to 0 M NaCl in buffer C, and the PCNA-containing fractions were pooled, dialyzed against 20% sucrose, 25 mM NaCl, and buffer A and stored in aliquots at −70 °C until used.

Electrophoresis, Immunoblotting, and Chemical Cross-linking—SDS-PAGE of PCNA proteins was carried out on 12% polyacrylamide gels using the conditions described elsewhere (61). Western blotting was performed using nitrocellulose membranes as described earlier (62). Native gel electrophoresis was carried out on 8–25% gradient gels using the Phast System (Amersham Pharmacia Biotech) according to the manufacturer's recommendations.

Chemical cross-linking was carried out using ethylene glycol-bis-succinimidyl succinate as described previously (63).

Gel Filtration Analysis—Purified wt, mutant, and hybrid PCNA preparations (100 μg) were applied onto a Superose 12 HR10/30 (Amersham Pharmacia Biotech) gel filtration column equilibrated with 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 250 mM NaCl. The column was calibrated by eluting a sample of blue dextran and five native molecular mass markers, lactate dehydrogenase (145 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and cytochrome *c* (12 kDa). The elution volume for each marker was the time taken to elute the peak fraction of that sample. The peak fractions (300–500 μl) for each PCNA were collected, concentrated using Centricon™ microconcentrators (Amicon), and the presence of PCNA was confirmed by SDS-PAGE.

Stimulation and Processivity Assay for DNA Pol δ—The DNA pol δ stimulation assay used in this study was a slightly modified version of that described previously (12, 13). The reaction mixtures (12.5 μl) contained 30 mM HEPES/NaOH pH 6.6 buffer, 7 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml bovine serum albumin, $(dA)_{540}$, $oligo(dT)_{16}$, in a ratio of 19:1 in nucleotides, 40 μM dTTP, 0.17 pmol of [α-^{32}P]TTP, 0.15 unit of calf thymus pol δ, and different concentrations of PCNA. The reactions were incubated at 37 °C for 15 min, and 2 μl was spotted on DE81 paper (Whatman), washed in 0.5 M Na_2HPO_4, and the incorporated radioactivity was measured in a scintillation counter.

The processivity assay was essentially as described above except that the reactions contained limiting amounts of PCNA (0.5–5 ng) and 0.0075 unit of pol δ in the presence of 1.7 pmol of [α-^{32}P]TTP. The incorporated radioactivity was determined as described above, and the rest of the reaction mixture was analyzed on a 2% alkaline agarose gel and visualized by autoradiography.

RF-C ATPase Assays—RF-C ATPase activity was measured according to a protocol described previously (57). The reaction mixture (20 μl) contained 30 mM HEPES pH 7.5 buffer, 7 mM $MgCl_2$, 0.5 mM DTT, 0.1 mg/ml bovine serum albumin, 100 μM ATP, 3 pmol of [α-^{32}P]ATP (800 Ci/mmol), 0.25 pmol of RF-C, 0.4 μmol of hairpin DNA with 5′-single-strand extension, and different concentrations of PCNA. The reaction mixtures were incubated for 1 h at 37 °C and stopped by 10 mM EDTA. One-tenth (2 μl) of the reaction mixture was spotted onto a polyethyleneimine-cellulose plate that had been pretreated with 1 M formic acid. The plates were then developed in a mixture of 1 M formic acid and 0.5 M LiCl for 60 min. After autoradiography the amount of ADP produced was determined by scanning the plates on a Storm 860 PhosphorImager (Molecular Dynamics).

DNA Replication Assays—M13 DNA synthesis reactions were carried out by incubating singly primed M13mp18 DNA with pol δ, RF-C, PCNA, and RPA as described previously (56).

The SV40 DNA replication assays *in vitro* were performed as described previously (16) using S100, fraction IA, fraction II, large T antigen, and SV40 origin of replication containing plasmid, pSV011. The optimal amounts of extracts and proteins required in the assay were determined empirically by titration. Reaction mixtures were assembled using PCNA, pSV011, fractions IA and II, large T antigen, nucleotide mixture, and creatine phosphate/creatine phosphokinase on ice. The samples were incubated at 37 °C for 1 h; the controls were left on ice. After terminating the reaction with 20 mM EDTA, the ^{32}P incorporation was determined using DE81 paper. The remaining portion of the reaction mixture from either M13 or SV40 assay was deproteinated with proteinase K and extracted with phenol/chloroform. After precipitating with ethanol, the replication products were analyzed on an agarose gel, fixed in a mixture of 10% methanol + 10% acetic acid, dried, and autoradiographed.

Other Methods—The techniques used for genetic manipulation of bacteria were performed using standard protocols (64). Protein concentrations were determined by the BCA method (Sigma) using BSA as standard. Nuclease contamination in PCNA preparations was tested by incubating each PCNA protein (20 ng–2 μg) with 1 μg of pGEX-2T for 1 h at 37 °C followed by separation on an agarose gel. All gel pictures and blots were scanned and assembled using the Adobe PhotoShop version 5.

RESULTS

Expression and Purification of PCNA Mutants and Hybrids—We have produced 4 hPCNA (HU) and 5 cPCNA (CE) mutants, each bearing a mutation that created a unique restriction site within the coding region (Table I). The sites for mutation were selected, as far as possible, to fall in the region connecting different β sheets or α helices. Two of the hPCNA mutants, HU2 and HU4, had silent mutations and were used only for making hybrids; the other two, HU1, containing the mutation D21E in the A1B1 loop on the inner surface of the trimer, and HU3, containing the mutation D120E at the beginning of the IDC loop, were conservative changes. These mutants and hybrids were employed in functional analyses, whereas the cPCNA mutants were used only for preparing hybrids.

The pThPCNA and pTyPCNA plasmids expressing h- and cPCNA, respectively, were used in site-directed mutagenesis as described under "Experimental Procedures." The predicted structure of the hybrids is shown in Fig. 1 and Table II. The relative mobility of the wt h- and cPCNA on SDS-PAGE yielded an apparent molecular mass of 36 kDa and 29 kDa, respectively. The wt hPCNA, HU1, and HU3 had identical mobility on SDS-PAGE, whereas the electrophoretic mobilities of the hybrids varied between that of wt h- and cPCNA (Fig. 2). All constructs produced soluble PCNA proteins when grown in bacteria on a smaller scale; however, when a large scale cultures were employed, four of the constructs, HC2, HC3, HC4, and CH1, lost their ability to produce soluble protein effi-

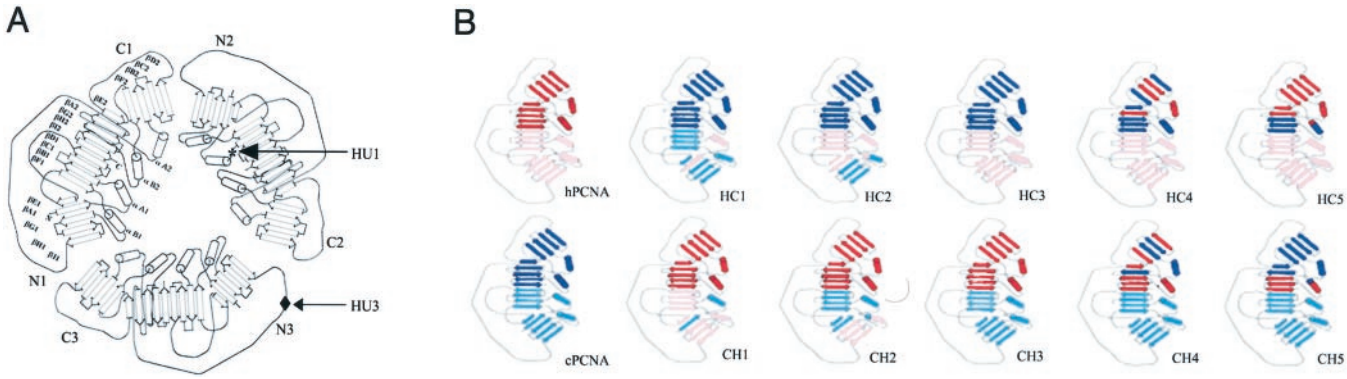

FIG. 1. **Schematic representation of the polypeptide folding in a native PCNA molecule.** *Panel A*, the elements within the two topologically identical domains N1 and C1 are labeled using the nomenclature described by Krishna *et al*. (17). The location of the mutation D21E in HU1 is shown by an *asterisk*, which, in the folded structure, lies on the inner surface of the trimeric ring. The mutation D120E in HU3, shown by a *diamond*, is located in the IDC loop. *Panel B*, structure of monomeric PCNA for the wt human, *S. cerevisiae*, and the hybrids. The hPCNA polypeptide is shown by a combination of *red* and *pink*; cPCNA is shown by a combination of *cynol* and *blue*. The N termini of the h- and cPCNA are shown, respectively, by *pink* and *cynol;* the C termini are shown by *red* and *blue*.

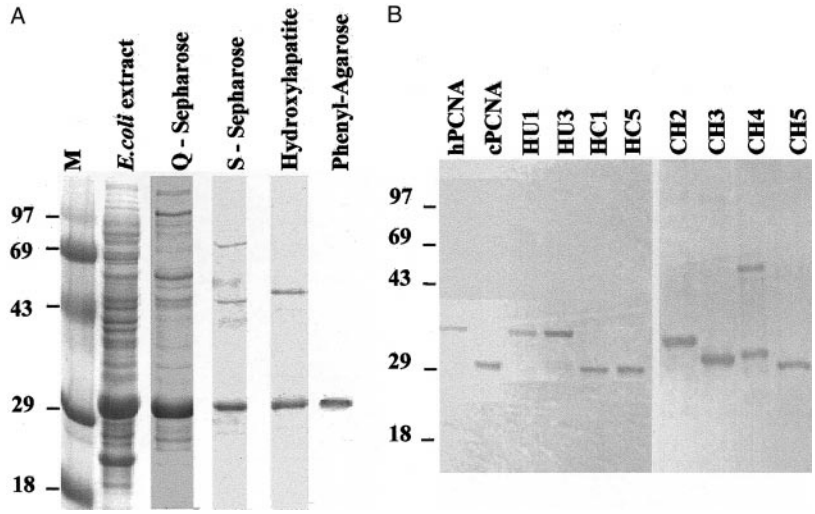

FIG. 2. **Purification of recombinant wt, mutant, and hybrid PCNA from *E. coli*.** Plasmid constructs containing wt, mutant, and hybrid PCNA were transformed in BL21(DE3) *E. coli* cells, and protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside. The lysate from a 200-ml culture was adjusted to 0.2 M NaCl, filtered through 0.22 μM before subjecting to Q-Sepharose, S-Sepharose, hydroxyapatite, and phenyl-Sepharose columns as described under "Experimental Procedures." The purification of PCNA at various stages of the protocol was followed by subjecting a small fraction through SDS-PAGE and staining the gel with Coomassie Blue. *Panel A*, a representative gel of the PCNA hybrid CH3 during different stages of purification. *Panel B*, silver-stained SDS gel of the purified PCNA proteins used in this study. A 10-μl sample containing 3–5 μg of purified PCNA was analyzed in each case.

ciently, which could have been the result of inclusion body formation. These constructs were therefore excluded from this study.

Soluble PCNA extracted from large scale cultures were purified to near homogeneity as judged by silver staining of SDS-PAGE gels (Fig. 2). Starting from a 200-ml culture, the yield of purified PCNA was 1–2 mg from different constructs except for HU3 and CH4, which gave only 400 and 360 μg of purified protein, respectively. The lower yield of CH4 could be caused by the aggregation tendency of this hybrid as reported for other mutants (23, 45); however, the same did not apply to HU3 that was predominantly trimeric in solution (see Fig. 3). These preparations were free of nucleases because no degradation of plasmid DNA was observed by up to 200 μg/ml PCNA (not shown), which was about 5-fold higher than the amount used in DNA replication assays.

Immunoblotting of the hybrids with anti-PCNA antibodies (65) produced reactivity of PC-9 with CH2, CH3, and CH4, but not with HC1, HC5, and CH5, whereas PC-10 reacted with CH2 but did not react with HC1, HC5, CH3, CH4, and CH5 (data not shown). The mutants HU1 and HU3 reacted with both of the antibodies, as did the wt hPCNA. The reactivity of these antibodies with the hybrids is consistent to their epitope locations reported earlier (66).

PCNA Mutants and Hybrids in Solution Can Assemble into Oligomers—To test whether the PCNA mutants and hybrids can form trimers in solution we determined their elution profile on a gel filtration column calibrated with five native proteins of known molecular sizes. Wt h- and cPCNA preparations separated into a major peak corresponding to an apparent molecular mass of 98 kDa (trimer) and a minor peak corresponding to 29 kDa (monomer). The mutants HU1 and HU3 and hybrids HC5 and CH3 also gave a major trimer peak and a minor monomer peak. The hybrids HC1 and CH5 produced more monomers than trimers, and in CH2 most of protein eluted at the position of a dimer. The hybrid CH4 had a small proportion of trimers, but most of the protein eluted in the void volume. The proportion of oligomeric species in different PCNA preparations is listed in Table III. The data suggest that in solution our PCNA preparations, with the exception of CH4, exist in equilibrium among monomers, dimers, and trimers. Thus our initial expectation that hybrids of PCNA from the two species would assemble into trimers was vindicated. However, the tertiary structure of some of the hybrids was distorted as indi-

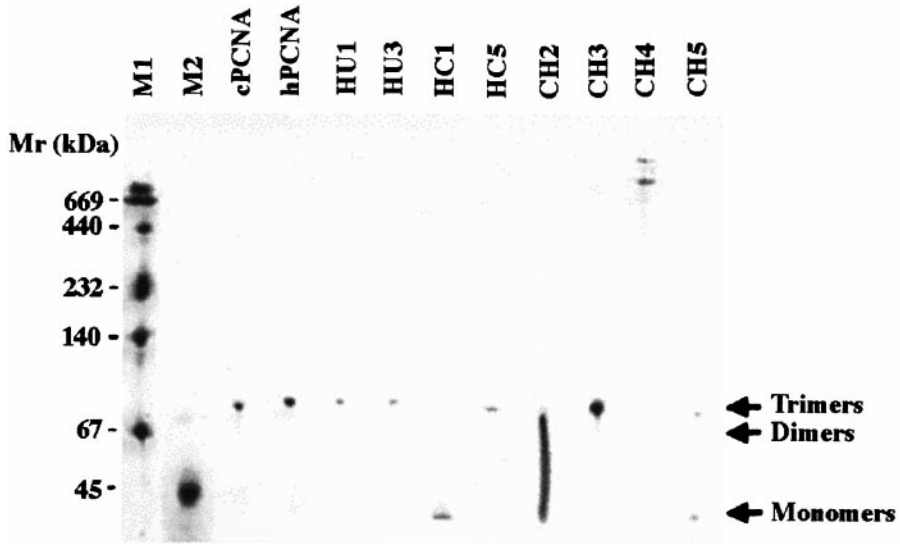

FIG. 3. **Analysis of PCNA proteins on a native gel.** Wt PCNA (h- and cPCNA), mutants (HU1 and HU3), and the hybrid proteins were dialyzed in buffer A containing 25 mM NaCl and 20% glycerol. 1 μg of each PCNA protein and molecular markers were taken in 8 μl of loading buffer, and 4 μl of this mixture was run on an 8–25% polyacrylamide gel for 45 min and stained with Coomassie Blue. *Lanes M1* and *M2* indicate native protein markers: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), BSA (67 kDa), and ovalbumin (45 kDa).

TABLE III
Ratio of monomeric, dimeric, and trimeric PCNA recovered from gel filtration column

PCNA proteins	Trimers[a]	Dimers[a]	Monomers[a]
hPCNA	72	6	22
CPCNA	77	8	15
HU1	67	11	22
HU3	67	10	23
HC1	25	17	58
HC5	63	13	24
CH2	16	53	31
CH3	56	22	22
CH4[b]	21	0	0
CH5	40	6	54

[a] Percentage of total protein applied onto the column.
[b] Most of the protein (about 80%) eluted with the void volume.

cated by a lower proportion of trimers.

The pattern of PCNA oligomerization was investigated further by native gel electrophoresis using an Amersham Pharmacia Biotech Phast System. In this gel system the trimer band could be clearly distinguished from the fast moving dimer and monomer bands (45). Consistent with our gel filtration data we observed trimeric species in the wt h- and cPCNA, HU1, HU3, HC5, and CH3. The HC1 hybrid remained predominantly a monomer, and CH5 was a mixture of monomers and trimers. However, minor PCNA species were not detected in this system, which could be the result of the low sensitivity of Coomassie staining. The hybrid CH2 produced a smear with no defined band for monomers or trimers. This suggested that trimers in CH2 were unstable and that the PCNA molecule might spontaneously unfold and refold. CH4 was the only hybrid to have large aggregates with no monomer and only traces of trimer (Fig. 3, in the actual gel a trace of trimer was seen which is not very clear in the photograph). The aggregates in CH4 were much more homogeneous in size than for the Y114A mutation reported earlier (45). The presence of dimers in CH2 and large aggregates in CH4 was established further by ethylene glycol bis-succinimidyl succinate-induced cross-linking (data not shown).

Function of Mutants and Hybrids Using PCNA-induced DNA Polymerase δ Activity—The ability of the different PCNA proteins to stimulate calf thymus pol δ activity on a synthetic DNA template was studied. Incubation of pol δ with the wt hPCNA stimulated the enzyme in a dose-dependent fashion as reported previously (23). Compared with the hPCNA, the wt cPCNA did not express significant pol δ stimulatory activity (Fig. 4). The two mutants, HU1 and HU3, also stimulated pol δ in a dose-dependent fashion, but they had about 40% stimulatory activity compared with the wt hPCNA. This is interesting because the two mutations were located in different regions of the molecule. The D21E mutation in HU1 is located toward the N terminus, and the D120E mutation in HU3 is located in the IDC loop. These two sites appear to play a role in PCNA-induced stimulation of pol δ activity. As shown in Fig. 4, the hybrids HC1, HC5, CH2, and CH4, did not stimulate pol δ, but CH3 and CH5 stimulated the enzyme in a dose-dependent fashion. The degree of pol δ stimulation by CH3 was similar to that achieved with hPCNA, whereas CH5 had much reduced stimulatory activity. The ability of the wt, mutant, and hybrid PCNA to enhance the processivity of pol δ was determined by analyzing the size distribution of the replication products on alkaline agarose gel electrophoresis. As shown in Fig. 5, the hybrids HC1, HC5, CH2, and CH4 did not induce processive DNA synthesis, which was consistent with them being unable to interact efficiently with pol δ. The PCNA proteins that stimulated pol δ, HU1, HU3, CH3, and CH5, appeared processive, but their activity, except for CH3, was reduced compared with the wt hPCNA. This suggested that HU1, HU3, and CH5 were impaired in their initial interaction with pol δ but not in the processive DNA synthesis. The data indicate involvement of IDC loop and both the N- and C-terminal domains in the interaction of PCNA with pol δ. The processivity of pol δ with CH3 was not very different from that with hPCNA, but it was certainly more active than the wt hPCNA (see Fig. 5). Taken together, the data suggest that formation of PCNA·pol δ complex involves multiple site interactions.

Most of the PCNA Hybrids Were Active in RF-C-mediated DNA Clamping—The interaction between RF-C and PCNA in the presence of DNA and ATP results in ATP hydrolysis and the formation of a sliding clamp. The ATPase activity of RF-C is stimulated by PCNA in the presence of primed DNA. The RF-C ATPase assay thus measures the ability of PCNA to interact with RF-C and DNA. We examined the loading of PCNA mutants and hybrids onto primed DNA by RF-C. The assay was conducted at four different PCNA concentrations (100, 200, 400, and 800 ng) and to compare the data from different sets, the PCNA-dependent stimulation of RF-C ATPase activity for different preparations was expressed as percentage of the wt hPCNA (Fig. 6). The RF-C stimulation with cPCNA was similar to that in the absence of PCNA, suggesting that cPCNA was inactive in this assay. All PCNA hybrids tested showed PCNA-dependent stimulation of RF-C ATPase, but to different extents. The least stimulation was

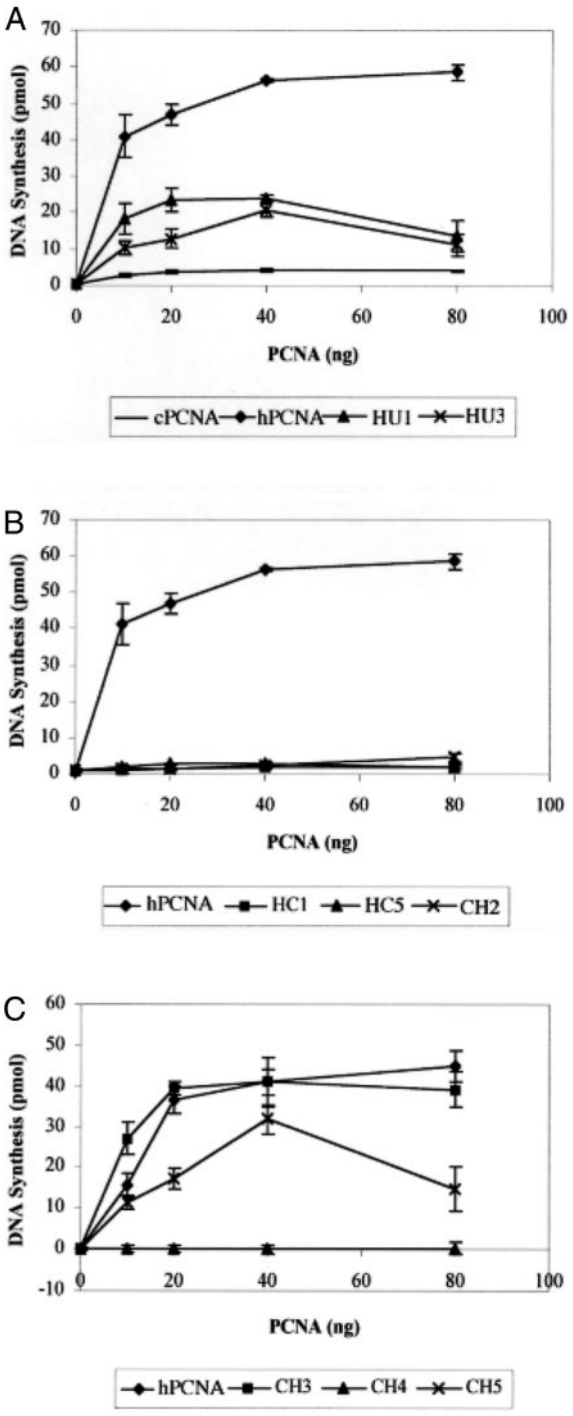

FIG. 4. **Function of PCNA variants with calf thymus DNA pol δ as determined by PCNA-induced stimulation of DNA polymerase activity.** Reactions were carried out as described under "Experimental Procedures" with increasing concentrations of PCNA, and incorporation of radioactivity after incubation at 37 °C for 15 min was determined by spotting 2 μl of the reaction mixture on DE81 paper. The amount of dTMP (in pmol) polymerized was plotted against the amount of PCNA used in the reaction. Each graph in *panels A*, *B*, and *C* represents results of a minimum of at least three independent experiments. The *standard error bars* represent 95% confidence intervals on the means. The PCNA proteins used in different reactions are shown by individual *symbols* in each graph.

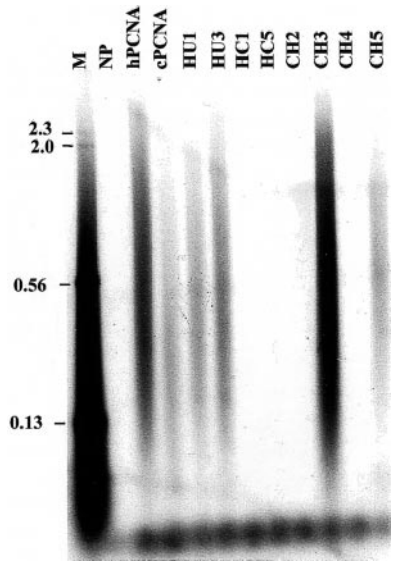

FIG. 5. **Effect of PCNA mutants and hybrids on the processivity of DNA pol δ.** In this set of experiments the reaction components were adjusted such that the dTMP incorporation corresponded to a DNA synthesis of less than 0.3 nucleotide from each primer end. A reaction mixture containing 0.5 ng of the indicated PCNA protein, 0.0075 unit of pol δ, and 1.7 pmol of [α-^{32}P]TTP in a total volume of 50 μl was incubated at 37 °C for 15 min. The products were analyzed on a 2% agarose gel under alkaline conditions and revealed by autoradiography. The length of the products represents the processivity of DNA pol δ in the presence of indicated PCNA proteins. *Lane M* contained DNA fragments obtained with λDNA digested with *Hin*dIII, and *NP* was a control reaction without PCNA.

seen with the hybrids HC1 and HC5, both showing about 10% of PCNA-dependent RF-C stimulation compared with the wt hPCNA. The two mutants HU1 and HU3 and the other hybrids, CH2, CH3, and CH5, with the exception of CH4, achieved 40–70% of RF-C stimulation. As with the wt PCNA, the percentage of RF-C stimulation did not increase with PCNA concentration for any of the hybrid PCNA preparations, except for the hybrid CH4. In CH4 the PCNA-dependent stimulation of RF-C ATPase was proportional to the PCNA concentration and was much higher than for the wt hPCNA.

To determine the ATP turnover for the interaction of RF-C with PCNA, we performed RF-C ATPase assays at 800 ng of PCNA for 15 and 30 min. Previous studies (9, 23, 67) have shown that an incubation of 30 min was sufficient for ATP hydrolysis during RF-C·PCNA·DNA complex formation. Although an incubation of 30 min was sufficient for ADP production to reach steady state, the level of ATPase activity with mutants and hybrids, with the exception of CH4, was less compared with hPCNA (data not shown). This is consistent with the dose-dependent experiments described above and shows that the PCNA preparations, with the exception of HC1 and HC5, were active in RF-C dependent clamping of DNA.

PCNA Mutants HU1 and HU3 Showed Diminished Ability to Complement M13 DNA Replication in Vitro—Having shown the effects of the mutants and hybrids on both pol δ stimulation and RF-C loading, we tested their ability to support replication of a singly primed circular DNA, M13mp18. In this reaction assembly of the holoenzyme is achieved, and the assay measures the overall interaction of PCNA with RF-C and DNA as well as with pol δ. From the incorporation of [^{32}P]dAMP, formation of a quaternary complex of wt hPCNA with RF-C, pol δ, and the DNA was evident. The cPCNA was inactive in this assay (Fig. 7*A*). The mutants HU1 and HU3 supported DNA replication, but the hybrids were inactive. The overall activity of both mutants was similar to each other but only about 30% compared with the wt hPCNA. However, an analysis on an alkaline agarose gel revealed that the replication products with HU1 were shorter than those obtained with hPCNA (Fig. 7*B*). HU3 replication products were even shorter than with HU1, and they resembled those described previously when PCNA or RF-C was omitted from the replication reaction (9, 25). Clearly,

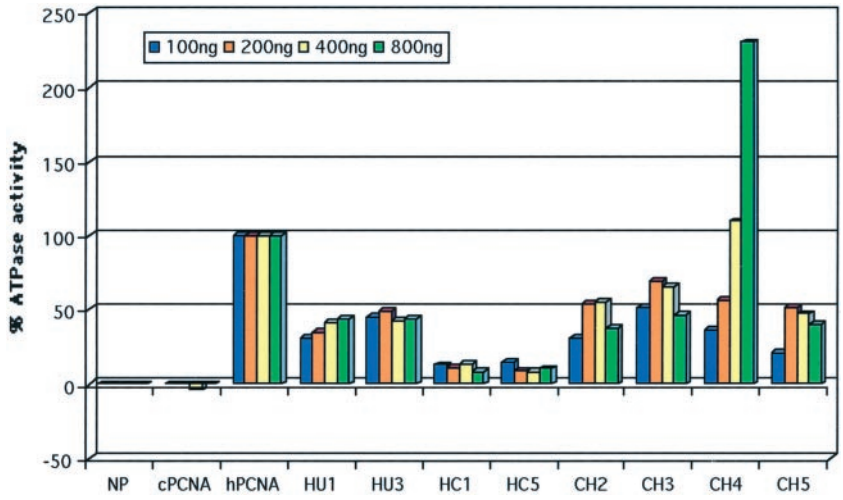

FIG. 6. **Characterization of RF-C ATPase activity induced by PCNA mutants and hybrids.** The RF-C-catalyzed ATPase assays were performed in a 20-μl reaction containing 0.4 μmol of hairpin DNA and 0.25 pmol of RF-C as described under "Experimental Procedures." The amount of ADP produced by RF-C in the absence of PCNA (*NP*) and in the presence of increasing amounts (100, 200, 400, and 800 ng) of each of the PCNA protein after incubation at 37 °C for 1 h was determined using a Storm 860 PhosphorImager. The degree of stimulation of RF-C ATPase activity by different PCNA preparations was compared with the wt hPCNA. The activity of hPCNA under identical conditions was taken as 100%.

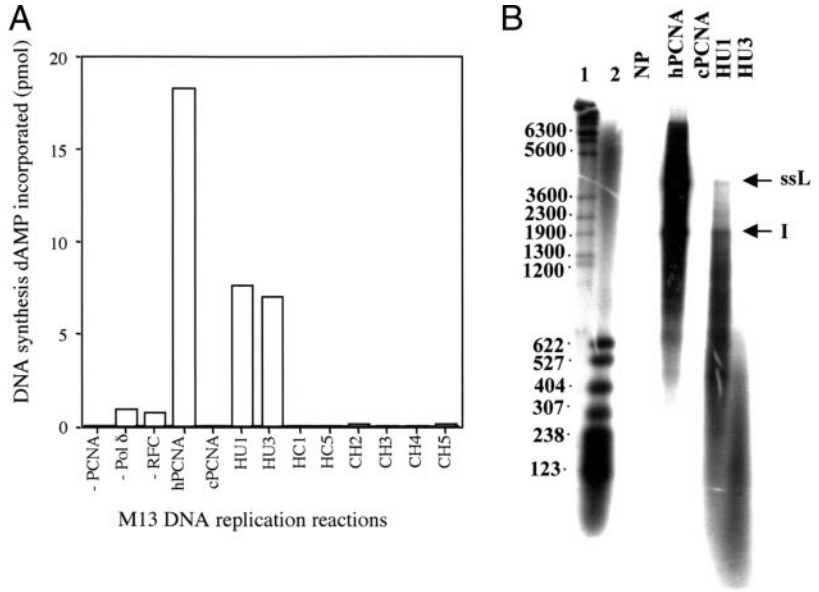

FIG. 7. **Influence of PCNA mutants and hybrids on M13 DNA replication *in vitro*.** DNA synthesis on singly primed M13 template was carried in 30 mM HEPES/KOH pH 7.5 buffer, 7 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT using 4 μg/ml M13mp18 DNA, 10 ng of pol δ, 10 μg/ml RPA, and 1 unit of RF-C in a 20-μl reaction mixture with a 20 μg/ml concentration of the indicated PCNA. *Panel A*, amount of dAMP (in pmol) incorporated in the presence of different PCNA proteins. *Panel B*, replication products were analyzed on a 2% agarose gel run under alkaline conditions and visualized by autoradiography. *Lanes 1* and *2* contained DNA fragments of known sizes by digesting λDNA with *Bst* II and the plasmid pBR322 with *Msp*I, respectively. *Lane NP* shows the reaction products in the absence of PCNA. *Lanes hPCNA* and *cPCNA* contained, respectively, the wt human and *S. cerevisiae* PCNAs. The single-stranded full-length linear DNA (*ssL*) and a possible pause site (*I*) are shown by *arrows*.

the effect of mutation in HU3 was more dramatic than in HU1 and highlighted the role of the IDC loop in the assembly of the pol δ holoenzyme. Furthermore, the inability of CH3 and CH5 hybrids to induce DNA synthesis in this assay suggests that the intermolecular interactions at a replication fork are perhaps not the same as those required for the stimulation of pol δ and RF-C using synthetic substrates.

Human-S. cerevisiae PCNA Hybrids Were Inactive in SV40 DNA Replication in Vitro—A more functional characterization of the PCNA mutants and hybrids was carried out by reconstituting SV40 DNA replication *in vitro* with purified PCNA and partially fractionated cell extracts. In the reconstitution experiments, replication of DNA was dependent on the presence of large T antigen and the SV40 origin of replication, indicating that replicative rather than repair synthesis was occurring (data not shown). The level of DNA synthesis achieved by the mutants and hybrids was determined from the amount of dAMP (in pmol) incorporated during a 1-h incubation. None of the mutants, hybrids, or the cPCNA attained a level of replication achieved with the wt hPCNA (Fig. 8). Analysis of the replication products on an agarose gel revealed three different categories, A, B, and C (Fig. 8). The replication products with hPCNA and HU1 shown in category C were almost identical. HU3 and cPCNA gave rise to replication products similar to those reported due to uncoupled leading strand synthesis (Fig. 8; group B). Hybrids HC1, HC5, CH2, CH3, CH4, and CH5 did not support DNA replication, and the products seen were due to pol α-primase activity (Fig. 8; group A). Thus the wt hPCNA and HU1 were able to accomplish complete DNA synthesis, and HU3 and the hybrids were inactive.

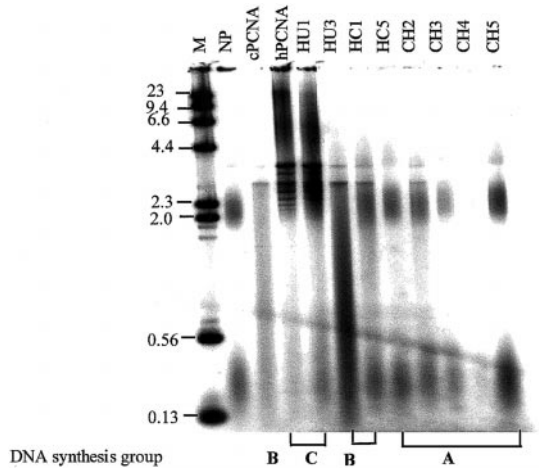

FIG. 8. **Influence of PCNA mutants and hybrids on the SV40 DNA replication *in vitro*.** The SV40 DNA replication *in vitro* was performed using S100 cell extract, fractions IA, II, purified large T antigen, and a SV40 origin containing plasmid pSV011 as described under "Experimental Procedures" with different PCNA proteins indicated in the figure. PCNA was mixed with 6 μg/ml pSV011, 600 μg/ml fraction II, 79 μg/ml large T antigen, 900 μg/ml fraction IA, 40 mM HEPES, pH 8, 8 mM $MgCl_2$. 0.5 mM DTT, 25 μM dATP, 0.1 mM each dCTP, dGTP, dTTP, 3 mM ATP, 0.2 mM CTP, GTP, UTP, 40 mM creatine phosphate, 0.12 unit creatine phosphokinase in a total volume of 12.5 μl. The samples were incubated at 37 °C for 1 h, and the controls were left on ice. After stopping the reaction with 20 mM EDTA, the mixture was freed of proteins, and the replication products were analyzed on an agarose gel. The gel was fixed, dried, and autoradiographed. *Lane M* refers to λ*Hin*dIII DNA size markers. *NP* is a control reaction in the absence of PCNA. The replication products were classified into groups A, B, and C based on the pattern seen on the gel.

We also investigated the possibility that complete DNA replication in these reactions might be PCNA dose-dependent, hence the replication reactions were carried out at three different concentrations of five of the PCNA hybrids. No obvious difference was observed in any of the reactions with increasing amounts of PCNA (data not shown). This suggested that the lack of complete DNA replication described above was not caused by insufficient amounts of PCNA in these assays.

DISCUSSION

Previous mutagenesis studies have shown that deletion of any part of PCNA can distort its tertiary structure, rendering it incapable of association into trimers. Taking a lead from previous work, where cPCNA was unable to complement hPCNA in SV40 DNA replication *in vitro* (53), when both proteins existed as trimers in solution (17, 55), we hypothesized that hybrids of the two proteins would be equivalent to large deletions within hPCNA without disrupting the trimeric ring. This would allow identification of functional regions in hPCNA involved in the assembly of the pol δ holoenzyme at a replication fork. We therefore produced two human PCNA mutants, HU1 (D21E) and HU3 (D121E), and six hybrids of human and *S. cerevisiae* PCNA (see Fig. 1). We have characterized these proteins for their ability to oligomerize and interact with DNA, pol δ, and RF-C using M13 and SV40 DNA replication *in vitro*.

From the crystal structure of PCNA, the intermonomer interactions involving βI1 and βD2 sheets appear important in trimer formation (17, 55). Consequently, mutations located in βI1, such as Y114A in hPCNA (45) and S115P in cPCNA (46), are reported to disrupt trimer formation. However, trimer formation by CH3 suggests that the presence of the βD2 sequence of human and the βI1 of *S. cerevisiae* at the intermonomer interface does not interfere with trimeric structure. In CH3, almost the complete domain 1 of cPCNA was fused to domain 2 of hPCNA. However, replacing βA2, βB2, and βC2 sheets of hPCNA in CH3 by the corresponding sheets of cPCNA, as in CH4, distorted the tertiary structure (Figs. 1 and 3). Similarly, replacing the N-terminal sequences as in CH2 and HC1 led to a lower proportion of trimers. These data indicate that intramolecular interactions between certain β sheets of h- and cPCNA in domain 1 and 2 may not be compatible with trimer formation. The C terminus of hPCNA in CH5 gave a higher proportion of monomers than the converse hybrid HC5, indicating a role for the C terminus in monomer-trimer equilibrium. The N and C termini in PCNA are buried into the structure, and deletion of these residues (23, 44) or replacing them by another PCNA sequence, as described in this study, is likely to disrupt inter-β sheet interactions. Our data are therefore consistent with a recent study where mutations L68S and G69D in βF1 of *Schizosaccharomyces pombe* PCNA (pPCNA) produced only dimers, suggesting that other parts of the molecule, besides intermonomer interface, play a role in the stabilization of trimers (51).

The two mutants HU1 and HU3 stimulated pol δ and RF-C by about 40% compared with the wt hPCNA (see Figs. 4 and 6), suggesting a role for Asp-21 and Asp-120 in these interactions. The mutation D21E in HU1 is located just outside αA1, and this region has never been implicated in pol δ or RF-C stimulation. In the crystal structure, however, Asp-21 is close to the central loop (^{41}DSSHV^{45}), which is shared by pol δ and RF-C (23, 24). Furthermore, the proximity of Asp-21 to helix αA1, which formed part of the central ring for DNA clamping, might also affect pol δ and RF-C stimulation. The mutation D120E in HU3 was part of IDC loop (^{118}MDLDVEQLGIPEQEYSC^{134}), which has been implicated in pol δ stimulation in several studies (23, 49). However, the IDC loop by itself was not sufficient for pol δ stimulation because the hybrid HC5 containing the IDC loop of human origin was inactive. More than 50% reduction in RF-C stimulation with HU3 may indicate a role for the IDC loop in RF-C-induced clamping. Involvement of the IDC loop in RF-C stimulation has not been suggested in the literature. However, a mutation close to the IDC loop, A112T, and another within the loop, S135F, have been shown to suppress the cold-sensitive mutants in Cdc44p, the largest subunit of *S. cerevisiae* RF-C (67). Although the mechanism for the suppression of *cdc*44 cold sensitivity is not clear, the data indicate a role for the IDC loop in the process.

All hybrids were able to assemble into trimers, albeit to different extents, but only CH3 and CH5 stimulated pol δ. The hybrid CH5, with the last 50 amino acids replaced by the corresponding region of the hPCNA (see Fig. 1), stimulated pol δ, but the converse hybrid HC5 was inactive. This indicates that the C-terminal region of hPCNA was a high affinity site for pol δ. This is consistent with previous work where the pol δ binding site in hPCNA (23) and in pPCNA (68, 69) has been located at the C terminus. However, the inability of CH2 and CH4 to stimulate pol δ when both had a C-terminal sequence of hPCNA could be due to a lower proportion of trimers in these preparations (see Fig. 3). This suggests that a certain proportion of PCNA trimers in solution was necessary in addition to a pol δ binding site for effective polymerase stimulation. It is interesting to note that hybrid CH3, which formed perfect trimers, also showed high pol δ stimulation and appeared to be more active than the wt hPCNA (see Figs. 1, 4, and 5). A PCNA mutant with such characteristics has not been described in the literature. The pol δ stimulation assay measures the interaction of PCNA with pol δ and the DNA in the absence of RF-C. The interaction of PCNA with DNA depends on the charge distribution on the central helices in PCNA which is conserved across species. All trimeric PCNA hybrids would have DNA clamping similar to hPCNA. The much higher pol δ stimulatory

activity associated with CH3 could therefore indicate a stretch of cPCNA in CH3 acquiring a conformation with much higher affinity for pol δ.

The data on RF-C stimulation with CH2, CH3, and CH5 suggest that increasing the proportion of cPCNA sequence does not change the ATPase activity, implying that the major RF-C binding was located at the C terminus (Fig. 6 and Table II). This is consistent with previous mutagenesis studies (23, 70). However, the C terminus of neither pPCNA (68) nor cPCNA (48) contains a RF-C binding site, which is consistent with our finding that the hybrids HC1 and HC5 with the C terminus of cPCNA showed the least RF-C binding (see Fig. 6). For RF-C stimulation, the conformation of residues rather than a trimeric structure appears to be more important. For instance, the hybrid CH2 that was predominantly a dimer in solution expressed RF-C stimulation as much as by HU1, HU3, and CH5, which were mainly trimeric (Fig. 6). This suggests that a trimeric PCNA may not be a prerequisite for RF-C-mediated clamping of DNA. However, it is possible that binding of RF-C to PCNA could induce trimeric assembly and subsequent clamping. Jonsson *et al.* (45) have shown that Y114A PCNA aggregates did not compete with the trimeric PCNA for pol δ and RF-C, and based on this they concluded that RF-C did not have a role in aiding trimer formation. However, it may be that for RF-C to aid trimer assembly the RF-C binding must be preserved, and the aggregated PCNA might have lost these sites. Although monomeric PCNA mutants have been shown inactive in RF-C mediated clamping *in vitro* (23, 46), one of them, S115P, assembled the pol δ holoenzyme in the presence of 6% polyethylene glycose and also supported *S. cerevisiae* cell growth, suggesting that nontrimeric PCNAs that have retained their ability to associate can be loaded by RF-C (46). RF-C-induced loading of CH2 as shown in this study indicates that RF-C can sequester nontrimeric PCNA in presence of DNA. RF-C-mediated loading of the two dimeric mutants L68S and G69D described recently is not known as they were not used in the loading assay (51). The basis for the very high PCNA-dependent RF-C ATPase activity observed in the PCNA hybrid CH4 is not clear. It could be caused by structural alterations introduced by swapping over the polypeptide segments.

Assembly of the pol δ holoenzyme complex at a replication fork is a reflection of the ability of PCNA to load on to DNA by RF-C and subsequent association of pol δ into a highly processive enzyme. Thus interaction among different components of the ternary complex including RF-C, PCNA, pol δ, and DNA can be measured in one holoenzyme assay. Using the M13 replication assay we found that only HU1 supported some replicative activity, whereas HU3 along with the hybrids was inactive. This indicated that stimulation of pol δ and RF-C-induced DNA clamping by the mutants and hybrids CH3 and CH5 could not activate DNA synthesis at a replication fork. It may be argued that hybrids CH3 and CH5 cannot interact simultaneously with pol δ and RF-C in this assay because identical sites are involved. However, pol δ and RF-C do interact simultaneously with the wt hPCNA perhaps because they can share redundant structure, which may be lost in the hybrids. Another possibility could be that assembly of the pol δ holoenzyme involves binding of other proteins and that loss of this binding in the hybrids could block DNA synthesis. Involvement of other proteins in holoenzyme assembly has been suspected previously from the IDC loop mutants unable to interact with pol δ but had no influence on DNA replication (48). Besides PCNA, pol δ, and RF-C, the only other protein in M13 assay is RPA. Although a direct interaction between RPA and PCNA has not been reported, a recent study has shown that RPA forms a common touch-point with pol δ, RF-C, and PCNA at a replication fork. Among these, an association of RPA with the RF-C·PCNA complex has been proposed to play an important role for the holoenzyme function (71). It is possible that the complex between RF-C and PCNA mutants or hybrids acquires a conformation that is not compatible with RPA binding. Alternatively, RPA may have cryptic sites for PCNA which are accessible only at the replication fork. Incomplete accessibility of these sites in PCNA variants could have prevented RPA binding. Our results therefore suggest a role of RF-C and RPA far beyond loading of PCNA and coupling the unwinding of the double helix with the initiation of DNA synthesis (72, 73). The results from SV40 DNA synthesis were similar to the M13 assay except for HU1, which was more active in SV40 than in the M13 assay. The discrepancy between the two assays could be caused by a factor(s) in the SV40 reaction mixture able to compensate for the loss of binding in the M13 assay. This is likely given the fact that SV40 DNA replication required a far more complex mixture of proteins. Taken together, the data from SV40 and M13 DNA replication assays suggest that the PCNA hybrids and the mutant HU3 were unable to assemble a functional DNA pol δ holoenzyme.

The three sites that we have identified on hPCNA located on N- and C-terminal regions and the IDC loop appear to play an important role in the stimulation of pol δ, RF-C, and also in the assembly of the holoenzyme. Besides PCNA, the proteins involved in the holoenzyme assembly pol δ, RF-C, and RPA are oligomers of heterosubunits. Mammalian DNA pol δ is an oligomer of three different subunits, 125 kDa, 48 kDa, and a recently identified 66-kDa subunit (74). RF-C has five subunits, 140, 36, 37, 38, and 40 kDa, but only the largest subunit binds PCNA (57). RPA is also a heterotrimer of p70, p34, and p11 (75). PCNA provides a platform for dynamic assembly and disassembly of these proteins at a replication fork. To enable an ordered flow of events during DNA replication it is perhaps important that there is more than one binding site for each protein on PCNA.

In summary, we have shown that the trimeric structure of PCNA is maintained by associations among several β sheets located in domains 1 and 2 of the molecule. We have identified three regions on hPCNA, located at the N- and C-terminal domains and the IDC loop, which are required to stimulate pol δ and RF-C activity, but the site at the C-terminal domain appears to be the high affinity site for the two proteins. Interestingly, PCNA dimers, which were inactive in pol δ stimulation but showed significant RF-C-induced loading, suggested that trimeric PCNA may not be a prerequisite for RF-C-mediated clamping of DNA. Our data indicate participation of other proteins, one of them could be RPA, for successful DNA synthesis at a replication fork. The identification and roles of these proteins at a replication fork will enable us to have a complete understanding of the biochemical steps necessary for the assembly of pol δ holoenzyme.

Acknowledgments— We are grateful to GKT Dental Institute for providing the space and facilities for this project. Ian Goldsmith of Clare Hall Laboratories, Imperial Cancer Research Fund, kindly supplied synthetic oligonucleotides used in this study. We also thank Dr. R. Evans and Dr. C. Joannou, Division of Biomolecular Sciences, King's College London, for helping us with the gel filtration and Phast electrophoresis experiments and Patricia Purkis for technical help. We also acknowledge Drs. R. Evans and A. Yeudall for helpful comments on this manuscript.

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