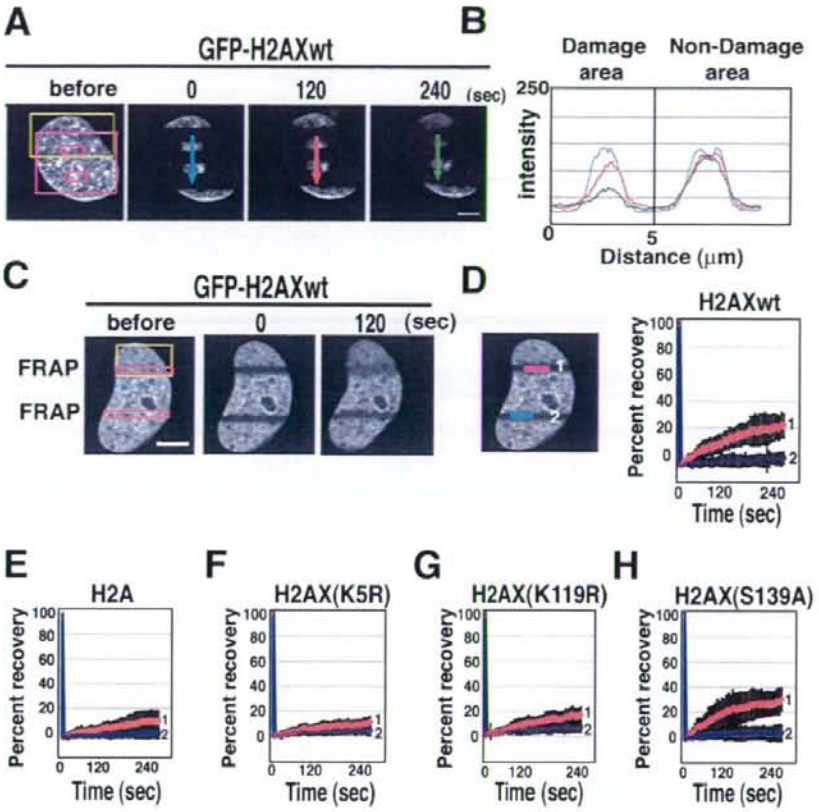

FIG. 6. In vivo dynamics of H2AX regulated by ubiquitination and acetylation of H2AX after induction of DSBs by microirradiation. (A) iFRAP analysis of GFP-H2AX in combination with microirradiation. Confocal images taken at indicated times after microirradiation are shown. DSBs were induced in GM02063 cells expressing GFP-H2AX by laser UVA microirradiation in the area indicated by a yellow box. Immediately after the induction of DSBs, fluorescence was bleached in the areas indicated by red boxes with the 488-nm laser line of an Ar laser. Scale bar, 5 μm. (B) Graph representation of fluorescence intensity versus distance along the arrows depicted in panel A. (C) FRAP analysis of GFP-H2AX after laser UVA microirradiation. GFP-H2AX was imaged before microirradiation, immediately after bleaching, and 120 s after bleaching (see the video in the supplemental material). Scale bar, 5 μm. (D) Quantitative analysis of fluorescence recovery curves after bleaching of irradiated (1, red line) and control (2, blue line) areas. Values represent averages ± standard errors for 14 cells. (E to H) Fluorescence recovery curves for GFP-H2A.1 ($n = 11$) (E), GFP-H2AX(K5R) ($n = 12$) (F), GFP-H2AX(K119R) ($n = 10$) (G), and GFP-H2AX(S139A) ($n = 15$) (H) in GM02063 cells over time in the microirradiated (red lines) and control (2, blue lines) regions.

from damaged chromatin into the microirradiated and photobleached area. Although we cannot exclude the possibility that a gross chromatin movement also contributed to the H2AX dynamics, such a gross movement alone is not sufficient to explain the observed H2AX dynamics. In support of this conclusion, the amount of γ-H2A(X) is lower near the DSBs than at distal sites in yeast (43). In fact, H2A(X) release upon DNA damage was also recently observed in yeast (S. Gasser, personal communication).

H2AX release in the early response to DSBs is regulated by acetylation-dependent ubiquitination. Following the establishment that H2AX is released from damaged chromatin upon DSBs, we next investigated the involvement of H2AX modifications in the DSB-induced release. Using FRAP, we examined the dynamics of GFP-tagged H2AX(K5R), H2AX(K119R), and H2AX(S139A) in GM02063 cells upon DSBs caused by microirradiation. Compared to wild-type H2AX (27.6% ± 8.2% at 260 s; Fig. 6D), both GFP-H2AX (K5R) (11.5% ± 4.4% at 260 s; Fig. 6F) and GFP-H2AX(K119R) (17.5% ± 6.3% at 260 s; Fig. 6G) showed reduced mobility following DNA damage ($P < 0.005$). The fluorescence recovery of H2AX (S139A) (32.8% ± 8.5% at 260 s) appeared comparable to that of wild-type H2AX (Fig. 6H). We also confirmed the reduced mobility of GFP-H2AX(K5R) and GFP-H2AX(K119R) after microirradiation in GM02063 cells by iFRAP analysis (data not shown). These results suggest a significant role of K5 acetylation and K119 ubiquitination in DSB-induced H2AX release. Conversely, the phosphorylation of H2AX(S139) does not appear to play a role in the release of H2AX following DNA damage.

TIP60 and UBC13 regulate H2AX release following DNA damage. To examine the role of TIP60 and UBC13, we per-

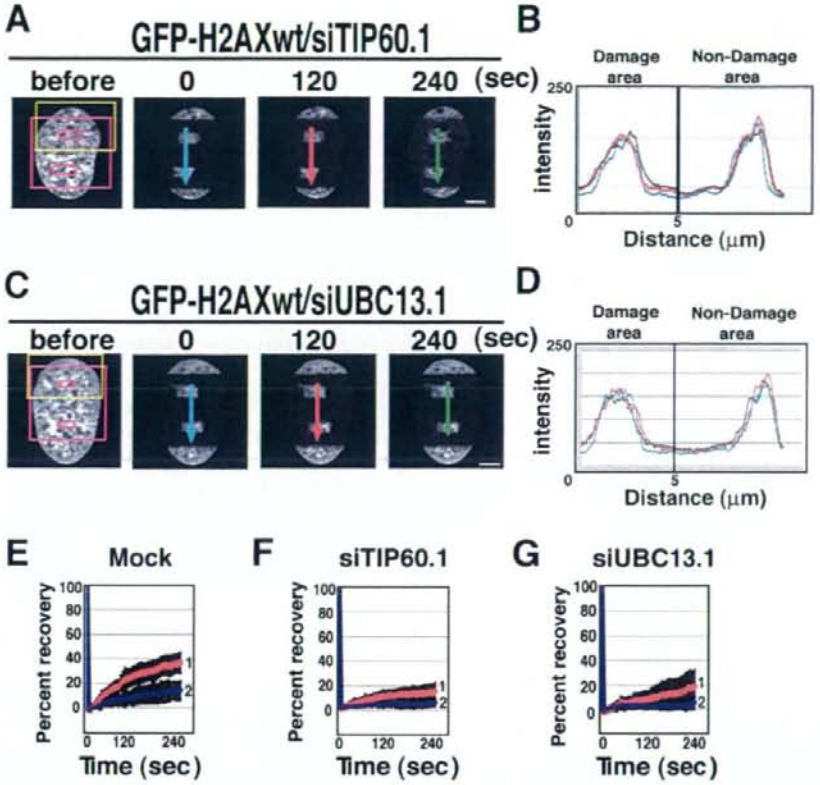

FIG. 7. TIP60 and UBC13 are required for the release of H2AX upon DNA damage. (A to D) iFRAP analysis of GFP-H2AX after microirradiation in GM02063 cells expressing TIP60 (siTIP60.1)- or UBC13 (siUBC13.1)-specific siRNAs was performed as described for Fig. 6A and B. Depletion of TIP60 or UBC13 suppressed the DSB-induced release of GFP-H2AX. Scale bars, 5 μm. (E to G) Fluorescence recovery curves for GFP-H2AX in the microirradiated (red lines) and control (blue lines) regions in mock-transfected cells ($n = 15$) (E) and for GFP-H2AX in GM02063 cells expressing TIP60-specific siRNA (siTIP60.1) ($n = 10$) (F) or UBC13-specific siRNA (siUBC13.1) ($n = 12$) (G).

formed iFRAP and FRAP analyses in TIP60 or UBC13 knockdown cells. Depletion of TIP60 by siRNA significantly reduced the diffusion of GFP-H2AX following microirradiation in iFRAP experiments (Fig. 7A and B). The involvement of TIP60 in the DSB-induced release of H2AX was confirmed by the reduced fluorescence recovery of GFP-H2AX in microirradiated regions of single nuclei in TIP60 knockdown cells (14.3% ± 7.7% at 260 s; $P < 0.001$; Fig. 7E and F; also see Fig. S2C in the supplemental material). Similar results were obtained in cells expressing a dominant-negative TIP60 HAT mutant (data not shown). UBC13 knockdown GM02063 cells also showed a significant reduction in GFP-H2AX mobility following microirradiation in iFRAP and FRAP experiments (20.1% ± 13.8% at 260 s; $P < 0.05$; Fig. 7C, D, and G). Taken together, these results are consistent with the conclusion that TIP60 and UBC13 control the damage-induced release of H2AX via the acetylation-ubiquitination pathway in the earlier stage of DNA repair.

The regulation of chromatin reorganization immediately after DNA damage by TIP60 in conjunction with UBC13 via the release of H2AX suggests the involvement of TIP60 in the subsequent DNA repair process. In support of this conclusion, UBC13 is reported to be required for the recruitment/activation of the ubiquitin ligase function of BRCA1 and the subsequent formation of RAD51 nucleoprotein filaments at DSBs (64). To investigate the role of TIP60 in the DNA repair process, we examined RAD51 focus formation after IR in GM02063 cells expressing the TIP60 HAT mutant. RAD51 focus formation after IR was significantly disturbed in TIP60 HAT mutant-expressing cells (see Fig. S7 in the supplemental material). Although we cannot exclude the possibility that TIP60 regulates RAD51 focus formation independently of H2AX release in the early step of homologous recombination repair, it is possible that the TIP60-UBC13 complex is involved in the DNA repair process, especially homologous recombination repair, via H2AX release.

DISCUSSION

In this study, we found that TIP60 HAT interacts with H2AX upon DNA damage and that H2AX is not only acetylated but also ubiquitinated just after the induction of DSBs in human cells. DSB-induced acetylation regulated by TIP60 is

required for the ubiquitination of H2AX. We identified the ubiquitin-conjugating enzyme UBC13 as a novel binding partner for TIP60 in the ubiquitination of H2AX following DSB formation. Interestingly, experiments using FRAP/iFRAP in conjunction with microirradiation indicated that the release of H2AX from damaged chromatin depends on the acetylation-ubiquitination pathway. Specifically, our findings indicate that the TIP60-UBC13 complex regulates the release via acetylation-dependent ubiquitination in the early stage of the DNA damage response.

We showed here that immediately after microirradiation, DSBs facilitate the interaction of TIP60 with UBC13 (Fig. 5B and C). Because TIP60-induced acetylation of H2AX is required for the polyubiquitination of H2AX upon DNA damage, the acetylation of H2AX may be involved in the recruitment of UBC13 to the damage site, or it may modulate the activity of UBC13 to facilitate the polyubiquitination of H2AX, leading to the release of H2AX. In our preliminary experiments, depletion of TIP60 by use of siRNA did not suppress the interaction of UBC13 with H2AX upon DNA damage (data not shown), suggesting that the acetylation of H2AX by TIP60 is not essential for the recruitment of UBC13 into the damage site. Therefore, the enzymatic activity of UBC13 may be induced by TIP60 through an unknown mechanism mediating H2AX polyubiquitination after the induction of DSBs.

At present, it is not clear how the polyubiquitination of H2AX regulates the release of H2AX. Because polyubiquitination by UBC13 often regulates protein function or protein-protein interactions (33), damage-induced polyubiquitination of H2AX by TIP60-UBC13 could be a signal for the recruitment of histone chaperone or chromatin-remodeling factors. The other possibility is that the structural change of H2AX by polyubiquitination might lead to the decreased affinity of H2AX for nucleosomes. Further studies are required to determine which of these possibilities is correct.

The DmTIP60-p400/Domino complex regulates the exchange of phospho-H2A.v with an unmodified H2A.v in vitro (22). Because p400 is included in the purified TIP60 complex and is required for UV-induced apoptosis in human cells (54), p400 may be involved in the release of H2AX in cooperation with the TIP60-UBC13 complex, leading to cell cycle progression or apoptosis. A previous study also showed that the TIP60-TRRAP complex is involved in homologous recombinational repair by acetylating histone H4 after the induction of DSBs (39). Although it remains unclear whether the TIP60-TRRAP complex participates in the release of H2AX, histone H4 acetylation by the TIP60-TRRAP complex may be needed to facilitate the polyubiquitination of H2AX by UBC13 during homologous recombinational repair. Therefore, to address the significance of the TIP60-UBC13 complex in H2AX release, it will be important to identify factors involved in this process within the TIP60 complex following the induction of DSBs.

A number of potential functions of H2AX in DNA damage response or DNA repair have been suggested. γ-H2AX has been proposed to function as a docking site for repair protein complexes to bind to broken DNA ends (5, 46). γ-H2AX may also keep the broken DNA ends tethered together with assembled repair factors to prevent aberrant repair of DSBs such as chromosomal translocations, because the loss of one H2AX allele results in a haploinsufficiency that compromises genomic integrity and enhances genomic instability in the absence of p53 (5, 7). However, it is unclear whether the phosphorylation of H2AX is involved in the alteration of the chromatin structure to facilitate the access of repair proteins to the damaged chromatin. The release of H2AX via the acetylation-ubiquitination pathway immediately after the induction of DSBs may be involved in the opening of damaged chromatin for the access of repair proteins around DSBs. Thus, H2AX could play two distinct roles in the early steps of the DNA damage response: (i) phosphorylation by ATM or DNA protein kinase catalytic subunit to form γ-H2AX foci for the accumulation of repair factors and (ii) acetylation-dependent ubiquitination by TIP60-UBC13 to facilitate the release of H2AX for the alteration of chromatin structure at the damage site. Recently, RAP80 has been shown to bind ubiquitin polymers at DSBs for the recruitment of BRCA1 (17, 45, 59). Therefore, ubiquitinated H2AX could also provide a docking site for repair proteins such as γ-H2AX.

In addition to the direct role of TIP60 in chromatin reorganization, TIP60 plays other roles in cell cycle control and apoptosis by acetylating MYC or ATM (41, 47, 54). Recently, TIP60 was shown to acetylate p53 directly in the DNA damage response and to regulate the transcriptional regulation of the target genes of p53 (48, 50). Because DSBs facilitate the interaction of TIP60 with UBC13, p53 may also be polyubiquitinated by UBC13 during transcriptional regulation. It is also possible that some other signaling molecules could be acetylated and polyubiquitinated by TIP60-UBC13 upon the formation of DSBs. If so, acetylation-dependent polyubiquitination by TIP60-UBC13 in the DNA damage response may be required not only for the release of H2AX but also for transcriptional regulation in cell proliferation, cell cycle control, or apoptosis.

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Dynamics of human replication factors in the elongation phase of DNA replication

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ABSTRACT

In eukaryotic cells, DNA replication is carried out by coordinated actions of many proteins, including DNA polymerase δ (pol δ), replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and replication protein A. Here we describe dynamic properties of these proteins in the elongation step on a single-stranded M13 template, providing evidence that pol δ has a distributive nature over the 7 kb of the M13 template, repeating a frequent dissociation–association cycle at growing 3′-hydroxyl ends. Some PCNA could remain at the primer terminus during this cycle, while the remainder slides out of the primer terminus or is unloaded once pol δ has dissociated. RFC remains around the primer terminus through the elongation phase, and could probably hold PCNA from which pol δ has detached, or reload PCNA from solution to restart DNA synthesis. Furthermore, we suggest that a subunit of pol δ, POLD3, plays a crucial role in the efficient recycling of PCNA during dissociation–association cycles of pol δ. Based on these observations, we propose a model for dynamic processes in elongation complexes.

INTRODUCTION

In eukaryotic cells, DNA replication is carried out by coordinated actions of many proteins. It has been demonstrated that the elongation process can be reconstituted with distinct protein factors, DNA polymerase δ (pol δ), replication factor C (RFC), proliferating cell nuclear antigen (PCNA) and replication protein A (RPA) (1–3). These replication factors, in addition to the polymerase α-primase complex (pol α), are required components of the *in vitro* simian virus 40 (SV40) replication system (4). The pol δ heterotetrameric complex, consisting of 125, 66, 50 and 12 kDa proteins (5), is the major polymerase involved in chromosomal replication in eukaryotic cells. RFC is composed of one large subunit (145 kDa) and four smaller ones (40, 38, 37 and 36 kDa) (6,7) and has DNA-dependent ATPase activity, loading sliding clamp PCNA onto DNA (6,8). PCNA itself is a homotrimeric ring-shaped protein with a molecular mass of 29 kDa for each monomer, which encircles double-stranded DNA (9,10). The likely role of PCNA in pol δ replication is in stabilizing the pol δ–DNA interaction to maintain the processivity of the polymerase (11–17). RPA is a heterotrimeric single-stranded (ss) DNA-binding protein, consisting of 70, 32 and 14 kDa proteins, required for the initiation and elongation phases of DNA replication (18).

The initiation phase of DNA replication with these protein factors has been investigated intensively, and it has been demonstrated that their actions are well coordinated (19–26). An important process is the switch from pol α to pol δ (24), mediated by RFC (20,22,24,26).

After an elongation complex is once established, concerted actions of these protein components could still be required. However, the architecture and actions within elongation complexes remain obscure. It is generally believed that an elongation complex containing pol δ and PCNA, but not RFC, is somehow extremely stable (27). However, we have obtained surprising results, revealing a markedly dynamic picture for elongation complexes consisting of pol δ, PCNA and RFC. We thus have evidence that pol δ frequently repeats a dissociation–association cycle at growing 3′-hydroxyl ends. RFC appears to remain at the primer terminus throughout the elongation phase, probably holding PCNA from which pol δ has detached, with the potential to catalyze unloading of PCNA. Once pol δ dissociates from a growing 3′-hydroxyl end, a significant fraction of PCNA may remain at the primer terminus through interaction with RFC, while the remainder may be unloaded by RFC or slide out of the primer terminus. RFC persisting around primer termini then may reload PCNA from solution to restart DNA synthesis. In addition, characterization of the dynamic properties

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of the same protein factors with a subassembly of pol δ lacking the POLD3 subunit revealed a crucial function of POLD3 in the efficient recycling of PCNA.

MATERIALS AND METHODS

Plasmids

The expression plasmid for human PCNA was as described earlier (28) and that for human RPA, p11d-tRPA (29), was a generous gift of Dr Marc S. Wold (University of Iowa College of Medicine, Iowa City, Iowa). Human cDNAs for pol δ and RFC were amplified from a HeLa cDNA library introducing a cutting site of NdeI at the start codon. The expression plasmids are listed in Supplementary Table 1, and details for their construction are described in the Supplementary Data.

Proteins

All proteins used in this study were overproduced in *Escherichia coli* cells (Supplementary Table 1) and purified by conventional column chromatography. During all purification steps, induced proteins were monitored by SDS–PAGE followed by staining with Coomassie Brilliant Blue R-250 (CBB) or western blotting. Protein concentrations were determined by Bio-Rad protein assay using BSA (Bio-Rad) as the standard. The procedures are described in the Supplementary Data.

Pol δ holoenzyme assays on single-stranded (ss) mp18 DNA

DNA polymerase activity was measured with reference to incorporation of [α-^{32}P]dTMP. The standard reaction mixture (25 μl) contained 20 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM each of dGTP, dATP, dCTP and [α-^{32}P]dTTP, 33 fmol (240 pmol for nucleotides) of singly primed ss mp18 DNA (the 36-mer primer, CAGGGTTTTCCCAGTCACGACGTTGTA AAACGACGG is complementary to 6330–6295 nt) (30), 1.0 μg (9.1 pmol) of RPA, 86 ng (1.0 pmol as a trimer) of PCNA, 75 ng (260 fmol) of RFC and 90 ng (380 fmol) of pol δ. In the reactions with HincII, 10 U of the enzyme (Takara Bio Inc.) were introduced into the standard reaction mixture. Reaction mixtures lacking pol δ were pre-incubated at 30°C for 1 min, then reactions were started by addition of pol δ. After incubation at 30°C for 10 min, reactions were terminated with 2 μl of 300 mM EDTA, and the mixtures were immediately chilled on ice. Five-microliter samples were spotted on DE81 paper (Whatman), which was washed three times with 0.5 M Na_2HPO_4. The amount of incorporated [α-^{32}P]dTMP was determined as the radioactivity retained on the paper (31). For electrophoretic analysis of replication products, 5 μl samples were mixed with 1 μl of loading buffer (150 mM NaOH/10 mM EDTA/6% sucrose/0.1% bromophenol blue), and electrophoresed on 0.7% alkaline-agarose gels as described (32). To detect the products by Southern blotting, the newly synthesized strands were hybridized with a 5′-^{32}P-labeled oligonucleotide (GCACCCCAGG CTTTACACTTTATGCTTCCGGCTCGTATGTTGTG TGGAATTGTGAGCGGATAACAATTTCACACAGG AAACAGCTATGACCATGATTAC). For linearization of ss mp18 DNA before reactions, an oligonucleotide, CAGGGTTTTCCCAGTCACGACGTTGTAAAACGAC GGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGA CTCTAGAGGATCCCCGGGTACCGAGCTCGAATT, was annealed to the template.

Isolation of PCNA on DNA bound to magnetic beads

The 5′-biotinylated primer (CTCTCTCTCTCTCTCTCT CTCAGGGTTTTCCCAGTCACGACGTTGTAAAAC GACGG) was annealed to 33 fmol ss mp18 DNA, immobilized onto a 15-μl suspension of streptavidin magnetic beads, M280 (Dynabeads) in 20 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT by incubation at room temperature for 30 min and chilled on ice. The buffer condition of the mixture was adjusted to that of the pol δ standard assay. For the reaction with HincII, 10 U of the enzyme (Takara Bio Inc.) were introduced into the standard reaction mixture. Reaction mixtures lacking pol δ were pre-incubated at 30°C for 1 min, then pol δ or buffer (as control) was introduced into the reaction mixture. After incubation at 30°C for 10 min, reactions were terminated with 2 μl of 300 mM EDTA, and then the mixtures were immediately chilled on ice. Subsequent washes were carried out at 4°C using a Dynal magnet with 40 μl of wash buffer [20 mM HEPES–NaOH (pH 7.5), 0.5 M NaCl, 0.2 mg/ml BSA, 1 mM DTT, 1 mM EDTA] four times. The beads were boiled in sample loading buffer and proteins were separated on a SDS 4–20% gradient polyacrylamide gel, blotted onto a nitrocellulose membrane, and probed with an anti-PCNA antibody (Santa Cruz, sc-7907). Detection was achieved with an ECL chemiluminescence kit (GE Healthcare Life Science). Different exposures of the blot were photographed with a CCD camera and quantified.

RESULTS

Reconstitution of DNA synthesis

We initially tried to establish procedures to purify the replication proteins (pol δ, RFC, PCNA and RPA), at quantities sufficient for detailed biochemical studies. Because it has been shown that bacterial systems are very powerful for large-scale production of PCNA and RPA as complexes (24,28,29), we developed expression systems for heterotetrameric pol δ and heteropentameric RFC in *E. coli* and the complexes were then purified by conventional column chromatography (Figure 1A).

First, we measured activities of purified proteins on singly primed ss mp18 DNA. In this assay, all of the protein components, RPA, PCNA and RFC, were found to be required for maximum activity of pol δ (Figure 1B) (6,33,34). In the reaction for 10 min, we detected a long product, probably corresponding to the full-size mp18 DNA (7.2 kb) (Figure 1B), and the amount increased with further incubation (Figure 1C). The size of the products was heterogeneous, indicating several pausing sites. Omitting PCNA or RFC from the reaction mixture

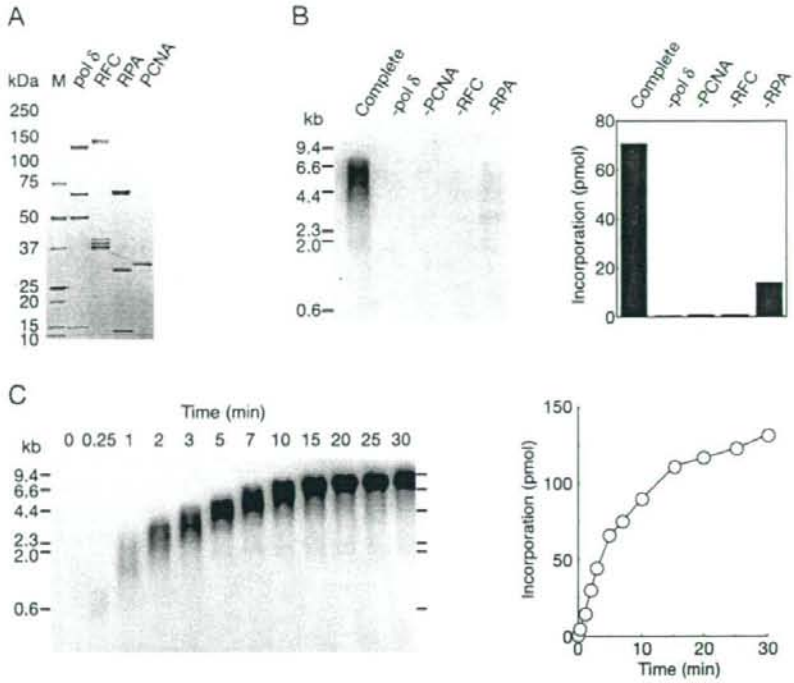

Figure 1. Reconstitution of DNA replication with recombinant replication factors on singly primed ss mp18 DNA. (**A**) SDS–PAGE analysis of purified recombinant proteins. Pol δ (2.4 μg), RFC (1.5 μg), RPA (1.2 μg) and PCNA (0.8 μg) were loaded on a SDS 4–20% gradient polyacrylamide gel and stained with CBB. (**B**) Requirement of replication factors for synthesis of singly primed ss mp18 DNA. Reactions were carried out for 10 min under the conditions described in the Materials and Methods section or omitting one replication factor. Products were analyzed by 0.7% alkaline-agarose gel electrophoresis as described in the Materials and Methods section. Incorporation of dNMP was measured as described in the Materials and Methods section. (**C**) Time course of the reaction of DNA synthesis. The reaction products were analyzed by the same procedures as for (B).

resulted in virtually no incorporation of dNMP (Figure 1B). In the absence of RPA, heterogeneity in the length of the products was emphasized (Figure 1B) (6,34,35). Under the experimental conditions used, efficient dNMP incorporation was observed within the first 5 min, and then DNA synthesis continued at slower rates (Figure 1C). These results indicated that the recombinant proteins efficiently interacted with each other in the holoenzyme assay.

Differential modes of action of protein components on mp18 replication

Theoretically, proteins act during replication in two distinct modes (32). Some proteins remain associated continuously with the growing 3′-hydroxyl end (acting processively), and others repeat a cycle of association and dissociation (acting distributively). The distributive factors have to be reloaded many times from solution during elongation, while the processive factors are loaded only once at the initiation.

How each of the proteins acts during replication on ss mp18 DNA can be investigated (32) as described below. If a protein acts distributively, then the product size is expected to vary proportionally with the concentration of the protein in the reaction mixture; the average size of products should be shorter at lower concentrations of the proteins because association of the protein to primer termini would be random and all primer termini would be utilized with the same efficiency (Figure 2A). On the other hand, if a protein acts processively, limiting its concentration in solution would be expected to affect only the frequency of the first assembly event (initiation), but not the size of products (Figure 2B). If the association and dissociation rates were much faster than other chemical steps, the binding process would not be rate limiting, and the incorporation rate of dNMP would not vary (Figure 2A and B, right panels). In the following experiments, we investigated the mode of action of proteins by analyzing size distributions of products by electrophoresis on agarose gels under denaturing conditions.

First, the concentration of pol δ was varied in the presence of saturating amounts of the auxiliary proteins, and the product size was analyzed by alkaline-agarose gel electrophoresis (Figure 2C). The results showed that the amount and length of the products increased as more pol δ was added to the reaction mixture (Figure 2C), implying that pol δ frequently dissociated from the growing 3′-hydroxyl end and reassociated randomly with free 3′-hydroxyl ends (34–36). The dissociation might be

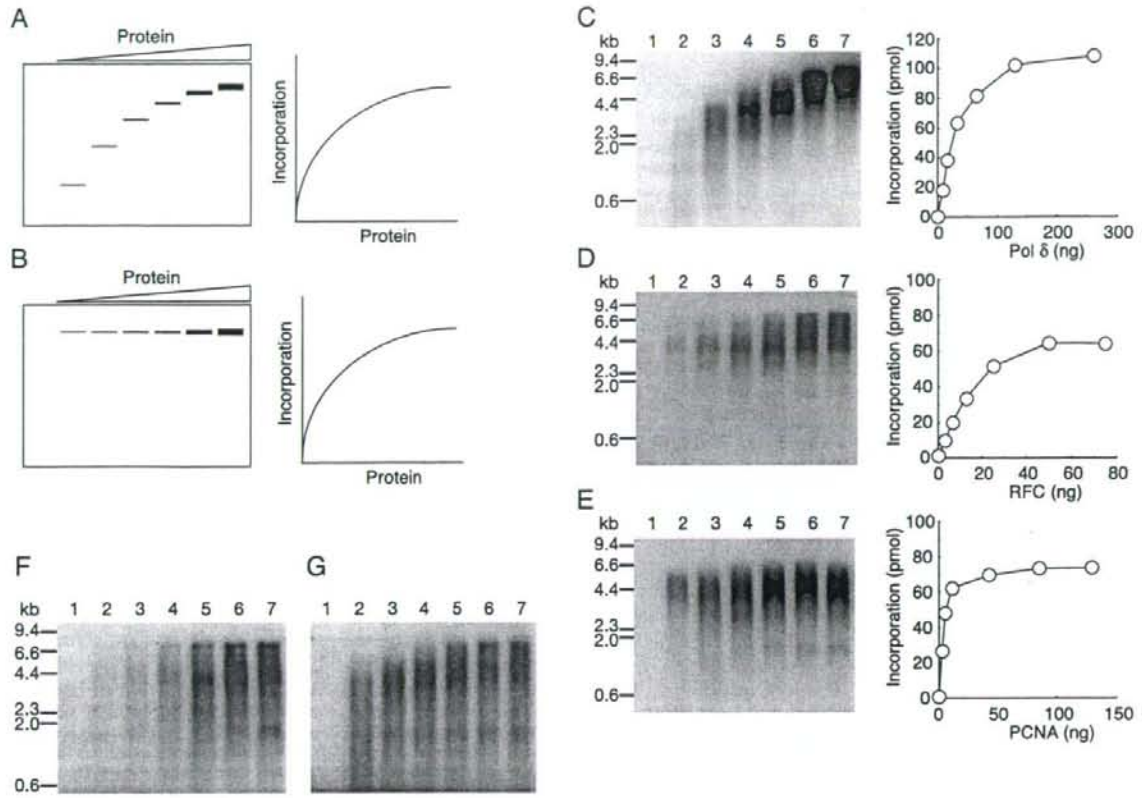

Figure 2. Analysis of the modes of action of replication factors in ss mp18 DNA replication. (**A** and **B**) Expected results of titration of a distributive factor (A) or a processive factor (B) on the DNA replication. The left panels represent gel images of alkaline-agarose gel electrophoresis, and the right panels represent graphs of quantified data for reaction products. See the text for details. (**C–E**) Titration of replication factors, pol δ (C), RFC (D) and PCNA (E). Reactions were carried out for 10 min under the conditions described in the Materials and Methods section except for variation in the amounts of single protein factors. Reaction products were analyzed by 0.7% alkaline-agarose gel electrophoresis and the newly synthesized DNA were visualized by the incorporated [α-^{32}P]dTMP (left panels). Incorporation of dNMP were measured as described in the Materials and Methods' section (right panels). Titration of pol δ (C); 0 ng (lane 1), 8.1 ng (lane 2), 16 ng (lane 3), 33 ng (lane 4), 65 ng (lane 5), 130 ng (lane 6) and 260 ng (lane 7). Titration of RFC (D); 0 ng (lane 1), 3.1 ng (lane 2), 6.3 ng (lane 3), 13 ng (lane 4), 25 ng (lane 5), 50 ng (lane 6) and 75 ng (lane 7). Titration of PCNA (E); 0 ng (lane 1), 2.7 ng (lane 2), 5.4 ng (lane 3), 11 ng (lane 4), 43 ng (lane 5), 86 ng (lane 6) and 129 ng (lane 7). (**F** and **G**) Titration of RFC (F) and PCNA (G) in the reactions using the 5′-^{32}P-labeled primer. The labeled primer was annealed to ss mp18 DNA, and [α-^{32}P] dTTP was replaced with cold dTTP in the reaction mixtures. Amounts of proteins used in the titration were the same as for D and E.

caused by the secondary template structure (35). Therefore, we conclude that pol δ is not completely processive in this assay system.

Next, we changed concentrations of RFC (Figure 2D). The result was different from the case of pol δ. Essentially, the concentration of RFC affected only the amount of products, but not their size (Figure 2D) (6,37), although increase of size at higher concentrations of RFC was noted (Figure 2D, lanes 6 and 7). The processive nature of RFC might be explained in two alternative ways. One is that the sole role of RFC is loading of PCNA at the initiation step (38). Once PCNA is loaded, due to stable association with DNA, RFC may no longer be required. If so, even after dissociation of pol δ from the growing 3′-hydroxyl end, once loaded PCNA may remain at the primer terminus and assist reassociation of pol δ. The other explanation is that once RFC binds to a primer end and loads PCNA to DNA, it may travel with PCNA and pol δ (26,39,40). In the latter case, after dissociation of pol δ, RFC may continue to hold PCNA at the primer terminus, and if the RFC unloaded PCNA (41–43) or if PCNA slide out of the primer terminus (44), the remaining RFC could reload PCNA quickly from solution. Therefore, it seems to be a very critical question, how the concentration of PCNA affects the size of the products. If PCNA behaves as a processive factor, RFC should be required only for the initiation step to load PCNA. On the other hand, if PCNA behaves as a distributive factor, then RFC would have to be traveling with growing 3′-hydroxyl ends as a processive factor for loading PCNA anytime during elongation.

Thus, we subsequently examined effects of varying PCNA concentrations on the product size distribution. When the concentrations of PCNA were low, sizes of the

products became slightly decreased and smears <2 kb appeared (Figure 2E, lanes 2–4). However, large products (4–5 kb) were also detected, even at the lowest concentration of PCNA (Figure 2E, lane 2). In these assays, full-size products are excessively represented by their greater incorporation of radioactive nucleotides. To avoid this problem, we used a 5′-^{32}P-labeled primer. Note that the size distribution of the products in the PCNA titration, different from that in RFC titration, was more clearly visualized with a 5′-^{32}P-labeled primer than with incorporation of [α-^{32}P]dTMP (Figure 2F and G). The results revealed that the concentration of PCNA did not affect the initiation step, because the amount of utilized primer was not appreciably decreased with lower concentrations of PCNA (Figure 2G). On the other hand, the initiation was limited at a low concentration of RFC (Figure 2F), suggesting inefficient turnover of RFC. We consider that after dissociation of pol δ from the growing 3′-hydroxyl end, some PCNA does not remain at the primer terminus, rather sliding out or being unloaded by RFC. In both cases, another PCNA might be reloaded from solution. Therefore, the results suggested that PCNA is partially distributive in our assay system, and also suggested that the processive nature of RFC is due to a stable association at the primer terminus during elongation of DNA replication. These issues were addressed intensively in subsequent experiments.

DNA replication on linearized ss mp18 DNA

For further analysis of PCNA and RFC actions during elongation, we examined the effects of linearization of mp18 DNA after initiation of DNA replication by introduction of a restriction enzyme, HincII, into reaction mixtures (Figure 3). A unique cutting site of HincII in the template DNA is located 29-bases downstream from the 3′-hydroxyl end of the primer. Soon after initiation of DNA replication, the region should be converted to double-stranded DNA and become cleavable by HincII (Figure 3A). In standard reaction mixtures, we introduced 10 U of HincII that can digest more than 98% of double-stranded mp18 DNA, for 15 s. As shown in Figure 3B, the sizes of all the products became uniformly much shorter in the presence of HincII and incorporation of dNMP was decreased by half (Figure 3B), probably due to a defect in elongation, rather than initiation, because the average size of products was also reduced by almost a half (Figure 3B). We considered that the decreased efficiency of DNA synthesis was probably the consequence of dissociation of PCNA, suggesting that some PCNA once assembled into the elongation complex might not remain at primer termini during elongation, and with a circular template, the majority of detached PCNA slides back to be reutilized.

The results shown in Figure 2 suggest that RFC remains at growing 3′-hydroxyl ends and reloads PCNA from solution during elongation. This implies that DNA synthesis can be resumed again after PCNA is once dissociated from the primer termini. If this were indeed the case, the size of the products should increase in a time-dependent manner. The results shown in Figure 3C indicate that incorporation increased and the products became longer depending on the incubation time, but with a slower rate, suggesting reloading of PCNA from solution. Because pausing likely promotes the dissociation of pol δ from PCNA (35), some PCNA also would be released from the primer termini at pausing sites. As expected these became more prominent, resulting in a more heterogeneous size distribution of products (Figure 3C).

Then, we further tested whether the concentration of RFC could affect the size of products in the presence of HincII (Figure 3D). Again, only the amount, but not the size distribution, was affected, although increase of the size at higher concentrations of RFC was observed, as in the reaction without HincII (Figure 2D). In contrast, the concentration of PCNA partially affected the size distribution of products (Figure 3E). The difference between RFC and PCNA titration was not clear by visualization with incorporation of the radioactive nucleotides. However, the 5′-^{32}P-labeled primer, as shown in Figure 2F and G, was unavailable because of the restriction cutting. To solve the problem, newly synthesized strands were visualized, instead, by Southern hybridization with an oligonucleotide annealed to the newly synthesized strand just downstream of the HincII site. Results confirmed the difference in size distributions of the products between in the PCNA titration and RFC titration (Figure 3F and G). Since some PCNA slides out of the DNA (Figure 3B) and is reloaded from solution many times by RFC to continue DNA synthesis (Figure 3C), the size of the products would depend on the concentration of RFC, if this acted distributively. Therefore, our results again suggested that RFC acts processively, binding in the initiation of DNA replication and traveling with pol δ.

Inefficient elongation by spontaneous loading of PCNA from template DNA ends

It is known that PCNA is spontaneously loaded from a double-stranded end of template DNA in an RFC-independent manner, and supports elongation with pol δ (44). Therefore, the restart reactions after dissociation of PCNA observed in Figure 3C could be due to spontaneous loading of PCNA at the ends. To determine the efficiency of RFC-independent restart, ss mp18 DNA was linearized first, then subjected to reactions in the absence of RFC. In this assay, a primer covering HincII site was annealed to ss mp18 DNA (Figure 4A). HincII was introduced in standard reaction mixtures, and then the reactions were started by addition of pol δ after pre-incubation for 1 min (Figure 4A). The time course of the reaction revealed the extension rate to be much slower (Figure 4B) than that of RFC-dependent reactions (Figure 3C) and the primer ends were hardly extended beyond the pausing site around 4 kb (Figure 4B). Next, we examined effects of varying PCNA concentrations on the size distribution of the products. The results demonstrated that the length of the products is uniformly short at a low concentration of PCNA (Figure 4C), suggesting that PCNA once assembled with pol δ is not stable

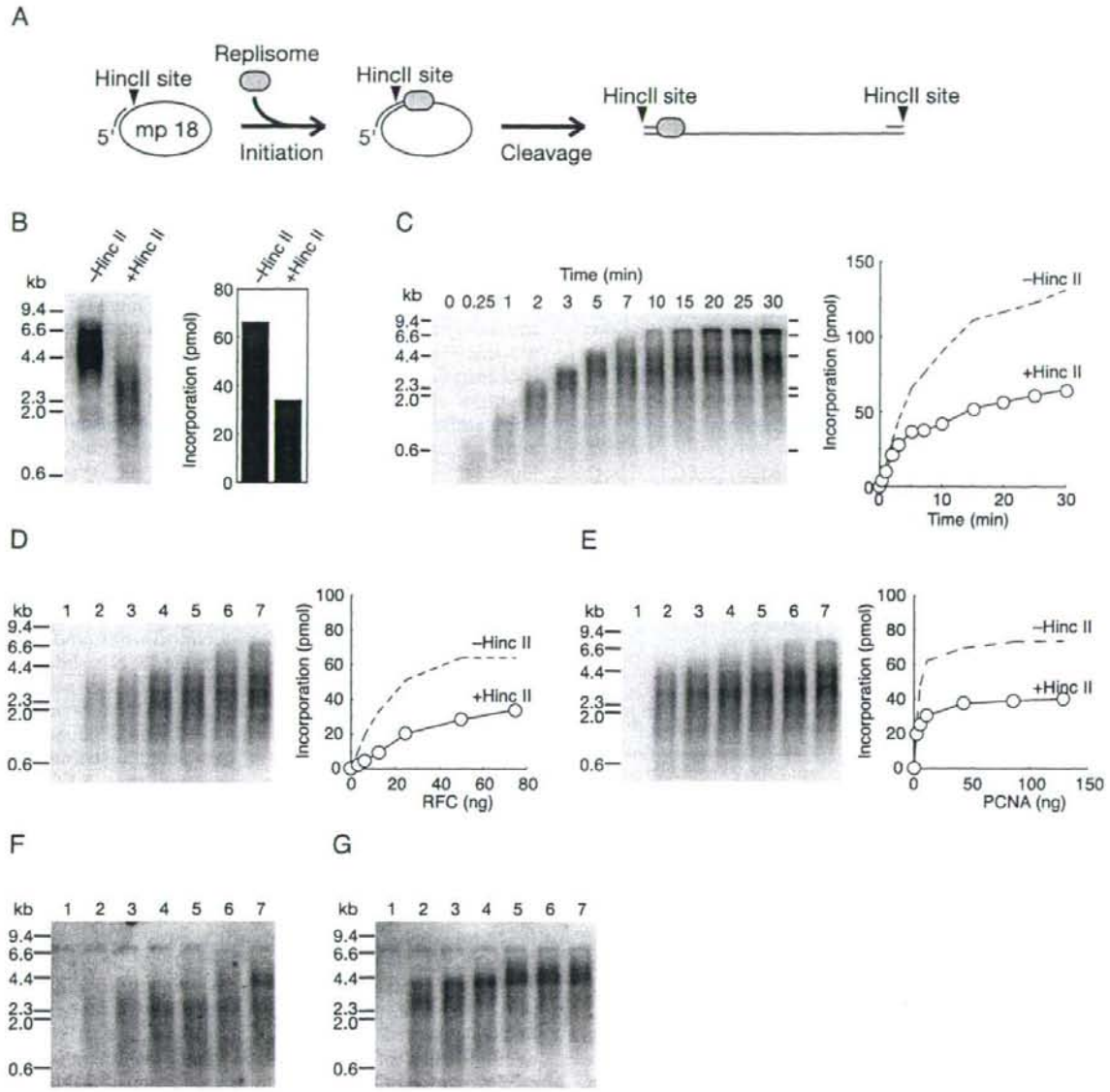

Figure 3. Effects of linearization of newly synthesized DNA just after initiation of DNA replication. (**A**) Schematic representation of the experimental design. A restriction enzyme, HincII, was introduced into the reaction mixtures. A unique cutting site in the template DNA is located 29-bases downstream from the 3′-hydroxyl end of the primer. After the region was converted to double-stranded DNA by initiation of DNA synthesis, indicating assembly of elongation complexes, the DNA becomes cleavable by HincII. (**B**) Effects of HincII on synthesis of ss mp18 DNA. (**C**) Time course of DNA synthesis in the presence of HincII. (**D** and **E**) Titration of RFC (D) and PCNA (E) in the presence of HincII. Amounts of PCNA and RFC used in the titration were the same as used for Figure 2 (see legend of Figure 2). Autoradiograms of 0.7% alkaline-agarose gels (left panels) in which the newly synthesized DNA was visualized by the incorporated [α-^{32}P]dTMP, and incorporation of dNMP were measured as described in the Materials and Methods section (right panels). Dotted lines represent results without HincII of Figure 2B. (**F** and **G**) Titration of RFC (F) and PCNA (G) in the presence of HincII. [α-^{32}P] dTTP was replaced with cold dTTP in the reaction mixtures and the newly synthesized strands were visualized by Southern blotting with a 5′-labeled oligonucleotide, which is complementary to newly synthesized strand just downstream of HincII site. Amounts of proteins used in the titration were the same as for (D) and (E). Reactions in (B, D–G) were carried out for 10 min under the conditions described in the Materials and Methods section with addition of HincII (10 U/25 µl of reaction mixture).

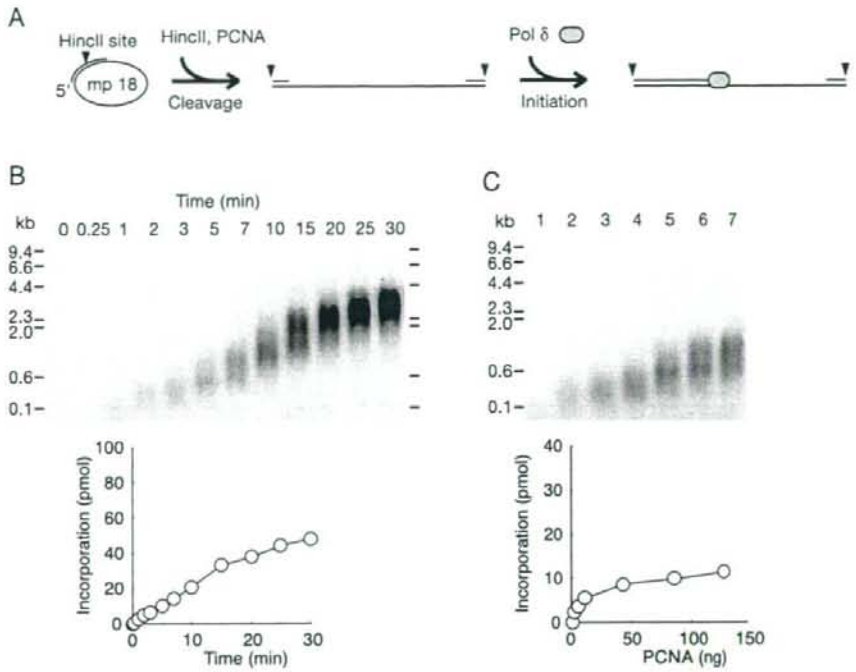

Figure 4. Effect of PCNA loaded spontaneously at ends of template DNA. (**A**) Schematic representation of the experimental design. A primer that covered HincII site was annealed to ss mp18 DNA. HincII (10 U) was introduced into standard reaction mixtures (25 μl) under the conditions described in the Materials and Methods section except for omitting RFC. After pre-incubation for 1 min, reactions were started by addition of pol δ. (**B**) Time course of DNA synthesis in the absence of RFC. (**C**) Titration of PCNA in the reactions without RFC. Amounts of PCNA used in the titration were the same as for Figure 2 (see legend of Figure 2). Reactions were carried out for 10 min. Autoradiograms of 0.7% alkaline-agarose gels in which the newly synthesized DNA were visualized by the incorporated [α-^{32}P]dTMP, and incorporation of dNMP were measured as described in the Materials and Methods section.

during elongation. These results revealed differences between RFC-dependent and -independent reactions, indicating a requirement of RFC for efficient restart after dissociation of PCNA on linearized DNA.

Effects of dilution of the elongation complexes

A different method of analysis was used to investigate the action of protein factors. Here, after the replication complex was assembled, the reaction mixture containing the complexes was diluted in pre-warmed reaction mixtures containing all the protein components at the same concentration, except one component. As with the previously described method, if the protein in question acts distributively, one would expect to observe a shift to shorter products with diluted reaction mixtures (32).

As schematically shown in Figure 5A, the primer–template DNA was mixed with saturating concentrations of RPA, PCNA and RFC, and then 1 min later pol δ was added for formation of replication complexes. At 15 s after the initiation complex was presumably assembled, an aliquot of the reaction mixture was diluted 10-fold either into a pre-warmed reaction mixture containing all the auxiliary proteins and pol δ, or into a similar one omitting one or two of PCNA, RFC and pol δ (Figure 5A). Then, reactions were continued for further 10 min, and the products were analyzed by alkaline-agarose gel electrophoresis (Figure 5B). Dilution of either PCNA or pol δ resulted in a decrease in the size of the products (Figure 5B, lanes 2 and 4) as compared to the complete case (Figure 5B, lane 1). This effect was more pronounced when both PCNA and pol δ were diluted together (Figure 5B, lane 7); virtually no products were detected. On the other hand, dilution of RFC exerted no influence (compare lanes 1 and 3 in Figure 5B). Furthermore, when both PCNA and RFC were diluted together, the size distribution of products was almost identical to that with dilution of PCNA alone (compare lanes 2 and 5 in Figure 5B). These results indicated that PCNA and pol δ are supplied from solution, whereas RFC is not, during the elongation. Furthermore, we noted that when both RFC and pol δ were diluted together, the product size was decreased to a much greater extent than with dilution of pol δ alone (compare lanes 4 and 6 in Figure 5B). This suggested that when reassociation of pol δ is limited, RFC is needed from solution. The importance of this observation is discussed below.

We also tested if adenosine (3-thiotriphosphate) (ATPγS) affected elongation reactions. When ATP in the dilution mixture was replaced with ATPγS, elongation reactions were completely halted (Figure 5B, lane 9).

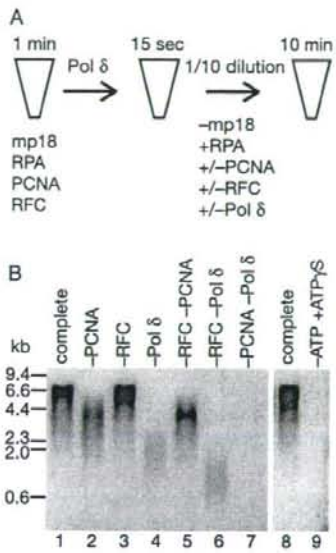

Figure 5. Effects on size distribution after dilution of elongation complexes. (**A**) Outline of the assay. At 15 s after the reaction was started by addition of pol δ under standard reaction conditions described in the Materials and methods section, 10-fold dilution was performed with pre-warmed reaction mixtures without template but containing all the protein components or omitting one or two of them. Both reaction mixtures, before and after dilution, contained [α-^{32}P]dTTP. After a further 10 min incubation, the reaction products were analyzed by 0.7% alkaline-agarose gel electrophoresis. (**B**) An autoradiogram of a 0.7% alkaline-agarose gel. The indicated proteins were omitted in the dilution mixtures. In the reaction shown in lane 9, 1 mM ATP in the dilution mixture was replaced with 2.5 mM ATPγS.

This suggested that the ATPase activity of RFC is required for the elongation phase of replication (24), consistent with our assumption that RFC remains around primer terminus, and holds, unloads and reloads PCNA.

Functions of the POLD3 subunit of pol δ

The role of the 66 kDa subunit, POLD3, of pol δ in the dynamic processes involved in elongation, and the biochemical properties of subassembly (pol δ*) lacking the POLD3 subunit are of great interest. First, we examined the efficiency of DNA synthesis of human pol δ* using purified proteins (Figure 6A). A comparison of activities with equivalent amounts of pol δ* and pol δ demonstrated inefficiency of pol δ* under the standard reaction conditions with singly primed ss mp18 DNA (Figure 6B), decrease and heterogeneity in length of the products being observed with emphasized pausing sites (Figure 6B and C). The shorter products were shifted to longer ones at higher concentrations of pol δ* (Figure 6C), as with pol δ (Figure 2C). When the missing subunit, POLD3, was introduced into the reaction with pol δ*, the activity was restored to the level with pol δ (Figure 6B), indicating that the lower activity of pol δ* was due to the missing function of POLD3 subunit, rather than denaturation of proteins caused by incomplete assembly. The evidence presented here is consistent with reports for yeast counterparts (30,45) and human pol δ (36,46).

Next, we tested whether variation in the concentrations of RFC and PCNA might affect the size of products in reactions with pol δ*. RFC was without influence, again suggesting stable association (Figure 6D). In contrast, the size of products varied with the concentration of PCNA (Figure 6E), in line with a requirement for a continuous supply of PCNA from solution for efficient DNA synthesis. Notably, with low concentrations of PCNA, all the products were uniformly small in reactions with pol δ*, exhibiting an entirely distributive nature. Furthermore, we tested the effect of linearization on mp18 DNA after initiation of DNA replication by addition of HincII (Figure 6F). DNA synthesis with pol δ* was very sensitive to linearization of DNA, and increasing concentrations of PCNA slightly restored the defect (Figure 6F). However, the majority of intermediates on elongation could not overcome the first pausing site (around 0.6–1 kb), even at the highest concentration of PCNA (Figure 6F). These results suggested frequent dissociation of PCNA during elongation with pol δ*.

Amounts of PCNA loaded on mp18 DNA during elongation

Since an excess PCNA was required for efficient DNA synthesis with pol δ* (Figure 6E), we considered whether PCNA might accumulate on DNA during elongation. If so, the frequency of sliding back to the primer terminus would increase and an increase in the local concentration of PCNA would help interactions with pol δ* at the primer terminus. To measure the amount of PCNA loaded on DNA directly, a primer containing an extended 5′ tail with one biotin molecule was annealed to ss mp18 DNA (Figure 7A). The primed ss mp18 DNA was then attached to magnetic beads and DNA replication reactions were carried out under standard reaction conditions (Figure 7A). The 5′ tail did not exert any influence on DNA synthesis (data not shown). In the reactions, 33 ng of pol δ and 140 ng of pol δ* were used since these amounts lead to equivalent efficiency of DNA synthesis (~40 pmol of the incorporation of dNMP) and to the same size distribution of products (compare lane 3 of Figure 2C with lane 7 of Figure 6C). After reactions for 10 min, PCNA bound to beads was detected by western analysis (Figure 7B). In this assay, non-specific association of PCNA with beads or DNA was detected (Figure 7B, lane 2). When RFC was introduced into the reaction, an increase of binding of PCNA was observed. The increased amount of PCNA (difference between lanes 2 and 3 in Figure 7B) was 40 fmol, which was equivalent to that of primer template (33 fmol), suggesting specific loading to the primer terminus. Introduction of pol δ increased the amount of PCNA only slightly (Figure 7B, lane 4). The majority of PCNA was dissociated by introduction of HincII with a decreasing signal to background level (Figure 7B, lane 5), again indicating specific loading on the DNA. When pol δ* was used instead of pol δ, excessive accumulation of PCNA was unexpectedly not observed (Figure 7B, lane 6), suggesting that we cannot attribute the entire distributive nature of PCNA on the

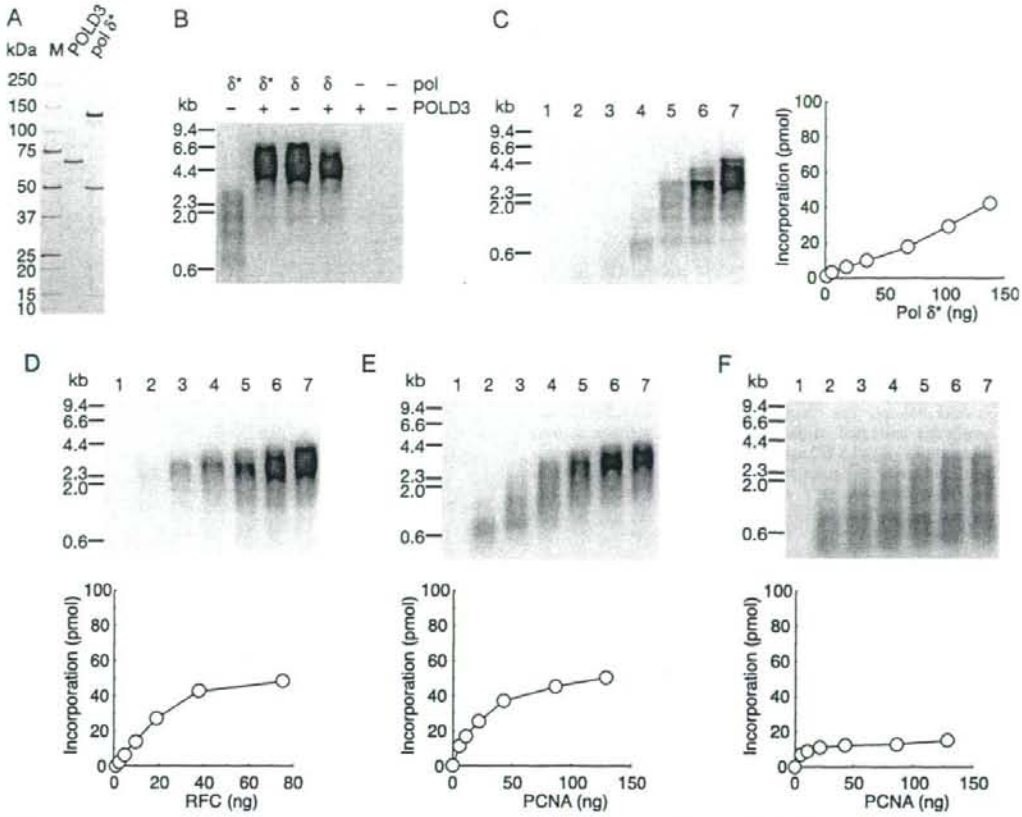

Figure 6. Dynamics of replication factors in the elongation phase of DNA synthesis with pol δ*. (**A**) SDS–PAGE analysis of purified recombinant proteins. Pol δ* (1.9 μg) and POLD3 (0.5 μg) were loaded on a SDS 4–20% gradient polyacrylamide gel and stained with CBB. (**B**) Reconstitution of pol δ with POLD3 and pol δ*. Reactions were carried out for 10 min under the conditions described in the Materials and Methods section except for pol δ* (70 ng) or pol δ (90 ng) in the absence (–) or presence (+) of POLD3 (20 ng). (**C–E**) Titration of pol δ* (C), RFC (D) and PCNA (E). Amounts of pol δ* were 0 ng (lane 1), 4.3 ng (lane 2), 17 ng (lane 3), 35 ng (lane 4), 70 ng (lane 5), 100 ng (lane 6), and 140 ng (lane 7). Amounts of RFC used in the titration were 0 ng (lane 1), 2.3 ng (lane 2), 4.7 ng, (lane 3), 9.4 ng (lane 4), 19 ng (lane 5), 38 ng (lane 6) and 75 ng (lane 7). Amounts of PCNA used in titration were 0 ng (lane 1), 5.4 ng (lane 2), 11 ng (lane 3), 22 ng (lane 4), 43 ng (lane 5), 86 ng (lane 6) and 129 ng (lane 7). (**F**) Titration of PCNA in the presence of HincII. Amount of PCNA is same as (E). Reactions in (C) were carried out for 10 min under the conditions described in the Materials and Methods section. Reactions in (D–F) were carried out for 10 min under the conditions described in the Materials and Methods section except for the amount of pol δ* (140 ng). Products were analyzed by 0.7% alkaline-agarose gel electrophoresis and incorporation of dNMP were measured as described.

reaction with pol δ* to only the decreasing affinity between PCNA and pol δ*. We therefore considered the possibility that loading and unloading of PCNA is equilibrated in both pol δ and pol δ* cases, and importantly, could be accelerated in the reaction with pol δ*.

DISCUSSION

Generally, proteins act during replication in two distinct modes, processively or distributively (32). The studies documented here showed very different dynamics of the various protein factors in the elongation phase of DNA replication.

DNA synthesis *in vitro* by pol δ has been investigated extensively. Previous studies have shown that pol δ itself is a very distributive enzyme, which turns into a processive polymerase when bound to the clamp, PCNA (11,12,15–17). However, even in the presence of PCNA, pol δ replicated M13 ss DNA through a number of dissociating and reassociating steps, as proposed for mammalian pol δ isolated from natural sources and overproduced in insect cells (34–36). Our results support the conclusion that human pol δ has a distributive nature for DNA replication in our model system *in vitro*.

The length of products synthesized by pol δ does not depend on the concentration of RFC (6,37). The processive nature of RFC might be explained in two alternative ways. One is that the sole role is loading of

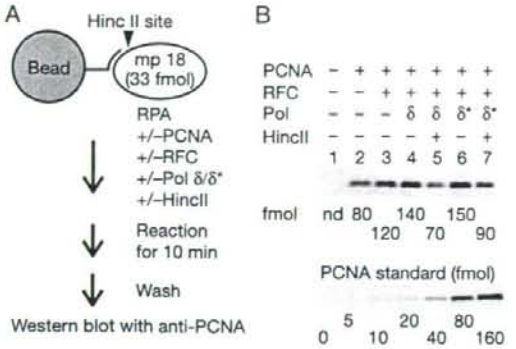

Figure 7. Amounts of PCNA loaded on DNA during elongation. (**A**) Outline of the assay. DNA was attached to magnetic beads via biotin–streptavidin linkage. The reactions were carried out for 10 min under the conditions described in the Materials and Methods section except for the amounts of pol δ (33 ng) and pol δ* (140 ng). (**B**) Western analysis. Chemiluminescence signals were detected with a CCD camera and quantified with reference to a standard curve for PCNA in the same blot.

PCNA at only the initiation step (35,38). Some biochemical data for yeast and human RFC support this possibility, because RFC has been found to dissociate from DNA after loading PCNA (38,47,48). The other explanation is that once RFC finds a 3′-hydroxyl end and loads PCNA, it then travels with PCNA and pol δ (39,40). In earlier work with yeast RFC, formation of tertiary complexes on DNA was also suggested (49). Later, Yuzhakov and colleagues (26) also reported that human RFC travels with pol δ, and a similar complex has been isolated from the elongation phase of SV40 replication (50). Our observations also provide support for continuous binding of RFC.

Because PCNA is a sliding clamp, it should be able to freely slide along double-stranded DNA and fall off at the ends (43,44,51). However, the fate of PCNA in the elongation phase of DNA replication is currently obscure. We here obtained evidence that some PCNA does not remain at the primer terminus after dissociation of pol δ. However, in titration experiments of PCNA, the longer products (around 4 kb) still remained even at low concentrations of PCNA, independent of the presence or absence of HincII (Figures 2E and G, 3E and G), indicating that significant fractions of the intermediates of elongation phase could continue DNA synthesis without supply of PCNA from solution. In such fractions, PCNA must remain at the primer terminus even after dissociation of pol δ. The observations support the conclusion that PCNA has a partially distributive nature in our DNA replication system with RFC *in vitro*. On the other hand, PCNA was entirely distributive in the RFC-independent reaction (Figure 4C). Taken the results together, we suggest the possibility that RFC could hold PCNA from which pol δ has detached.

We here propose a model for dynamics of replication factors during a dissociation–association cycle of pol δ (Figure 8). The elongation complex consists of RFC,

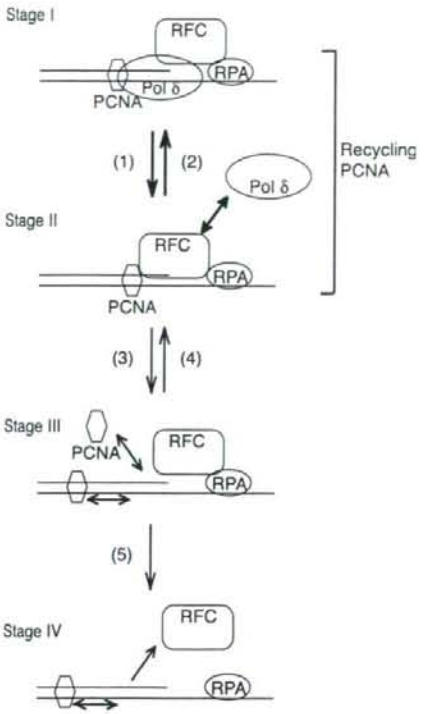

Figure 8. A model for dynamics of replication factors during pol δ dissociation–association cycles. The elongation complex consists of RFC, PCNA and pol δ in the elongation phase of DNA replication (Stage I). Pol δ contacts with PCNA and prevents RFC from dissociating. Contribution of RFC–RPA interaction for stable association in the complex has been proposed (26). Pathway 1, dissociation of pol δ leaving RFC and PCNA on DNA (Stage II). DNA–RFC–PCNA complex formation could be coupled with dissociation of pol δ, mediated by the POLD3 subunit. Pathway 2, reassociation of pol δ to form the elongation complex. The POLD3 subunit of pol δ might mediate efficient transfer of PCNA from RFC to pol δ. Pathway 3, unloading or sliding of PCNA out of the primer terminus, leaving RFC (Stage III). RFC probably interacts with RPA for retaining around primer terminus (26). Pathway 4, reloading of PCNA from solution or PCNA sliding back along the DNA to reform the DNA–RFC–PCNA complex. Pathway 5, dissociation of RFC from DNA (Stage IV). The main pathways are shown as thick arrows.

PCNA and pol δ (Figure 8, stage I). Pol δ dissociates frequently from growing 3′-hydroxyl ends and PCNA during elongation of DNA replication (Figure 8, pathway 1). Then, PCNA from which pol δ has detached would be held by the remaining RFC (Figure 8, stage II). If pol δ reassociated quickly (Figure 8, pathway 2), RFC could remain around the primer terminus, and travel with pol δ and PCNA during elongation of DNA replication. In this cycle, PCNA is not released out of the replication complex, which implies 'recycling PCNA' (Figure 8). Since the DNA–RFC–PCNA complex (stage II) is not stable (52), the RFC could unload PCNA, or release the PCNA out of the primer terminus (Figure 8, pathway 3). Probably, the unloading reaction does not predominate, as shown in yeast RFC (53). If PCNA were available from

solution or along the DNA, RFC could incorporate PCNA into the complex (Figure 8, pathway 4). Otherwise, RFC would dissociate from the primer terminus (Figure 8, pathway 5). At higher concentrations greater than saturated amounts of RFC, the size of products was increased (Figures 2D and F, 3D and F). Probably, with such concentrations, RFC is sufficient for initiation and remaining RFC in solution could help to overcome the pausing site, implying dissociation of RFC at strong pausing sites. However, this was negligible under normal replication conditions, and was detectable in reactions omitting pol δ, as shown in lane 6 of Figure 5B. This suggested that pol δ prevents dissociation of RFC. In intensive studies of PCNA-loading reactions with yeast RFC, no stable RFC–DNA complex was detected in the absence of PCNA and only became detectable in the presence of ATPγS, and RFC dissociated quickly from DNA after loading PCNA (47,48). Therefore, it has been considered that RFC is absent in elongation complexes. Our results may explain the discrepancy regarding the prevention of dissociation of RFC by sequential loading of PCNA (pathway 4) and pol δ (pathway 2) in the replication assay, but not PCNA loading assays. Therefore, we consider that our model is consistent with the previous observations (38,47,48). Gomes *et al.* (47) has also proposed a loading pathway, in which PCNA–RFC complex first forms an ATP-dependent ring-opened complex and subsequently associates with DNA and delivers PCNA to the template–primer junction. RFC associated with the DNA cannot recruit PCNA nor load it at termini, first having to dissociate coupled with ATP hydrolysis (47). Our model is consistent with the loading mechanism. RFC is probably detached from the primer terminus before loading PCNA, but associated around primer terminus via interaction with RPA (stage III) as proposed previously (26).

Here, we further examined the dynamics of protein factors in the reaction with pol δ* to elucidate functions of the POLD3 subunit. This, together with its budding and fission yeast counterparts, Pol32 and Cdc27, respectively, has a PCNA-binding domain that is responsible for processive DNA synthesis on M13 ss DNA (54–57). We demonstrated that the size of products varies depending on the concentration of PCNA exhibiting an entirely distributive nature (Figure 6E). If PCNA were accumulated on the DNA reflecting an increase amount in solution, the requirement of large excess of PCNA in solution could be simply due to decreasing affinity between pol δ* and PCNA. We failed to demonstrate excessive accumulation of PCNA on the DNA (Figure 7B). Rather, the concentration of PCNA in solution little affected that on DNA. Therefore, it is impossible to attribute the entire distributive nature of PCNA on the reaction with pol δ* to simply a decreasing affinity between PCNA and pol δ*. We consider that the defect with pol δ* could be due to not only decreased affinity to PCNA, but also failure in recycling of PCNA after dissociation. In the reactions with pol δ, significant fractions of intermediates in the elongation stage could continue DNA synthesis without supply of PCNA from solution (Figure 2E), indicating efficient recycling of PCNA (Figure 8, pathways 1 and 2). In contrast, such a fraction was not detected in reactions with pol δ* (Figure 6E), presumably due to predominant loss of PCNA from the primer terminus. This could be a consequence of unloading of PCNA by RFC, since excessive accumulation of PCNA was not observed (Figure 7B) under conditions whereby excess loading of PCNA from solution was expected (Figure 6E). Yeast studies have predicted a second function of subunits enhancing processivity of pol δ in a manner independent of the PCNA-binding site (56). We propose that the previously unknown function of POLD3 subunit is stimulation of recycling of PCNA in the dissociation–association cycle with pol δ.

It has been shown that RFC binds non-specifically to DNA with a potential for loading PCNA on the double-stranded DNA, independent of the primer ends (58). Is the processivity of RFC observed in this work only apparent and due to non-specific loading of PCNA? If PCNA molecules were loaded anywhere on the double-stranded DNA and accumulated on the DNA, it could explain that size of the products of DNA synthesis depends on the concentration of PCNA, especially in reactions with pol δ* (Figure 6E). However, we failed to detect excessive accumulation of PCNA on the DNA (Figure 7B). Furthermore, spontaneously loaded PCNA from the end of DNA did not well support elongation, even an excessive amount of PCNA was present in the RFC-independent reactions (Figure 4), suggesting that loading of PCNA at primer terminus is crucial for the efficiency. We speculate that PCNA loaded non-specifically on the DNA is probably ineffective, just like spontaneously loaded PCNA. Although we could not exclude the possibility of contribution of non-specific loading of PCNA to the apparent processivity of RFC, it is probably not predominant in our reaction conditions.

PCNA functions as a platform not only for elongation but also for Okazaki fragment processing through interaction with protein factors, FEN1 and DNA ligase I. Recently, the Kunkel laboratory has provided evidence that pol ε is active in DNA synthesis on the leading strand (59), suggesting the pol δ is active on the lagging strand (59,60). Consistent with this, stable association of RFC in elongation complexes with pol δ for efficient utilization of PCNA could thus be of benefit to maturation of Okazaki fragments. Consistently, physical interactions between RFC and DNA ligase I have been demonstrated (61), implying a functional significance of stable association of RFC in elongation complexes on the lagging strand.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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DNA Damage-induced Ubiquitylation of RFC2 Subunit of Replication Factor C Complex*[S]

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Many proteins involved in DNA replication and repair undergo post-translational modifications such as phosphorylation and ubiquitylation. Proliferating cell nuclear antigen (PCNA; a homotrimeric protein that encircles double-stranded DNA to function as a sliding clamp for DNA polymerases) is monoubiquitylated by the RAD6-RAD18 complex and further polyubiquitylated by the RAD5-MMS2-UBC13 complex in response to various DNA-damaging agents. PCNA mono- and polyubiquitylation activate an error-prone translesion synthesis pathway and an error-free pathway of damage avoidance, respectively. Here we show that replication factor C (RFC; a heteropentameric protein complex that loads PCNA onto DNA) was also ubiquitylated in a RAD18-dependent manner in cells treated with alkylating agents or H_2O_2. A mutant form of RFC2 with a D228A substitution (corresponding to a yeast Rfc4 mutation that reduces an interaction with replication protein A (RPA), a single-stranded DNA-binding protein) was heavily ubiquitylated in cells even in the absence of DNA damage. Furthermore RFC2 was ubiquitylated by the RAD6-RAD18 complex *in vitro*, and its modification was inhibited in the presence of RPA. The inhibitory effect of RPA on RFC2 ubiquitylation was relatively specific because RAD6-RAD18-mediated ubiquitylation of PCNA was RPA-insensitive. Our findings suggest that RPA plays a regulatory role in DNA damage responses via repression of RFC2 ubiquitylation in human cells.

Cellular DNA is continuously damaged by a vast variety of endogenous and exogenous genotoxicants. When genomic DNA is damaged, cells respond by activation of complex signaling networks that delay cell cycle progression, induce repair of lesions, activate damage tolerance pathways, and trigger apoptosis or senescence (1, 2). It is hypothesized that DNA damage-inducible signaling pathways serve important tumor-suppressive roles and prevent mutations that could lead to malignancy. Various genotoxins elicit different forms of DNA damage and result in distinct signal transduction pathways and biological outcomes. Distal steps of DNA damage-induced checkpoint signaling pathways that result in inhibition of the cell cycle are relatively well understood (3, 4). However, molecular details of proximal signaling events and lesion-specific DNA damage recognition events are less clear.

DNA replication and repair require the coordinated actions of multiple proteins on small regions of DNA. A limited number of proteins serve to coordinate multiple replication and repair events. Some proteins function commonly in DNA replication and repair and frequently have a crucial role in both processes. Three such examples are replication protein A (RPA),[2] proliferating cell nuclear antigen (PCNA), and replication factor C (RFC). RPA was originally identified as a eukaryotic single-stranded DNA-binding protein essential for *in vitro* replication of SV40 DNA (5, 6). PCNA is a trimer of three identical subunits arranged head-to-tail to generate a ringlike structure with a large central cavity for encircling DNA. It is well established that PCNA provides a mobile platform to serve as anchor and processivity factor for DNA polymerases during chromosomal replication (7, 8). PCNA is loaded onto the primer-template junction in an ATP-dependent manner by a multiprotein clamp loader, RFC (9, 10). RFC binds preferentially to double-stranded/single-stranded junctions with a recessed 3′-end, which is the DNA target for PCNA loading.

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[2] The abbreviations used are: RPA, replication protein A; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; Pol, polymerase; RLC, RFC-like complex; HA, hemagglutinin; PBS, phosphate-buffered saline; HU, hydroxyurea; MMS, methyl methanesulfonate; Sup, supernatant; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; AP, apurinic/apyrimidinic.

RPA, PCNA, and RFC are key proteins that play central roles in DNA replication, participating in competitive polymerase switching during lagging strand synthesis. The DNA polymerase α-primase complex (Pol α) that synthesizes an RNA-DNA hybrid primer requires contact with RPA to remain stably attached to the primed site. For processive DNA synthesis to follow, Pol α must be replaced by DNA polymerase δ (Pol δ). Replacement of Pol α by Pol δ is initiated by interactions between RFC and RPA that disrupt Pol α-RPA interactions and result in removal of Pol α from DNA. After RFC loads PCNA onto the primed site, Pol δ associates with PCNA by displacing RFC. The switching process is indeed coordinated by RPA via cooperative interactions with PCNA and RFC (11, 12). RPA, RFC, and PCNA also play key roles in DNA repair by interacting with many DNA repair enzymes (13–15). Such interactions are believed to play roles in DNA damage recognition and in recruiting and positioning of DNA repair enzymes.

RFC consists of five different subunits, which are homologous to one another and are members of the AAA^+ family of ATPases (16, 17). The RFC1(p140) subunit is sometimes referred to as the "large subunit" as it contains both N- and C-terminal extensions beyond its region of homology with the four "small" subunits. The four small RFC subunits are designated RFC2(p40), RFC3(p36), RFC4(p37), and RFC5(p38) in mammals. Three protein complexes with resemblance to RFC that are involved in maintaining genome stability have been described recently. These RFC-like complexes (RLCs) share four common small subunits (RFC2–5), and each carries a unique large subunit (RAD17, CTF18, or ELG1) replacing the RFC1. These RLCs are involved in the checkpoint response (RAD17-RFC), sister chromatid cohesion (CTF18-RFC), and maintenance of genome stability (ELG1-RFC) (18, 19).

DNA damage sensors and repair proteins must react in a rapid and efficient manner to execute their functions. Frequently the regulation of these proteins involves post-translational modifications, such as phosphorylation and ubiquitylation, to help modulate the assembly and disassembly of complexes and to assist targeting and the regulation of enzymatic activity in a timely manner. For example, RPA is hyperphosphorylated upon DNA damage or replication stress by several checkpoint kinases (20). Hyperphosphorylation alters RPA-DNA and RPA-protein interactions (15, 21). Recent studies in the DNA repair field have highlighted the expanding role of ubiquitylation in the regulation of diverse DNA repair processes and pathways. One of the most striking examples of how ubiquitylation can affect protein function is that of PCNA in the budding yeast *Saccharomyces cerevisiae*. Following DNA damage, PCNA can be monoubiquitylated or polyubiquitylated on the Lys-164 residue, and each modification results in a different outcome with respect to DNA synthesis and repair (22, 23). Monoubiquitylated PCNA directs translesion synthesis via error-prone DNA polymerases, whereas polyubiquitylated PCNA is associated with an error-free DNA repair pathway (22, 23). Mammalian PCNA also undergoes monoubiquitylation after UV irradiation, and monoubiquitylated PCNA preferentially binds to translesion synthesis polymerases that contain one or two copies of ubiquitin-binding domains (24–27).

In contrast to RPA and PCNA, damage-dependent modification of RFC has not been described. Recent studies have significantly broadened the scope of the role of ubiquitylation to include regulatory functions in DNA repair and damage response pathways. Therefore, in this study we investigated whether the clamp loader RFC is likewise subjected to regulated modification. We examined the modification of all subunits in RFC and RLCs. We demonstrated that RFC2 and RFC4 are ubiquitylated following treatment of cells with alkylating agents. The ubiquitylation was partially dependent on RAD18. Surprisingly RPA inhibited the RAD18-dependent ubiquitylation of RFC2. Our results suggest that RFC regulates the DNA damage response pathway via interaction with RPA and ubiquitylation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—To generate pCDNA·RFC2(p40)FLAG and pCDNA·RFC2(p40)HA, human p40 coding region was amplified by PCR as an EcoRI-XhoI fragment. The PCR product was inserted into the EcoRI-XhoI site either of pCDNA-C-FLAG or pCDNA-C-HA. To generate pCAGGS·RFC2(p40), the human p40 coding region was amplified by PCR as a SalI-XhoI fragment. The PCR product was inserted into the XhoI site of pCAGGS. pCDNA-C-FLAG and pCDNA-C-HA was constructed by inserting the FLAG or HA epitope into the XhoI-XbaI site of pCDNA3.1. Expression plasmids containing human RFC1-FLAG, human FLAG-RAD17, human FLAG-CTF18, human FLAG-p38, human FLAG-p37, and human FLAG-p36 were constructed by inserting their cDNA described previously (28) into pCDNA3. Although N-terminally and C-terminally tagged forms of each RFC2 subunit were used, the presence of the epitope tag did not affect RFC2 regulation at least in the context of experiments reported in this study. pCAGGS·FLAG-Ubiquitin and pCAGGS·hRAD18 were constructed as described previously (25). The expression plasmids for human RFC and PCNA were described earlier (29, 30), and that for human RPA, p11d-tRPA (31), was a generous gift of Dr. Marc S. Wold (University of Iowa College of Medicine, Iowa City, IA). Mouse E1 expression vector RLC (32, 33) was a generous gift of Dr. Hideyo Yasuda (School of Life Science, Tokyo University of Pharmacy and Life Science, Tokyo, Japan). Human cDNAs for RAD6A and RAD18 amplified from a HeLa cDNA library by PCR introducing a NdeI site at the start codon were cloned together into pET20b(+) (Novagen) as an artificial operon. After cloning the PCR fragments, the nucleotide sequences were verified. All the expression plasmids of PCNA, RPA, RFC, E1, RAD6A, and RAD18 were designed for production of intact proteins without any affinity tags.

Cell Culture and Transfection—293A and HCT116 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HCT116 *RAD18*$^{-/-}$ cells were established as described previously (25). Cells were transfected with Lipofectamine Plus (Invitrogen) or Lipofectamine 2000 according to the manufacturer's protocol. 2.4 μg of plasmid DNA was used to transfect each 6-cm plate of cells. Transfected cells were treated with genotoxins 24 h post-transfection.

Genotoxin and Inhibitor Treatments—Asynchronous cell cultures were grown to approximately 80% confluency. For UV