Replication-Dependent Marking of DNA by PCNA Facilitates CAF-1-Coupled Inheritance of Chromatin

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Summary

Chromatin assembly factor 1 (CAF-1) is required for inheritance of epigenetically determined chromosomal states in vivo and promotes assembly of chromatin during DNA replication in vitro. Herein, we demonstrate that after DNA replication, replicated, but not unreplicated, DNA is also competent for CAF-1-dependent chromatin assembly. The proliferating cell nuclear antigen (PCNA), a DNA polymerase clamp, is a component of the replication-dependent marking of DNA for chromatin assembly. The clamp loader, replication factor C (RFC), can reverse this mark by unloading PCNA from the replicated DNA. PCNA binds directly to p150, the largest subunit of CAF-1, and the two proteins colocalize at sites of DNA replication in cells. We suggest that PCNA and CAF-1 connect DNA replication to chromatin assembly and the inheritance of epigenetic chromosome states.

Introduction

During replication of the eukaryotic cell genome, not only is the DNA replicated, but the newly synthesized DNA must also be assembled into chromatin. DNA replication-coupled chromatin assembly is essential for the inheritance of chromatin structures, without which epigenetically determined chromosomal states present in parent cells could not be propagated to their daughters (Brown, 1984; Weintraub, 1985; Lewin, 1998). It is therefore of considerable interest to study the mechanism and control of chromatin assembly during DNA replication.

Chromatin assembly factor 1 (CAF-1) is unique among histone chaperones because it preferentially assembles nucleosomes onto replicating DNA. CAF-1 was initially identified as an activity in a nuclear extract from human cells that promoted negative supercoiling of replicating DNA when added to a cell-free system for simian virus 40 (SV40) DNA replication (Stillman and Gluzman, 1985; Stillman, 1986). This factor also facilitates assembly of chromatin on templates that have undergone DNA repair (Gaillard et al., 1996, 1997). CAF-1 is a complex of three subunits, p150, p60, and p48 (Smith and Stillman, 1989), and in cells they can form a larger complex (CAC) that contains specific acetylated forms of histones H3 and H4 (Verreault et al., 1996). The composition and biochemical activity of CAF-1 are conserved among human, *Drosophila*, and yeast (Bulger et al., 1995; Kaufman et al., 1995, 1997; Verreault et al., 1996).

In the budding yeast *Saccharomyces cerevisiae*, the genes encoding the CAF-1 subunits *CAC1*, *CAC2*, and *CAC3* are not essential for cell viability, suggesting the existence of at least one other replication-coupled chromatin assembly mechanism (Kaufman et al., 1997). However, *cacΔ* mutants are defective in the stable inheritance of gene silencing at telomeres and at the mating type loci (Enomoto et al., 1997; Kaufman et al., 1997; Monson et al., 1997; Enomoto and Berman, 1998). These phenotypes suggested a functional interdependence between DNA replication-coupled chromatin assembly by CAF-1 and inheritance of epigenetically determined chromosomal states. However, how they are linked together is still uncertain.

The proliferating cell nuclear antigen (PCNA) was originally discovered as a cell cycle–regulated nuclear protein whose rate of synthesis correlated well with the proliferative status of the cell. Later, it was found to be an essential processivity factor for DNA polymerase δ and ε in eukaryotic DNA replication (Tan et al., 1986; Prelich et al., 1987a, 1987b; Burgers, 1991; Lee et al., 1991; Tsurimoto and Stillman, 1991; Podust and Hübscher, 1993; Waga and Stillman, 1998) and for nucleotide excision repair (Nichols and Sancar, 1992; Shivji et al., 1992). PCNA is a homotrimer that forms a closed ring structure around duplex DNA, similar to the β subunit of *E. coli* DNA polymerase III (Krishna et al., 1994). Many proteins interact with PCNA, either for DNA replication or repair (Jonsson and Hübscher, 1997; Kelman and Hurwitz, 1998). The best-characterized function for this protein is as a sliding clamp for DNA polymerases, allowing these enzymes to move along their template while PCNA remains topologically linked to the DNA. During replication, PCNA is loaded onto the DNA by replication factor C (RFC), an ATP-dependent clamp loader protein (Tsurimoto and Stillman, 1989, 1991; Lee et al., 1991; Jonsson and Hübscher, 1997; Kelman and Hurwitz, 1998).

During investigation of the mechanisms and control of CAF-1-dependent chromatin inheritance, we discovered that DNA can become noncovalently marked during DNA replication and that this marked, replicated DNA can function as a template for CAF-1-dependent assembly of chromatin. We present several lines of evidence demonstrating the involvement of PCNA in this replication-coupled marking process. These results suggest mechanisms for the inheritance of chromatin and stable chromosomal states, as well as possibilities for asymmetric distribution of gene regulatory complexes to sister chromatids during cell type specification. Because PCNA and CAF-1 also function in DNA repair, we suggest that any DNA synthesis may offer a mechanism for altering chromatin in both dividing and nondividing cells.

Results

Temperature-Dependent Stability of Replicated DNA as a Template for Chromatin Assembly

Little was known about how CAF-1 preferentially assembled chromatin during DNA replication or on templates

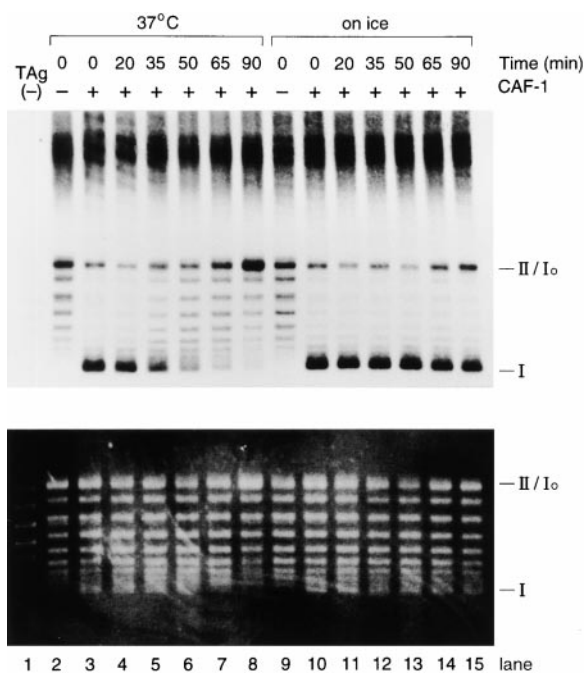*To whom correspondence should be addressed (e-mail: stillman@cshl.org).

Figure 1. CAF-1-Dependent Assembly of Chromatin on Replicated DNA

A large-scale SV40 DNA replication reaction was performed for 40 min, followed by addition of aphidicolin (7.5 μg/ml). The reaction mixture was divided into aliquots, which were incubated at 37°C or on ice for the indicated times, and aliquots were transferred to new tubes containing CAF-1 (lanes 3–8 and 10–15) or buffer only as a control (lanes 2 and 9) to allow the supercoiling reaction to proceed. The upper panel shows the newly replicated DNA as visualized by autoradiography, and the lower panel shows the total DNA, as visualized by staining with ethidium bromide. Migration of relaxed, covalently closed circular DNA (form I_o), nicked circular DNA (form II), and negatively supercoiled DNA (form I) are indicated. A reaction without added T antigen during the first 40 min incubation did not allow DNA replication (lane 1), and a reaction without CAF-1 in the second reaction did not allow supercoiling (lanes 2 and 9).

that had undergone DNA repair. During characterization of *Drosophila* CAF-1 (dCAF-1), however, an observation gave us a hint of how to address this problem. We found that dCAF-1 could be added to the DNA replication reactions even after DNA replication was blocked by aphidicolin, and yet preferential chromatin assembly still took place on the replicated DNA when dCAF-1 was added (Kamakaka et al., 1996). The temporal separation of DNA replication and replication-dependent chromatin assembly was also observed by others (Fotedar and Roberts, 1989). These observations suggested that the replicated DNA was somehow marked for subsequent chromatin assembly. Our attempts to reproduce these findings using purified human cell CAF-1 and a human cell extract uncovered a temperature-dependent instability of the chromatin assembly template.

SV40 DNA replication was performed first for 40 min prior to adding aphidicolin, a DNA synthesis inhibitor. The reaction mixtures were incubated at 37°C or on ice for the indicated times, and then CAF-1 was added to allow supercoiling to proceed. The products were then isolated and separated on an agarose gel. The replicated DNA, labeled during the replication reaction with dATP
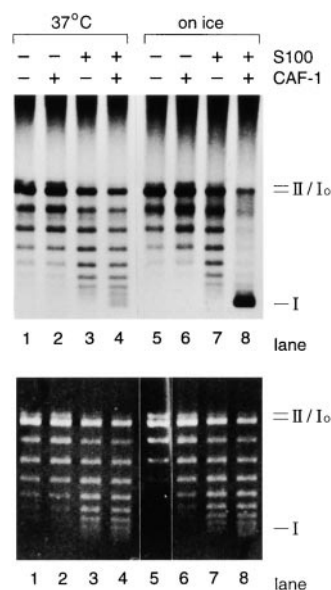

Figure 2. Chromatin Assembly of Newly Replicated and Isolated DNA

SV40 DNA replication was performed as for Figure 1. The reaction mixture was divided into two aliquots, which were then incubated for 90 min at either 37°C (lanes 1–4) or on ice (lanes 5–8). The DNA was then isolated by spin column chromatography and added to supercoiling reactions containing S100 extract and CAF-1 as indicated by + or −. The products were analyzed by gel electrophoresis. The upper panel shows an autoradiograph, and the lower panel shows an ethidium bromide–stained gel. Each form of DNA (form I, I_o, and II) is indicated, as in Figure 1.

in a T antigen–dependent manner, was visualized by autoradiography (Figure 1, top), and the total DNA, including unreplicated and unlabeled DNA, was visualized with ethidium bromide staining (Figure 1, bottom). At 37°C, the preferential supercoiling of labeled DNA was not affected by incubation for 20 min after the DNA replication reaction, but further incubation reduced or abolished supercoiling (Figure 1, lanes 2–8). These results were consistent with the previous results using dCAF-1 and a *Drosophila* embryonic cell extract (Kamakaka et al., 1996). In contrast, incubation of the replicated DNA for various times on ice did not affect preferential supercoiling at all (Figure 1, lanes 9–15). The labeled, replicated DNA was supercoiled, but the unreplicated DNA was not. This result suggested the existence of some coupling property that was activated during DNA replication, was unstable at 37°C, but was stable on ice.

Noncovalent Marking of DNA Is Required for the Coupling of Chromatin Assembly to DNA Replication

The dependence of chromatin assembly on DNA replication could be due to a *cis*-acting mark left on the replicated DNA. To test for this possibility, we loaded reaction mixtures onto a spin column to separate the DNA from the soluble proteins after incubation of the replication mixtures for 90 min at either 37°C or on ice, as in Figure 1. As shown in Figure 2, CAF-1 could not promote preferential supercoiling of labeled DNA that was preincubated at 37°C, even when high amounts of fresh extract were added to the supercoiling reactions (Figure

	− ATP														+ ATP														
	4°C						37°C								4°C						37°C						
RFC (pmol)	0	0	0	0	.25	.5	1	0	0	0	0	.25	.5	1	0	0	0	0	.25	.5	1	0	0	0	0	.25	.5	1
CAF-1	−	+	−	+	+	+	+	−	+	−	+	+	+	+	−	+	−	+	+	+	+	−	+	−	+	+	+	+
S100	−	−	+	+	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	+	+	−	−	+	+	+	+	+

lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
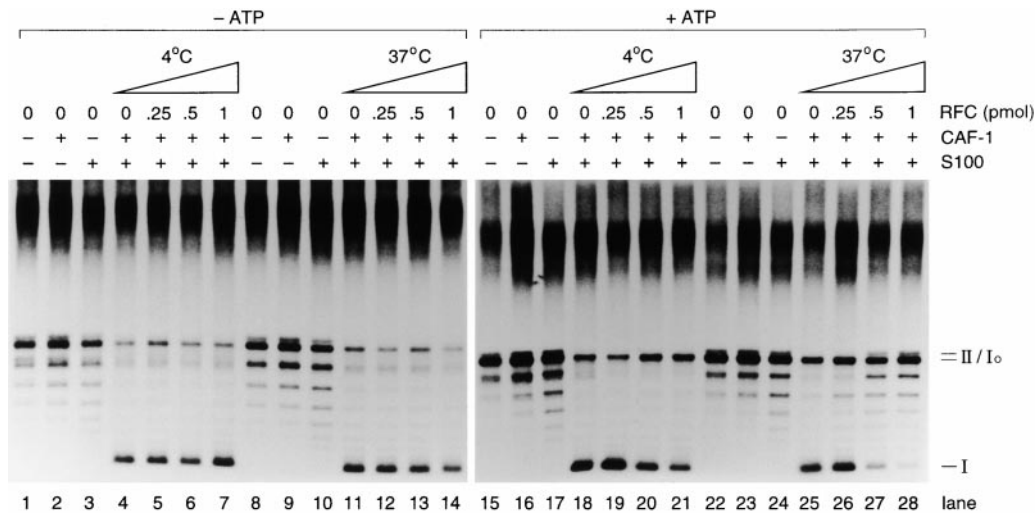
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Figure 3. Inhibition of Preferential Chromatin Assembly by Preincubation with RFC

Replicated DNA as in Figure 1 was purified by spin column chromatography and preincubated with buffer only (lanes 1–4, 8–11, 15–18, and 22–25) or the indicated amounts of purified RFC (lanes 5–7, 12–14, 19–21, and 26–28) at 4°C (lanes 1–7 and 15–21) or 37°C (lanes 8–14 and 22–28) for 25 min. The preincubations were performed either in the absence of ATP (lanes 1–14) or in the presence of 3 mM ATP (lanes 15–28). Supercoiling assays were performed either with or without human cell S100 extract (56 μg) and recombinant CAF-1 as indicated by + or −. Although an ethidium bromide–stained gel is not shown here, the replicated DNA was preferentially converted to form I. Each form of DNA (form I, Io, and II) was indicated as in Figure 1.

2, compare lanes 1–4). In contrast, when the replicated DNA was incubated on ice for 90 min, then isolated and incubated in a fresh extract with CAF-1, supercoiling was observed (Figure 2, lanes 5–8).

These results suggested that some property, imprinted reversibly on the DNA during replication, was required for coupling of chromatin assembly to DNA replication. The imprinted property, or mark on the DNA, could be a structural feature of the DNA itself, a protein loaded onto DNA, or both. For convenience, we term the DNA prepared by the spin column chromatography "replicated DNA."

The preferential supercoiling observed in Figure 2 was due to formation of an array of nucleosomes, because micrococcal nuclease digested these DNA products into 140–160 bp ladders of bands (data not shown). Both the S100 extract and CAF-1 were essential for chromatin assembly on replicated DNA (Figure 2, lanes 5–8), partially because the extract supplied newly synthesized histones H3 and H4, but other unidentified factors present in this extract were essential for CAF-1-mediated chromatin assembly (data not shown).

Treatment of Replicated DNA with RFC and ATP Abolishes Preferential Supercoiling

The separation of chromatin assembly from DNA replication provided the means to analyze the mechanism of chromatin assembly in more detail. We suspected that a protein common to both DNA replication and nucleotide excision repair (NER) might be required for the coupling DNA synthesis to chromatin assembly. Because PCNA topologically links with the DNA and is required for both DNA replication and NER, it was a prime candidate.

Replication factor C (RFC) is known to recognize primer–template junctions or nicked DNA and to load

PCNA in an ATP-dependent manner onto the DNA (Tsurimoto and Stillman, 1990, 1991; Lee et al., 1991). RFC can also unload PCNA from duplex DNA, although it takes more RFC than is required to load PCNA onto primed DNA (data not shown; Podust et al., 1995). Thus, we hypothesized that if PCNA were involved in marking of the replicated DNA for chromatin assembly, preincubation of the replicated DNA with RFC might unload the PCNA and block chromatin assembly by CAF-1.

The supercoiling reaction was performed with replicated DNA that had been treated with RFC under various conditions (Figure 3). Preincubation with increasing amounts of RFC in the presence of ATP at 37°C, but not at 4°C, significantly inhibited preferential supercoiling of the replicated DNA (Figure 3, compare lanes 27 and 28 with lanes 18–21, 25 and 26). In contrast, preincubation with increasing amounts of RFC in the absence of ATP, either at 37°C or at 4°C, did not inhibit the preferential supercoiling of replicated DNA (Figure 3, lanes 4–7 and 11–14). Addition of a large excess of CAF-1 over RFC could not compensate the inhibitory effect of RFC (data not shown). This excluded the possibility that RFC might be a competitive inhibitor of CAF-1 or that RFC might block CAF-1 activity by direct binding. Furthermore, RFC did not inhibit the supercoiling reaction itself, and it did not increase nicking of DNA (compare lanes 27 and 28 with 22, 23, or 24).

PCNA Associates with Replicated DNA, and Removal of PCNA Leads to Loss of Template Marking

A plausible explanation for the result shown in Figure 3 is that the loss of CAF-1-mediated chromatin assembly was due to the removal of PCNA from replicated DNA by RFC. To examine this possibility, the replicated DNA was isolated by spin column chromatography, incubated in the presence or absence of RFC (as in Figure
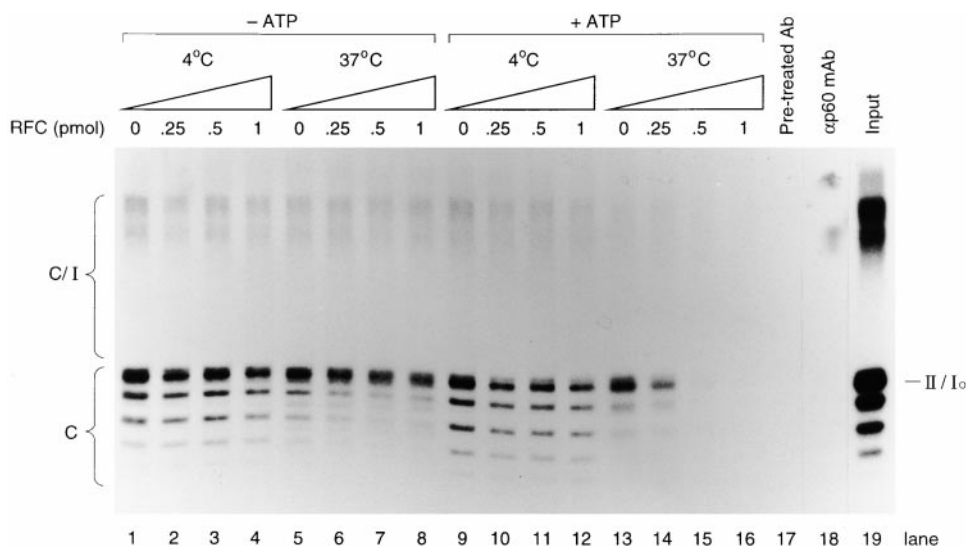
Figure 4. Removal of PCNA from Replicated DNA by RFC and ATP

SV40 DNA replication reactions, separation by spin column chromatography, and preincubation reactions with RFC were performed as described in Figure 3. Immunoprecipitations of DNA were performed with beads containing anti-PCNA antibodies at 4°C. Precipitated DNA was then isolated by protease treatment and extraction with phenol and subjected to agarose gel electrophoresis. Anti-PCNA antibodies beads blocked with a large excess of PCNA (pretreated Ab) or anti-CAF-1 p60 monoclonal antibody beads (αp60mAb) were used as negative controls (lanes 17 and 18). The same amount of replicated DNA (without RFC treatment) without immunoprecipitation was also loaded (Input, lane 19). Covalently closed circular DNA (C) and catenated dimers or DNA replication intermediates (C/I) are labeled.

3), and then the products were immunoprecipitated with anti-PCNA antibodies. The precipitated DNA was detected by agarose gel electrophoresis and autoradiography (Figure 4).

Covalently closed circular DNA (marked "C") as well as catenated dimers or replication intermediates (marked "C/I") were both precipitated by PCNA antibodies. However, the amount of immunoprecipitated DNA was severely reduced when the DNA was preincubated with increasing amounts of RFC in the presence of ATP at 37°C, demonstrating that RFC removed PCNA from the replicated DNA (Figure 4, lanes 1–16). PCNA antibodies blocked by an excess of PCNA, or anti-CAF-1-p60 antibodies, were used as controls, and neither precipitated significant amounts of the replicated DNA. This confirmed that the immunoprecipitation of labeled DNA was PCNA dependent (Figure 4, lanes 17 and 18). Taken together, these results demonstrated that PCNA remained associated with the completely replicated DNA and that RFC dissociated PCNA from the closed circular DNA in an ATP- and temperature-dependent manner.

The total radioactivity corresponding to completely replicated DNA (marked "C" in Figure 4) was quantitated using a Phosphor Imager. Using the value from lane 17 as background, the total radioactivity was reduced to 8.9% and 0.9% in lanes 15 and 16, respectively, of the amount present in lane 1 (set to 100%), while 72.9% and 48.1% were present in lanes 13 and 14, respectively. Dissociation of half of the PCNA from the replicated DNA did not affect coupled chromatin assembly (Figure 3, lane 26), but the severe reduction of PCNA resulted in the loss of CAF-1-mediated chromatin assembly (Figure 3, lanes 27 and 28). This result suggested that more PCNA was loaded onto the replicating DNA than was needed for CAF-1-coupled chromatin assembly. Indeed, only one small target site for NER was sufficient to start

CAF-1-dependent chromatin assembly, and once started, it proceeded to create a regular nucleosome array on the whole substrate DNA (Gaillard et al., 1997).

PCNA Antibodies Inhibit CAF-1-Mediated Chromatin Assembly
To further examine the involvement of PCNA, we tested whether anti-PCNA human autoantibodies could affect CAF-1-dependent chromatin assembly on replicated DNA. Chromatin assembly reactions were performed with isolated, replicated DNA, CAF-1, and S100 extract either in the presence or absence of anti-PCNA antibodies. Then the products were partially digested with micrococcal nuclease. As shown in Figure 5, replicated DNA incubated both with CAF-1 and S100 extract in the absence of sera was cleaved into a nuclease-resistant ladder of bands, indicating that an array of nucleosomes had been formed on the DNA in a CAF-1-dependent manner (Figure 5, lanes 1 and 2). The ladder of bands was also present when the replicated DNA was preincubated with nonautoimmune sera (Figure 5, lanes 5 and 8). In contrast, two human sera containing PCNA autoantibodies reduced ladder formation and thus inhibited CAF-1-dependent chromatin assembly (Figure 5, lanes 3 and 4). Depleting PCNA antibodies from the sera by prior mixing with PCNA-coupled beads blocked the inhibitory effect of these sera (Figure 5, lanes 6 and 7). These results suggested that the PCNA antibodies inhibited CAF-1-mediated chromatin assembly by masking PCNA that was associated with the replicated DNA. Together with the experiments shown in Figures 3 and 4, we conclude that PCNA is essential for coupling chromatin assembly by CAF-1 to DNA replication. One possible mechanism for PCNA to be involved in this process is for PCNA to function by interaction with CAF-1.
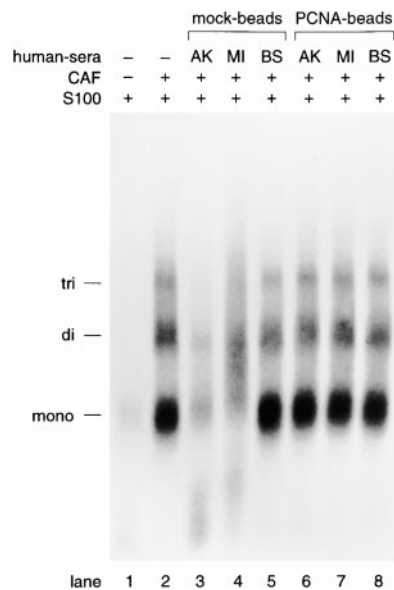
Figure 5. Inhibition of CAF-1-Mediated Chromatin Assembly by PCNA Antibodies

Replicated DNA prepared as in Figure 3 was premixed with buffer (lanes 1 and 2), human sera from autoimmune patients, AK (lanes 3 and 6), MI (lanes 4 and 7), or control human sera BS (lanes 5 and 8). The sera was also pretreated with PCNA coupled beads (lanes 6–8) or beads only (lanes 3–5), and depletion of PCNA reactive antibodies from AK and MI was very effective (data not shown). Chromatin assembly either with or without human S100 extract and recombinant CAF-1 is indicated by + or −. Although an equal amount of DNA was used for reactions in each lane, small DNA products were not trapped in the gel during drying. Reaction products were digested with 6 U of micrococcal nuclease at room temperature for 25 min. DNA was analyzed on 2.0% agarose gel. The migration of mononucleosome-, disome-, and trisome-length material is indicated.

Direct Interaction between CAF-1 and PCNA

Antibodies directed against either the p150 or p60 subunits of CAF-1 coimmunoprecipitated PCNA only in the presence of CAF-1, whereas anti-SV40 T antigen antibodies, used as a control, precipitated only background amounts of PCNA in a CAF-1-independent manner (Figure 6A). Moreover, anti-PCNA antibodies coimmunoprecipitated CAF-1 only in the presence of PCNA (Figure 6B). In the latter case, the efficiency of coimmunoprecipitation was low, even in the presence of an excess of PCNA compared to CAF-1, consistent with the observation presented in Figure 5 that these antibodies block CAF-1-dependent chromatin assembly on replicated DNA. Nevertheless, both results demonstrated that PCNA and CAF-1 directly bind to each other in vitro.

Binding assays using the separate p150, p60, or p48 CAF-1 subunits were performed to determine which subunits interacted with PCNA. Each CAF-1 subunit was synthesized by in vitro translation, and immunoprecipitation reactions in the presence or absence of PCNA were performed using anti-PCNA antibodies (Figure 6C). Only the p150 subunit interacted with PCNA, and this interaction was very efficient. Since both PCNA and p150 are very acidic proteins, with calculated pI of 4.60 and 5.65, respectively, the binding of PCNA and p150
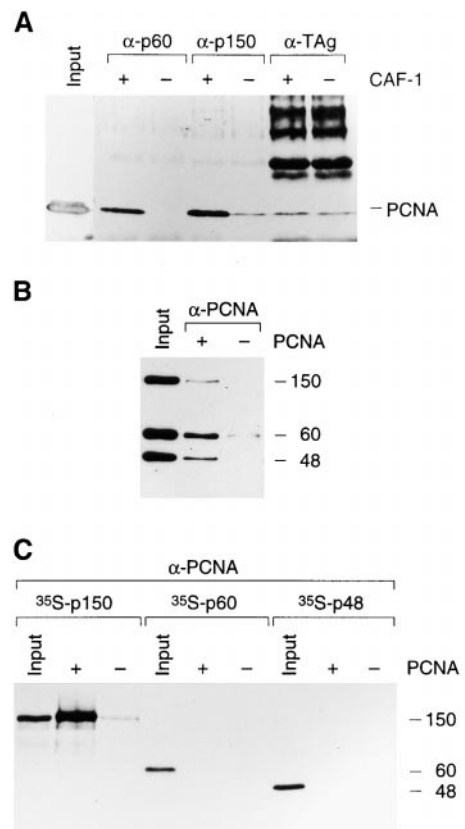

Figure 6. CAF-I and PCNA Directly Bind to Each Other

(A) Coimunoprecipitation of PCNA and CAF-1 with anti-CAF-1 antibody. Purified PCNA (105 pmol) was incubated with purified CAF-1 (7.5 pmol; +) or buffer only (−) and immunoprecipitated by anti-p60 mAb (SS24), anti-p150 mAb (SS1), or anti-TAg mAb (pAb 419). Coprecipitated PCNA was detected by Western blotting with anti-PCNA mAb (PC10).

(B) Coimunoprecipitation of CAF-1 and PCNA with anti-PCNA antibodies. Purified CAF-1 (7.5 pmol) was incubated with purified PCNA (105 pmol; +) or buffer only (−) and immunoprecipitated with anti-PCNA antibodies. Coprecipitated CAF-1 was detected by Western blotting with a mixture of anti-p150 mAb (SS1), anti-p60 mAb (SS24), and anti-p48 mAb (15G12).

(C) Binding of PCNA to the p150 subunit of CAF-1. In vitro translated p150, p60, and p48 were mixed with purified PCNA (50 pmol; +) or buffer only (−) and immunoprecipitated with anti-PCNA antibodies. The precipitated proteins were subjected to SDS-PAGE and autoradiography. Twenty percent of the proteins used in each binding assay was loaded as a marker (Input).

was not likely to be due to nonspecific electrostatic interactions.

Colocalization of CAF-1 with PCNA in Proliferating Cells

To examine the physical proximity of CAF-1 and PCNA in vivo, asynchronously growing HeLa cells were stained with antibodies directed against the CAF-1 p150 subunit or PCNA. Only a fraction of the cells exhibited a punctuate pattern of detergent-insoluble p150 and PCNA in the nucleus. Furthermore, PCNA and CAF-1 exactly colocalized in complexes that were tightly associated with chromatin, as judged from their resistance to Triton extraction (Figure 7). Both PCNA and CAF-1 have independently been localized to replication foci in S phase
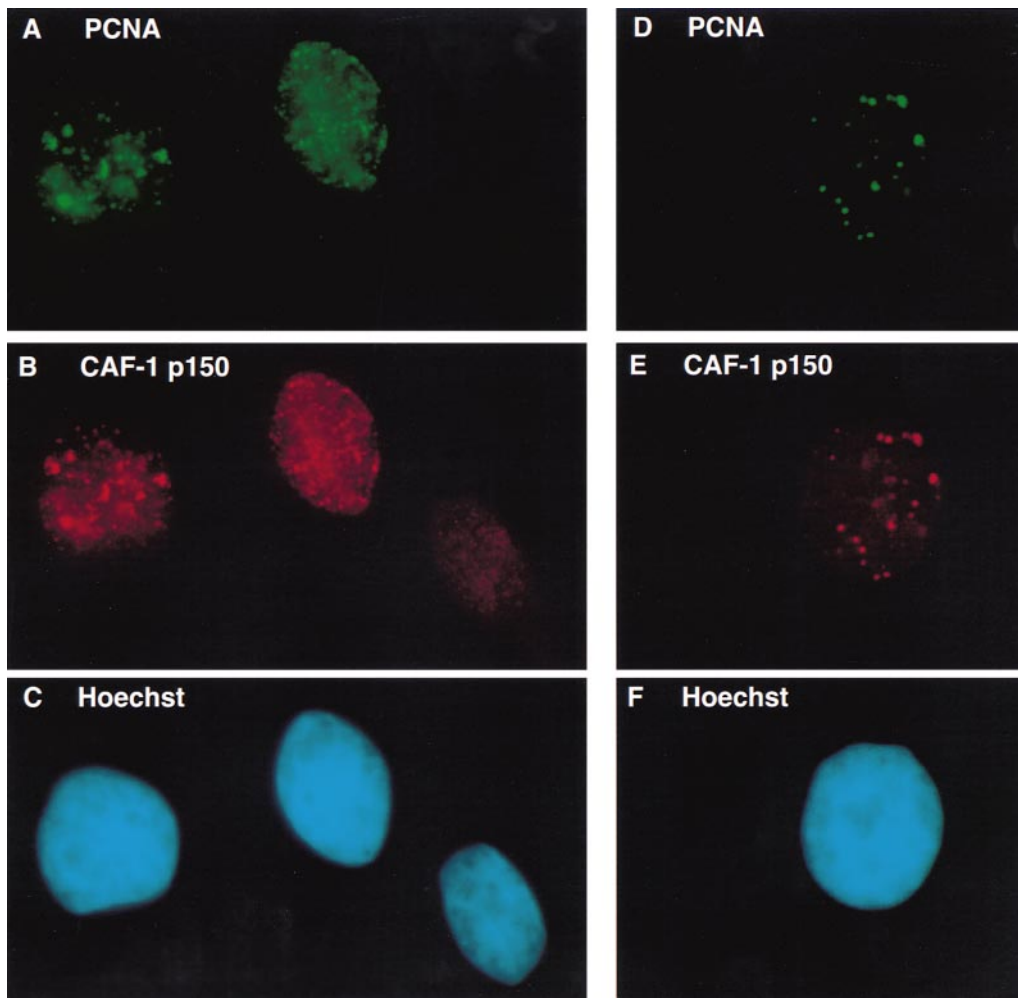
Figure 7. Colocalization of PCNA and CAF-1 in Cells

Asynchronous HeLa cells were stained with anti-PCNA antibodies, followed by a fluorescence isothiocyanate (FITC)-conjugated second antibody (A and D); with anti-CAF-1-p150 monoclonal antibody, followed by a Texas red–conjugated second antibody (B and E); and stained with Hoechst dye 33258 (C and F). Cells were pretreated with Triton X-100 before fixation with formaldehyde. Cells in early to mid S phase are shown in (A), (B), and (C), while cells in late S phase are shown in (D), (E), and (F). No immunofluorescence signal was detected when primary antibodies were omitted.

(Bravo and MacDonald-Bravo, 1987; Krude, 1995). Thus, based on the PCNA staining patterns, these complexes correspond to DNA replication foci and could be seen in early, mid, or late S phase (Figures 7A, 7B, 7D, and 7E). We conclude that PCNA and CAF-1 colocalize to sites of DNA replication in cells. Similar colocalization of PCNA and CAF-1 was observed in HeLa cells after UV irradiation (Martini et al., 1998).

These observations, coupled with the results in Figure 6, suggest that PCNA interacts with CAF-1 in vivo during S phase. In addition, complete colocalization of PCNA and CAF-1 in early, mid, and late S phase suggests that CAF-1 participates in replication-coupled chromatin assembly for the whole genome, not just for heterochromatic regions. A low amount of CAF-1 was observed in the cells that were not in S phase (e.g., the right cell in Figure 7B), and the nature of this association is under investigation.

Discussion

CAF-1- and PCNA-Dependent Chromatin Assembly

CAF-1 assembles nucleosomes during DNA replication in vitro on both the leading and lagging strands behind a replication fork (Stillman, 1986; Smith and Stillman, 1989). Nucleosome assembly is an ordered process, with histones H3 and H4 first loaded onto the DNA during replication in a CAF-1-dependent manner, and soon thereafter, histones H2A and H2B are added to form a mature nucleosome (Stillman, 1986; Smith and Stillman, 1991b). This is most likely how the bulk of de novo nucleosome assembly occurs during S phase (reviewed by DePamphilis and Wassarman, 1980; Grunstein, 1998). In the current paper, however, we demonstrated that nucleosome assembly could be temporally dissociated from passage of the DNA replication fork. Addition of CAF-1 after completion of DNA replication allowed
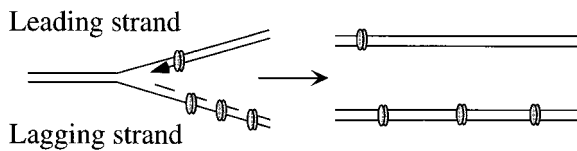
Figure 8. Asymmetric Marking of Sister Chromatids by PCNA
Because PCNA can remain associated with replicated DNA, the possibility exists that PCNA may asymmetrically and nocovalently mark the two sister chromatids.

nucleosome assembly on the replicated DNA, but not on the unreplicated DNA that was present in the same reaction. Thus, the replicated DNA was marked or imprinted for subsequent CAF-1-dependent processes. It was suggested that a component of the DNA replication fork mediated CAF-1 function (Smith and Stillman, 1989; Kelman and Hurwitz, 1998), and it now seems likely that PCNA is that factor.

The PCNA clamp is involved in the synthesis of both the leading and lagging strands at the DNA replication fork (Hübscher et al., 1996; Jonsson and Hübscher, 1997; Kelman and Hurwitz, 1998; Waga and Stillman, 1998). On the leading strand, PCNA associates with DNA polymerase δ and promotes continuous synthesis of long DNA strands in a processive manner. Thus, one PCNA clamp is needed per initiation event (Figure 8). On the other hand, during lagging-strand synthesis, DNA replication occurs by the discontinuous production of Okazaki fragments, a process that involves a PCNA-dependent polymerase switching mechanism (Tsurimoto et al., 1990; Waga and Stillman, 1994). In this case, one clamp needs to be loaded every Okazaki fragment or approximately every 100–200 bp (Figure 8). On both strands, PCNA is loaded by RFC at a primer–template DNA junction that is later recognized by the DNA polymerase. Analogous mechanisms exist for replication of the *E. coli* chromosome and the bacteriophage T4 chromosome (Nossal, 1992; Stillman, 1994; Kelman and Hurwitz, 1998). In all of these cases, when the polymerase completes DNA synthesis, it dissociates from the DNA and leaves the ring-shaped clamp topologically linked to the duplex, replicated DNA (Hacker and Alberts, 1994; Stukenberg et al., 1994). This implies that the rate-limiting step for removal of PCNA from the replicated DNA, and hence loss of the replication "imprint," is the ATP-dependent unloading of PCNA by RFC. Thus, RFC has the potential to regulate the duration of the PCNA marking on replicated DNA. We suggest that this might occur in a locus-specific manner in the genome, providing a mechanism for gene-specific modulation of chromatin structure.

We suggest that CAF-1-mediated nucleosome assembly normally occurs on both the leading and lagging strands immediately after passage of the DNA replication fork, as occurs in vitro when CAF-1 is present during DNA replication. Indeed, CAF-1 localizes to the sites of DNA replication in cells (Figure 7; Krude, 1995). But the data described here suggest that an alternative, post-replicative mechanism may also operate that could provide opportunities for asymmetric inheritance of chromatin states. If the PCNA that was used for Okazaki fragment synthesis were to remain associated with the lagging-strand product for some time after DNA replication, the amount of PCNA bound to the two sister chromatids would be inherently asymmetric (Figure 8). Since CAF-1 binding to PCNA can allow chromatin assembly after DNA replication, this situation would offer considerable opportunities for the establishment of an asymmetric chromatin structure on the two sister chromatids prior to division of proliferating stem cells. Such chromatin complexes, if inherited into the daughter cells, could provide the foundation for phenotypic asymmetry of sister cells during development.

One attractive feature about the cooperation between PCNA and CAF-1 in mediating chromatin assembly is the involvement of PCNA in other events that occur on DNA. PCNA is involved in a number of DNA repair pathways (Kelman and Hurwitz, 1998), and we have shown that nucleotide excision repair can be accompanied by CAF-1-dependent chromatin assembly (Gaillard et al., 1996). This type of chromatin assembly occurs bidirectionally from the site of DNA repair (Gaillard et al., 1997). Thus, it seems likely that CAF-1-dependent chromatin assembly does occur, even outside of S phase. We propose that genetically silent DNA synthesis, such as DNA synthesis started at regulated sites of DNA strand breaks, could be a major mechanism for chromatin modification in both proliferating and nonproliferating cells. For example, the DNA strand breaks initiated at the mating type locus in *S. cerevisiae* may provide one such example.

Links between DNA Replication, Replication-Coupled Chromatin Assembly, and Epigenetic Inheritance
Although chromatin states must normally be inherited to both daughter cells, it has long been suspected that DNA replication provides a window of opportunity for changes in chromatin structures that might affect gene expression (Brown, 1984; Svaren and Chalkley, 1990; Wolffe, 1991). Indeed, replication-coupled chromatin assembly suppresses basal transcription (Almouzni and Wolffe, 1993). Recent studies in the budding yeast *Saccharomyces cerevisiae* showed that CAF-1 is required for the stable inheritance of transcriptionally repressed chromatin structures at telomeres and *HM* loci (Enomoto et al., 1997; Kaufman et al., 1997; Monson et al., 1997; Enomoto and Berman, 1998). In the absence of CAF-1, the expression of genes near the telomeres is variegated in a population of cells. We suggest that the recruitment of CAF-1 by PCNA is required for suppression of this form of position effect variegation.

Support for a role for PCNA in suppression of position effect variegation comes from genetic studies in *Drosophila*. A mutant in *Drosophila* PCNA, *mus209*, is a suppressor of position effect variegation (Henderson et al., 1994) and exhibits DNA repair defects that overlap with the repair defects seen in yeast lacking CAF-1 (Kaufman et al., 1997). The *mus209* mutant also causes strong enhancement of homeotic transformation in a *trans*-heterozygous *crm; mus209* mutant (Yamamoto et al., 1997). The *cramped* (*crm*) gene is a *Polycomb*-group (Pc-G) gene, and some *crm* mutants show suppression

of position effect variegation. PCNA and the CRM protein colocalize in nuclei of proliferating cells. These phenotypes may reflect the role of PCNA in promoting efficient coupling between DNA replication and chromatin assembly.

The inheritance of epigenetically determined states in budding and fission yeasts may also occur by similar mechanisms. In the fission yeast *S. pombe*, replication proteins such as DNA polymerase α, chromatin proteins Swi6 and Clr4, and histone deacetylases are involved in the epigenetic inheritance of the mating type silent chromatin and centromeric heterochromatin (Ekwall et al., 1997; Grewal et al., 1998; Ivanova et al., 1998; reviewed by Klar et al., 1998). It is possible that PCNA and CAF-1 mediate these effects. In the budding yeast *S. cerevisiae*, position effect variegation occurs at telomeres (Gottschling et al., 1990; Aparicio et al., 1991), and the establishment of chromatin repression at the mating type loci requires passage through S phase (Miller et al., 1984). Furthermore, heterochromatin at the mating type loci (*HM*) is uniquely acetylated at lysine-12 of histone H4 (Braunstein et al., 1996), and a variety of mutations in the acetylated lysines of histones H3 and H4 result in defects in silencing at *HM* loci and telomeric reporter genes (Grunstein, 1998). Since CAF-1 associates with specific forms of acetylated histones (Verreault et al., 1996), the PCNA-CAF-1-linked chromatin assembly may be involved in assembly of heterochromatin and epigenetically determined chromosomal states.

PCNA interacts with the DNA-(cytosine-5)-methyltransferase at sites of DNA replication in mammalian cells, and this interaction likely mediates the inheritance of DNA methylation patterns (Chuang et al., 1997). We suggest that the PCNA molecules that remain associated with the replicated DNA might provide a common platform for both chromatin assembly and for DNA methylation. In some situations, this could allow preferential methylation of one sister chromatid over the other, as we have proposed for chromatin assembly.

Bacteriophage T4

Bacteriophage T4 encodes a protein called gp45 that functions like PCNA during DNA replication (Nossal, 1992). Interestingly, this DNA polymerase clamp mediates activation of transcription of genes whose expression is dependent on prior DNA replication (Herendeen et al., 1989). In this case, the sliding clamp that is placed onto the DNA by events at the DNA replication fork then functions as a mobile enhancer of gene transcription. Since we have shown that PCNA can remain associated with the DNA after DNA synthesis (either repair of replication), we wonder if a similar mechanism of enhancing gene transcription might occur in eukaryotic cells.

Future Perspectives

It was found recently that mutations in the *HIR* genes in *S. cerevisiae*, which regulate histone gene expression, act synergistically with mutations in the *CAC* genes to reduce gene silencing by heterochromatin at telomeres and the silent *HM* loci. The same *hir* mutations had no significant effect in the absence of *cac* mutations (Kaufman et al., 1998; Qian et al., 1998). This suggests

the existence of an alternative chromatin assembly pathway, which is acutely sensitive to histone levels in cells with both *cac* and *hir* mutations. It will be interesting to determine whether this CAC-independent pathway for chromatin assembly is also coupled to DNA replication via PCNA.

Experimental Procedures

SV40 DNA Replication and Purification of Replicated DNA by Spin Column Chromatography

SV40 DNA replication reactions were performed as described previously (Stillman, 1986; Smith and Stillman, 1989). The S100 extract from suspension cultures of human 293 cells, SV40 T antigen, and the plasmid DNA pSV011$^+$ were described previously (Stillman and Gluzman, 1985).

After DNA replication for 40 min and addition of aphidicolin (7.5 μg/ml), 50 μl of the reaction mixture was applied to a Miniprep Spun Column (Pharmacia Biotech) preequilibrated with 40 mM HEPES-KOH (pH 8.0), 0.5 mM DTT. The column was centrifuged 400 g for 2 min at 4°C in a swinging bucket rotor. The effluent after centrifugation, just as in prespun, was used for the following assays or stored at −70°C.

Supercoiling Assay with Replicated DNA Purified by Spin Column Chromatography

Effluent from a spin column (7.5 μl) was mixed with CAF-1 (100 fmol), S100 extract from human 293 cells (56 μg protein), and topoisomerases I (13 ng) and II (100 ng). The supercoiling reaction was performed at 37°C for 45 min (Figures 1, 2, and 5) or 35 min (Figure 3), and for another 15 min (Figures 1, 2, and 5) or 10 min (Figure 3) after addition of 400 ng of histones H2A.H2B dimers purified from human 293 cells (Simon and Felsenfeld, 1979). These histones H2A.H2B were added because the human S100 extract contains substoichiometric amounts of those histones compared with histones H3.H4. DNA was subjected to agarose gel electrophoresis after treatment with RNase A and self-digested pronase as described previously (Stillman, 1986).

Almost half of the ^{32}P-labeled DNA was recovered after the spin column separation, while less than 2% of thyroglobulin with a mass of 669 kDa was recovered, indicating that the separation was sufficient to remove most soluble proteins from the DNA. Further, no significant DNA synthesis was observed by dAMP incorporation assay, and no significant nucleotides were eluted.

Protein Purification and Antibodies

Recombinant human CAF-1 was purified from Sf9 insect cells. Recombinant baculoviruses expressing each of the three subunits of CAF-1 were used in a coinfection at a multiplicity of infection of 25, 12.5, and 10 for p150, p60, and p48, respectively. The infections were allowed to proceed for 44 hr at 27°C. CAF-1 was purified essentially as described (Kaufman et al., 1995; Verreault et al., 1996). Recombinant human RFC from insect Sf9 cells (Ellison and Stillman, 1998), recombinant human PCNA from *E. coli* (Fien and Stillman, 1992), and topoisomerase I and II from calf thymus (Tsurimoto and Stillman, 1989) were prepared as described.

Anti-PCNA human autoantibodies (AK and MI) were a generous gift from Dr. Eng M. Tan, Scripps Research Clinic. Control human sera was obtained from B. S. Anti-PCNA monoclonal antibody (PC10) was purchased from Pharmingen. Anti-CAF-1 p150 and p60 monoclonal antibodies were described previously (Smith and Stillman, 1991a). Anti-CAF-1-p48 monoclonal antibody was prepared from the hybridoma cell line 15G12, which was obtained from Dr. Eva Lee (Qian and Lee, 1995). Anti-SV40 T antigen monoclonal antibody (mAb419) was obtained from the Cold Spring Harbor Laboratory monoclonal antibody facility.

Immunoprecipitation of DNA with Human Antiserum Containing PCNA Antibodies

The replicated DNA preincubated with RFC was mixed with anti-PCNA antibodies (AK) and 10 μl of 50% slurry of GammaBind G-Sepharose (Pharmacia) at 4°C for 25 min. The beads were washed

four times quickly at 4°C with 600 μl of precooled buffer A150 (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10% glycerol, 150 mM NaCl, 0.01% NP-40). After washing, the beads were incubated with 2 μg/ml of self-treated pronase in 100 μl of 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.5% SDS at 37°C for 1 hr. The DNA was analyzed by electrophoresis in a 1.0% agarose gel after extraction with phenol-chloroform. The radioactivity was quantified by Fuji phosphoimager. As negative controls, human serum beads preincubated with 7.7 μg of human recombinant PCNA at 4°C for 1 hr and washed four times with 600 μl of buffer A150, and 10 μl of 50% slurry of anti-p60 mAb cross-linked with GammaBind G-Sepharose were used.

Micrococcal Nuclease Assay
Chromatin assembly reaction mixtures were digested with 6 Worthington units of micrococcal nuclease in the presence of 3 mM CaCl₂ at room temperature for 25 min. DNA was analyzed on 2.0% agarose gel after self-treated pronase as described previously (Stillman, 1986).

Purified PCNA (300 μg) was coupled with 50 μl of activated beads as described in the manufacture's protocol (Affi-Gel 15, Bio-Rad). Sera (2.5 μl) was mixed with 15 μl of PCNA beads at 4°C for 90 min, and supernatant was added to chromatin assembly reactions. Control beads blocked by 1 M ethanolamine (pH 8) were used as mock beads.

Coimmunoprecipitation Assays
Indicated amounts of recombinant CAF-1 and recombinant human PCNA were mixed in buffer A100 (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, 10% glycerol, 100 mM NaCl, 0.01% NP-40) with 10 μl of 50% slurry of anti-p150 mAb (SS1), anti-p60 mAb (SS24), and anti-T antigen mAb (mAb419) cross-linked with GammaBind G-Sepharose. After incubation at 4°C for 1 hr, the beads were washed five times with 600 μl of buffer A100 and subjected to SDS-PAGE and Western blotting analysis.

In vitro transcription/translation reactions for expression of full-length p150, p60, p48 proteins were performed using the TNT T7 Quick coupled reticulocyte lysate system (Promega). Amounts of full-length proteins were normalized per volume of lysate by analysis of SDS-polyacrylamide gels on a Fuji phosphoimager. Then translated proteins and purified PCNA were mixed with human sera and 10 μl of a 50% slurry of GammaBind G-Sepharose (Pharmacia) in buffer A100 at 4°C for 2 hr. The beads were washed four times with 600 μl of buffer A100 and subjected to SDS-PAGE analysis.

Immunofluorescence Staining
HeLa cells growing on glass coverslips were extracted with hypotonic buffer containing 0.1% Triton as described (Li et al., 1996). After the extraction, the cells were fixed in 2% formaldehyde for 15 min at room temperature. Then the cells were incubated with the primary antibodies for 90 min and the second antibodies (FITC- or Texas red–conjugated goat anti-human Ig G or goat mouse Ig G antibodies) for 30 min at room temperature. Samples were examined and photographed as described before (Li et al., 1996).

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