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FAST TRACK

Targeting of PCNA to Sites of DNA Replication in the Mammalian Cell Nucleus

Suryanarayan Somanathan, Thomas M. Suchyna, Alan J. Siegel, and Ronald Berezney*

Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260

Abstract We have examined the targeting of proliferating cell nuclear antigen (PCNA), an integral component of the mammalian replicative enzyme DNA polymerase δ, with sites of DNA replication by using confocal microscopy and computer image analysis. Labeling (5 min pulse) of DNA replication sites in normal human diploid fibroblast cells (NHF1) with BrdU was followed by immunostaining with PCNA antibodies. A striking degree of colocalization was seen between PCNA and the characteristic patterns of DNA replication sites of early, middle and late S-phase (Nakayasu and Berezney [1989] J. Cell. Biol. 108:1–11). These observations were confirmed by quantitative computer image analysis which revealed that approximately 90% of the PCNA-stained area overlapped with DNA replication sites in early S-phase. Pulse-chase experiments, involving in vivo labeling for replication followed by PCNA staining at later time points, suggested that PCNA disassembles from previously replicated sites and targets to newly active sites of DNA replication. To further study this phenomenon in living cells, stable GFP-PCNA transfectants under the control of a tetracycline-inducible promoter were created in mouse 3T6 cells. Like the endogenous PCNA, GFP-PCNA targeted to sites of replication (approximately 80% colocalization) and demonstrated similar dynamic changes following pulse-chase experiments in fixed cells. Studies of living cells revealed progressive changes in the GFP-PCNA distribution that mimic the replication patterns observed in fixed cells. We conclude that GFP-PCNA targets to DNA replication sites in living cells and is an effective marker for tracking the spatio-temporal dynamics of DNA replication as cells transverse the S-phase. J. Cell. Biochem. 81:56–67, 2001.

Key words: DNA replication sites in living cells; normal diploid human fibroblasts; confocal microscopy; computer image analysis; green fluorescent protein

In recent years research into the structural organization of the nucleus has pointed toward the existence of regions demarcated for the various nuclear processes including replication, transcription and RNA splicing [Berezney and Wei, 1998]. Several studies have demonstrated that during early S-phase of the cell cycle BrdU incorporation into mammalian cells stains a large number of punctate spots distributed throughout the nucleus [Nakamura et al., 1986; Bravo and Macdonald-Bravo, 1987; Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Mazzotti et al., 1990; Fox et al., 1991]. The replication foci change to a more perinuclear pattern during mid S- and onto a few large granular spots in late S-phase. Also termed "replication factories," these foci are regions in the nucleus where specific enzymes and replicational factors; such as the DNA polymerases, PCNA, RPA, RFC, DNA ligases, and DNA methyltransferases assemble to mediate DNA replication [Leonhardt and Cardoso, 1985; Leonhardt et al., 1992; Cook 1999]. The changes in the spatial and temporal organization of these replication sites are thought to reflect DNA synthesis that proceeds from copying transcriptionally active euchromatic DNA in early S to relatively inactive heterochromatic DNA later in S [Ma et al., 1998].

Abbreviations used: BrdU, 5-Bromodeoxyuridine; GFP, green fluorescent protein; NHF1, normal human fibroblast 1; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A.

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Thomas M. Suchyna's current address is Department of Physiology and Biophysics, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, NY 14214.

The first two authors equally contributed to the work.

*Correspondence to: Dr. Ronald Berezney, Department of Biological Sciences, State University of New York at Buffalo, Buffalo, NY 14260. E-mail: berezney@buffalo.edu

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The processes leading to the replication of the genome also involve the systematic association and dissociation of various enzymes and factors at the replication fork [Dutta and Bell, 1997; Tye, 1999]. Thus changes in the organization of the replication foci should reflect corresponding changes in the nuclear distribution of replicative factors. Proliferating cell nuclear antigen (PCNA), is a highly regulated protein necessary for cell cycle progression [Baserga, 1991]. It was initially described as a nuclear antigen in proliferating cells that reacted with the sera of patients with the autoimmune disorder systemic lupus erythematosus [Miyachi et al., 1978]. Bravo and Celis found a protein whose synthesis appeared to correlate with the S-phase of the cell cycle which they subsequently designated "cyclin" [Bravo and Celis, 1980; Bravo et al., 1982]. However, later studies revealed that PCNA was identical to cyclin [Mathews et al., 1984] and functions as a processivity factor for polymerase δ during DNA replication in vitro [Tan et al., 1986; Bravo et al., 1987; Prelich et al., 1987b].

With an apparent molecular mass of 36 kDa, PCNA is believed to be an integral component of the eukaryotic replication machinery based on its requirement for in vitro leading strand synthesis of simian virus 40 (SV 40) DNA [Prelich et al., 1987a; Prelich and Stillman, 1988]. In addition, depletion of PCNA from cells using anti-sense oligonucleotides or microinjected antibodies revealed that the protein was essential for in vivo cellular DNA replication [Jaskulski et al., 1988; Zuber et al., 1989]. Although inherently lacking the ability to bind DNA, a homotrimeric complex of PCNA can be loaded onto DNA at the primer–template junction in an ATP-dependent manner by the multi-protein complex-replication factor C (RFC) [Tsurimoto and Stillman, 1990, 1991; Lee et al., 1991; Cai et al., 1996]. Similar to its bacterial counterpart, the dimeric β-subunit complex of pol III, the homotrimeric complex of PCNA is proposed to form a closed ring structure with a hole in the center that encircles the DNA [Waga and Stillman, 1998].

Earlier studies on the distribution of PCNA in the nucleus revealed the existence of two populations during the cell cycle [Bravo and Macdonald-Bravo, 1987]. A soluble form of the protein sensitive to fixation with organic solvents was found to exist throughout all stages of the cell cycle. However, a detergent-resistant insoluble form of PCNA appeared only during the S-phase and was highly phosphorylated in human fibroblasts suggesting that this post-translational modification could be essential in binding to chromatin, during the transition from G1 to S-phase [Prosperi et al., 1994]. The soluble form of PCNA was hypophosphorylated and immunofluorescence microscopy showed diffuse staining throughout the nucleus. In contrast, the spatial distribution of the insoluble form of PCNA closely resembled those of replication foci [Bravo and Macdonald-Bravo, 1987].

The fact that staining patterns of the insoluble form of PCNA appeared similar to those of DNA replication sites implied that the protein associated at sites of replication [Madsen and Celis, 1985; Bravo, 1986]. Direct visualization of colocalization between replication sites and PCNA, however, was hampered by procedural constraints in BrdU detection involving DNA denaturation. This precluded doing double immunofluoresence staining for PCNA and BrdU in the same cell. Kill et al. [1991] attempted to surmount this problem by using biotin-11-dUTP as an analog, which had earlier been shown to label sites of replication in permeabilized cell systems [Nakayasu and Berezney, 1989]. The extent of colocalization of PCNA with replication sites in this study, however, was not complete [Kill et al., 1991]. Humbert et al. [1992] used a nuclease detection system for BrdU incorporation sites along with double channel confocal microscopy. The results are consistent with significant levels of colocalization of PCNA with replication sites but precise quantitation of colocalization was not performed.

Owing to these technical difficulties the association of PCNA at sites of active replication in intact cells remains to be clarified. To directly address this question, we have employed a modified double labeling procedure along with laser scanning confocal microscopy and computer image analysis to quantitate the colocalization between PCNA and replication sites in normal diploid mammalian cells. We then studied the localization of GFP-PCNA constituents in live cells undergoing DNA replication and monitored the dynamic rearrangements of the expressed protein in real time. We demonstrate in this study that PCNA associates at sites of replication throughout S-phase. In

addition, we find that PCNA dissociates from sites where replication has concluded and dynamically targets to sites of ongoing replication. The GFP-PCNA fusion construct targeted to sites of replication throughout S-phase of living cells and followed dynamics similar to that observed with the endogenous PCNA.

MATERIALS AND METHODS

Cell Culture

Mouse 3T6 fibroblasts (American Type Culture Collection [ATCC], Rockville, MD) were grown as monolayers in DMEM supplemented with 10% fetal bovine serum. Cells were grown at 37°C with 5% CO_2 and diluted ~1:40 at each passage. Normal diploid human fibroblast (NHF1) cells were a gift from Dr. David G. Kaufman at the University of North Carolina at Chapel Hill and were grown as indicated above.

Selection of a Cell Line Stably Transfected with a GFP-PCNA Construct

The methods of Gossen and Bujard [Gossen and Bujard, 1992; Gossen et al., 1995] were applied to obtain a stable cell line in mouse 3T6 cells where expression of GFP-PCNA is tightly regulated by use of a tetracycline-inducible system. This involved consecutive stages of transfection and selection to first generate stable cell lines expressing the reverse tetracycline transactivator (rtTA) and later the GFP-PCNA construct. Major steps in this strategy are described below.

Construction of pUHD(GFP-PCNA)10-3 plasmid. pUHD 10-3 response vector, containing the minimal promoter-tetracycline operator PhCMV*-1 of the tetracycline regulatory system, was graciously provided by Dr. Hermann Bujard of the University of Heidelberg, Germany. pEGFP N-1 vector containing the S65T GFP mutant cDNA was obtained from Clonetech [Palo Alto, CA]. pCG4 vector containing a 1.4 kb human PCNA cDNA insert was kindly provided by Dr. Renato Baserga of Thomas Jefferson University, Philadelphia, PA. pUHD 10-3 and pEGFP N-1 were digested with *Eco*R1 and *Xba*1 to produce a 3.15 kb pUHD 10-3 fragment cut in the multiple cloning site region, and a 0.7 kb GFP fragment from the pEGFP vector. These fragments were gel-isolated and combined in a ligation reaction to produce the pUHD(GFP) 10-3 vector. A PCNA cDNA insert with a *Sal*1 site at the N-terminus and an *Age*1 site at the C-terminus was produced by PCR amplification of the region between the two primers PCNA-N (5′ ACTGTCTGAGTCGACCTAGACTT TCCTCC-TTCC 3′) and PCNA-C (5′ TGATCACCGG-TTGAGATCCTTCTTCATCC TC 3′) and subsequently gel-isolated. This insert includes the entire PCNA cDNA sequence except for the final TAG stop codon. The PCNA cDNA insert and the pUHD(GFP) 10-3 vector were digested with *Sal*1 and *Age*1 and both were gel-purified. These two fragments were ligated to form the pUHD(PCNA/GFP) 10-3 vector containing a fusion cDNA where GFP is connected to the C-terminus of PCNA. The fusion protein produced by this vector has a five amino acid linker (Gly–Pro–Val–Ala–Thr) between the final Ser residue of PCNA and the initial Met of GFP.

Production of stable rtTA and PCNA/GFP expressing 3T6 cell lines. 3T6 cells were trypsinized and electroporated with 30 ng of pUHD 172-1 vector containing the reverse tetracycline transactivator (rtTA) cDNA and neomycin resistance (pUHD 172-1 provided by Dr. Hermann Bujard). The cells were plated on 6-well multiwell plates at different dilutions, and grown for 24 h in normal medium. Geneticin (0.5 g/ml) was then added followed by growth for 5 more days. Colonies were selected and maintained in selection media containing 0.2 g/ml of geneticin and tested for rtTA expression by secondary transient transfection with pUHD(GFP-PCNA) 10-3 vector. These cells, transiently expressing GFP-PCNA, were plated onto coverslips in multiwell dishes where they were incubated with different levels of doxycyclin (0, 5, 10, 50, 100, and 200 ng/ml) for 24 h. Colonies demonstrating the ability to increase GFP-PCNA expression levels (both fluorescence intensity and number of cells showing fluorescence) with increasing doxycyclin concentration were selected. One such colony (C13) was co-transfected with 5 μg of pUHD(GFP-PCNA) 10-3 vector, which does not express a drug resistance, and 25 ng of a hygromycin resistance vector. Cells were grown in non-selective media for 1 day and then switched to media containing 0.5 mg/ml hygromycin. Colonies were then selected and maintained in media containing 0.2 mg/ml hygromycin and 0.2 g/ml of geneticin. Colonies were tested for increases in GFP-PCNA expression levels in response to doxycyclin (0, 5, 10,

50, 100, 200 ng/ml) by monitoring fluorescence intensity and the percent of fluorescent positive cells. Colony 7-4, which demonstrated a dose-dependent increase in the level of fluorescence upon treatment with doxycyclin, and virtually no fluorescence in its absence, was selected for further studies.

Double Labeling of Replication Site and Endogenous PCNA

Single pulse experiments. Exponentially growing NHF1 were pulsed with BrdU (10 μM) for 5 min. Cells were then fixed in methanol for 30 min at −20°C, washed in phosphate-buffered saline and incubated with anti-PCNA monoclonal antibody (1:10 dilution, Sigma, Madison, WI) for 1 h. Following incubation with appropriate secondary antibodies, cells were briefly fixed with 4% paraformaldehyde for 5 min at room temperature. DNA was then denatured for BrdU detection by incubation with 4N HCl for 10 min on ice. Rat anti-BrdU-monoclonal antibody (1:10 dilution, Sera-Lab) along with biotin-labeled goat anti-rat secondary antibody (1:50 dilution, Jackson ImmunoResearch Laboratories) and Texas red conjugated to strepavidin (1:50 dilution, GIBCO, BRL) was used to label replication sites.

Pulse-chase experiments. Exponentially growing NHF1 cells were pulsed with BrdU for 5 min followed by a chase for 2 h and replication sites and PCNA detected as per the protocol described above.

Immunodetection of Replication Sites in Cells Stably Transfected with GFP-PCNA

The selected cell line (7-4) stably transfected with GFP-PCNA was plated onto glass coverslips and allowed to grow for at least 24 h. Cells were then washed in TBS followed by incubation in glycerol buffer containing 25% glycerol, 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, and 5 mM PMSF for 10 min at room temperature. Cells were then permeabilized in 0.25% Triton X-100 in glycerol buffer for 5 min on ice. Digoxigenin-dUTP incorporation was performed by incubating coverslips in DNA-synthesis buffer containing 25% glycerol, 150 mM NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1.8 mM ATP, 0.5 mM EGTA, 0.1 mM of dATP, dGTP, dCTP, and 25 μM digoxigenin-11-dUTP for 10 min at 37°C. Cells were then washed with TBS buffer containing 0.5% Triton X-100 and fixed with 3% paraformaldehyde for 10 min on ice. Digoxigenin-dUTP incorporation was detected with a mouse anti-digoxigenin antibody (Boehringer-Manneheim) followed by a goat-anti-mouse antibody conjugated to Texas red (Jackson ImmunoResearch Laboratories).

Microscope Stage Incubator for Real Time Studies

7-4 cells were plated onto Bioptech (Bather, PN) 35 mm coverslips and treated with 100 nM doxycyclin for 24 h. A coverslip was then mounted into the Bioptech FCS2 Foeht chamber which was maintained at 37°C and had a media flow through rate of ~12 ml/h produced by a peristaltic pump at its lowest setting. The media containing 100 nM doxycyclin and buffered with 10 nM Hepes at pH 7.2, was incubated in a 37°C water bath and continuously perfused to the Foecht chamber through tubing surrounded by a water jacket maintained at ~37°C.

Confocal Microscopy and Computer Image Analysis

All fixed cell images were produced by a Biorad MRC-1024 confocal microscope system equipped with a krypton-argon laser and Nikon upright (Optophot™) epifluorescence microscope. The objective used was a Zeiss 60X oil immersion planApo with 1.4 numerical aperture. The 488 nm (FITC or GFP) and 568 nm (Texas red) emissions from the krypton-argon laser were used for excitation of the fluorophores. For real time studies on living cells, the Bioptech Foecht chamber was mounted onto a Nikon Inverted (Diaphot™) microscope and the laser optics shifted to this system. Optical sections of 512 × 512 pixels × 8 bits/pixel were collected through the samples at 0.5 μm intervals. Confocal images were segmented using IP Lab software (Signal Analytics). Following three-color segmentation, the area occupied by each site and the extent of colocalization was determined based on the number of pixels within each segmented site.

RESULTS

PCNA Colocalizes with Active Sites of DNA Replication Throughout S-Phase

Normal diploid human fibroblast (NHF1) cells were pulsed for short intervals (5 min)

with the thymidine analog 5-bromodeoxyuridine (BrdU) and subsequently fixed and stained using antibodies against PCNA and the halogenated nucleoside. Using this approach we found that, following a 5 min BrdU pulse, the early S-phase replication patterns [Berezney and Nakayasu, 1989; Ma et al., 1998] strikingly colocalize with sites of PCNA staining (Fig. 1A). Similar results were obtained with cells labeled in mid- and late S-phase (Fig. 1B,C) [Berezney and Nakayasu, 1989; Ma et al., 1998].

We then applied a spot-based algorithm to determine the number of sites of PCNA within the nucleus and the extent of colocalization with sites of replication [Samarabandu et al., 1995; Ma et al., 1998]. We found that on an average there were about 1000 sites of replication and PCNA within the cell nucleus in early S-phase. $<90\%$ of the total PCNA-stained area colocalized with sites of replication during early S-phase (Fig. 4A). We failed to detect significant levels of PCNA in non-S phase cells (data not shown). These results demonstrate that endogenous PCNA tracks with sites of DNA replication throughout the S-phase.

PCNA Dissociates from Replication Sites Following Completion of DNA Synthesis and Reassembles at Sites of New Synthesis

Recent results indicate that active replication sites in mammalian cells have an average lifetime of <1 h [Manders et al., 1996; Jackson and Pombo, 1998; Ma et al., 1998]. We therefore performed pulse-chase experiments designed to directly determine whether PCNA dissociates from active DNA replication sites following completion of replication. We found that the BrdU-labeled sites—independent of whether they are labeled in early or late S-phase—are always completely separated from the PCNA sites labeled 2 h later (Fig. 2A–C). Since PCNA colocalizes with active DNA replication sites independent of the stage of S-phase (Fig. 1), these results suggest that within this 2-hour chase period, PCNA dynamically dissociates from previously active replication sites and is translocated to new sites of active replication. For example, when early S-phase replication patterns are labeled, subsequent PCNA sites show a labeling pattern similar but spatially distinct from the earlier S-pattern of replication sites (Fig. 2A) or have shifted to a mid S-type pattern, characterized by enhanced staining along the nuclear periphery (Fig. 2B). Similarly, when replication sites are labeled in early stages of late S, the subsequent PCNA labeling pattern typically is a late S-type that is spatially distinct from the earlier replication site labeling (Fig. 2C). In contrast, if replication sites are labeled very late in the S-phase as characterized by a limited number of very large staining foci, then no staining is seen by PCNA 2 h later (Fig. 2D bottom half). This is consistent with the cells having completed S-phase and entered into G_2 phase and further confirms the earlier observations that PCNA is not detected in mammalian cells in non-S stages of the cell cycle using our fixation conditions [Bravo and McDonald-Bravo, 1987]. In a small percentage of cases ($<10\%$) we observed no labeling of replication sites but PCNA staining 2 h later in a pattern resembling early S replication sites (Fig. 2D, top half). This is consistent with cells being in G_1 phase and entering S-phase following the 2-hour chase.

A GFP-PCNA Construct Targets to Sites of Replication

As a step toward studying the spatial organization and dynamics of PCNA in living cells we have constructed a stably transfected GFP-PCNA cell line whose expression is under control of an inducible tetracycline promoter (see Materials and Methods). Before performing studies in living cells, however, it was of paramount importance to determine whether significant amounts of the GFP-PCNA construct actually associate with sites of DNA replication. To overcome the deleterious effects of alcohol fixation and acid denaturation on GFP fluorescence, we labeled replication sites in stably transfected mouse 3T6 cells using an in situ procedure that incorporated digoxigenin-11-dUTP at sites of replication (see Materials and Methods). In earlier studies we have shown that this procedure labels sites of replication similar to those observed when cells are pulsed with BrdU in vivo [Nakayasu and Berezney, 1989; Wei et al., 1998, 1999]. The results from these double-labeling experiments show that the GFP-PCNA fusion construct targets to sites of replication throughout the S-phase (Fig. 3B,C). Approximately 80% of the GFP-PCNA signal colocalized with sites of replication in the early S-phase (Fig. 4B). Consistent with these findings the GFP-PCNA signal overlaps virtually completely with sites

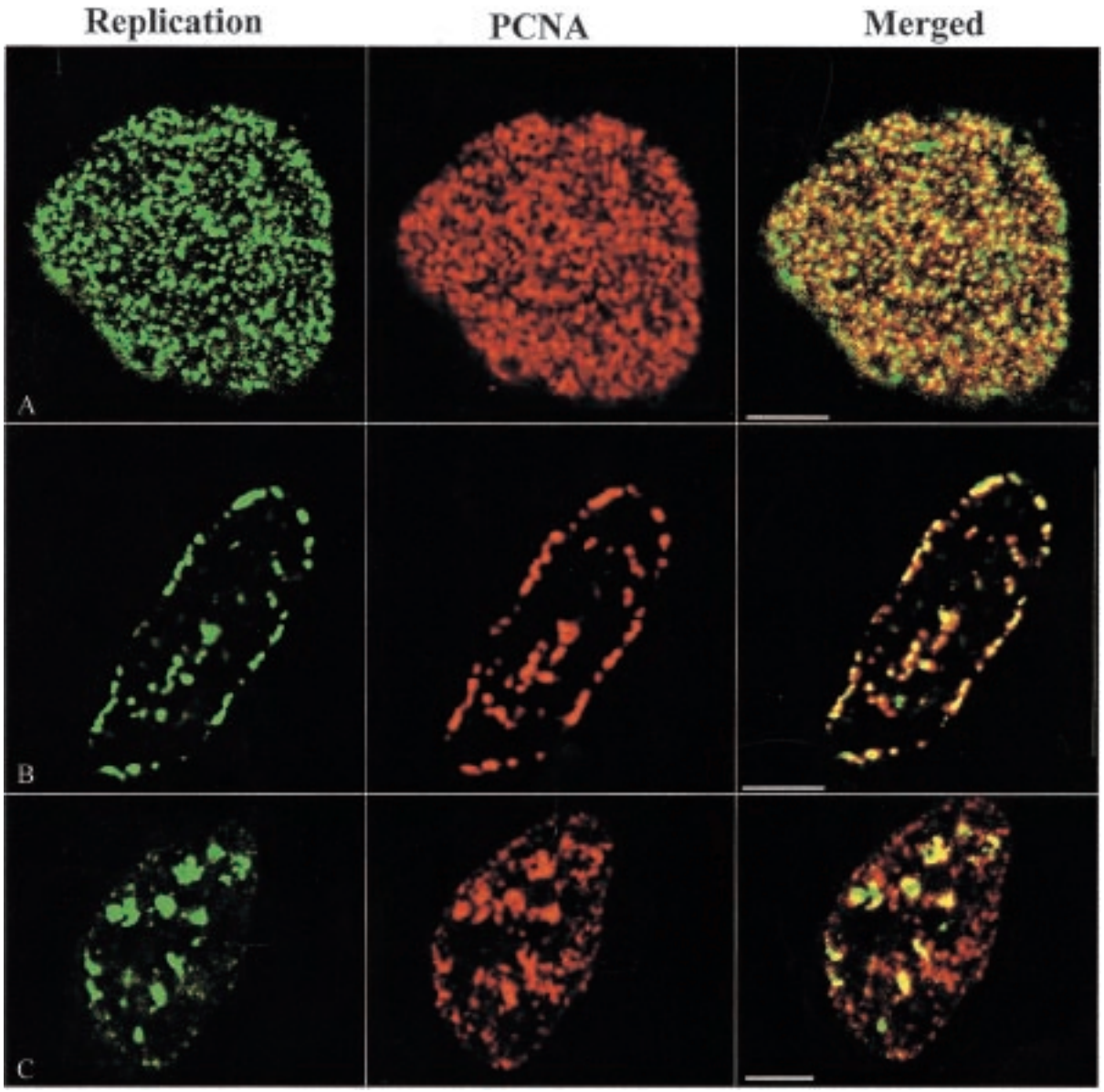

Fig. 1. Association of PCNA with sites of DNA replication. NHF1 cells were pulsed with BrdU for 5 min at different stages in S-phase: (**A**) early S; (**B**) mid S; (**C**) late S; and dual-labeled for replication sites (green) and PCNA (red). Bars, 5 μm.

of endogenous PCNA (results not shown). Moreover, non-replicating cells showed weak homogenous staining throughout the nucleus (Fig. 3A) and the expression of GFP, without fusion to PCNA, remained cytoplasmic (data not shown). We also found that expression of the fusion construct had no observable effect on the proliferating rates of these cells over several days (data not shown).

In evaluating the colocalization of endogenous PCNA and the GFP-PCNA construct with DNA replication sites it is important to stress that yellow color as an indicator of colocalization in the original microscopic images is only valid when the two signals, i.e., red and green, are of similar intensity. Thus if a relatively weak intensity for PCNA (green) was found over a replication site (red), the site would still appear red even though it contained significant PCNA. Indeed we observe that the intensity of PCNA staining is often not distributed homogeneously over individual replication sites and

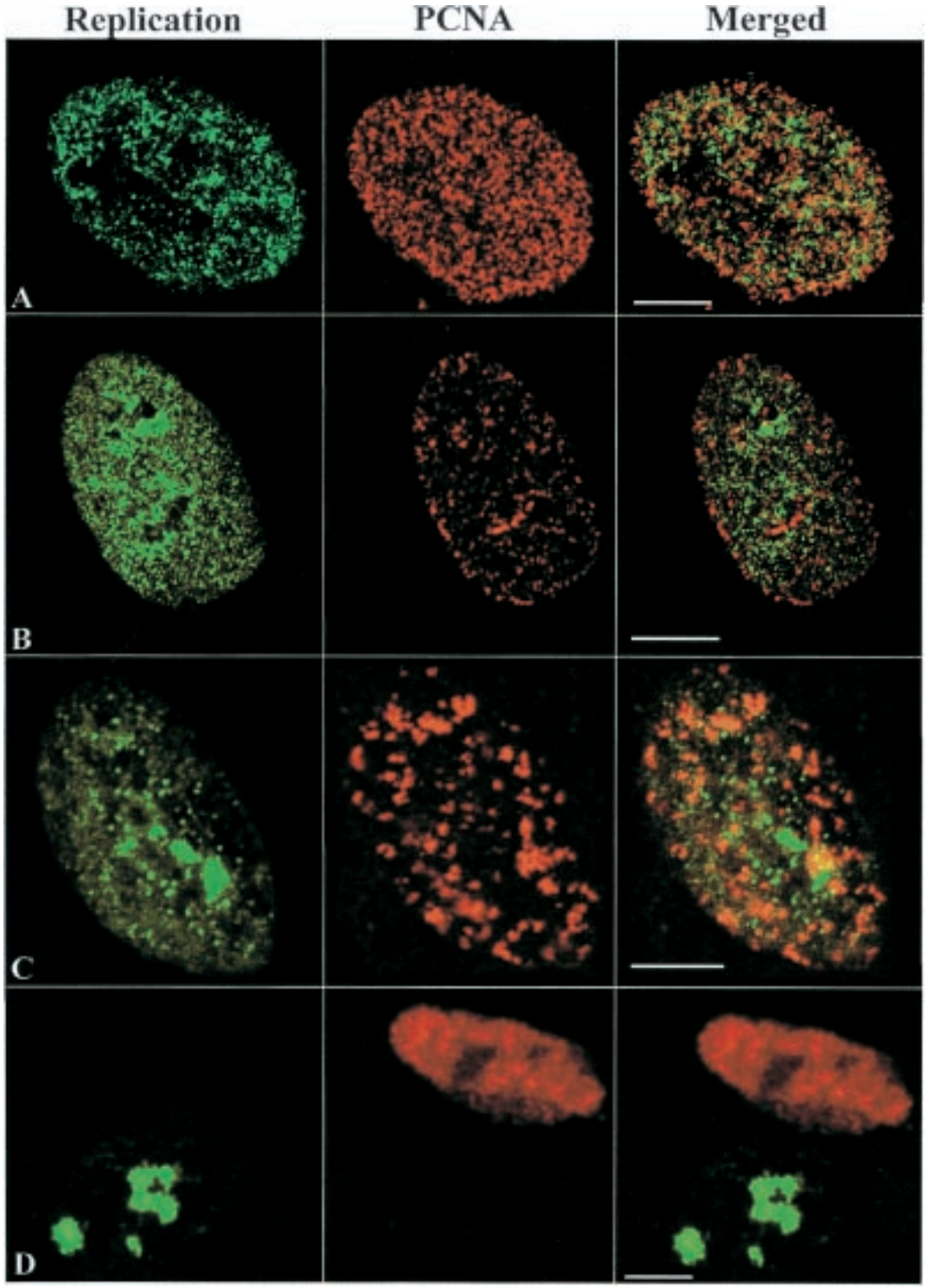

Fig. 2. Dynamic association of PCNA with DNA replication sites. NHF1 cells were pulsed with BrdU for 5 min. Following a chase of 2 h the cells were fixed and labeled for replication sites (green) and PCNA (red). The right panel shows cells that were labeled with BrdU at different stages in S-phase: (**A**) early S; (**B**) mid S; (**C**) late S. The subsequent PCNA (red) staining following the 2-hour chase is represented in the middle panel. Cells that showed only labeling for either of the two signals are represented in the bottom panel (**D**). Bars, 5 μm.

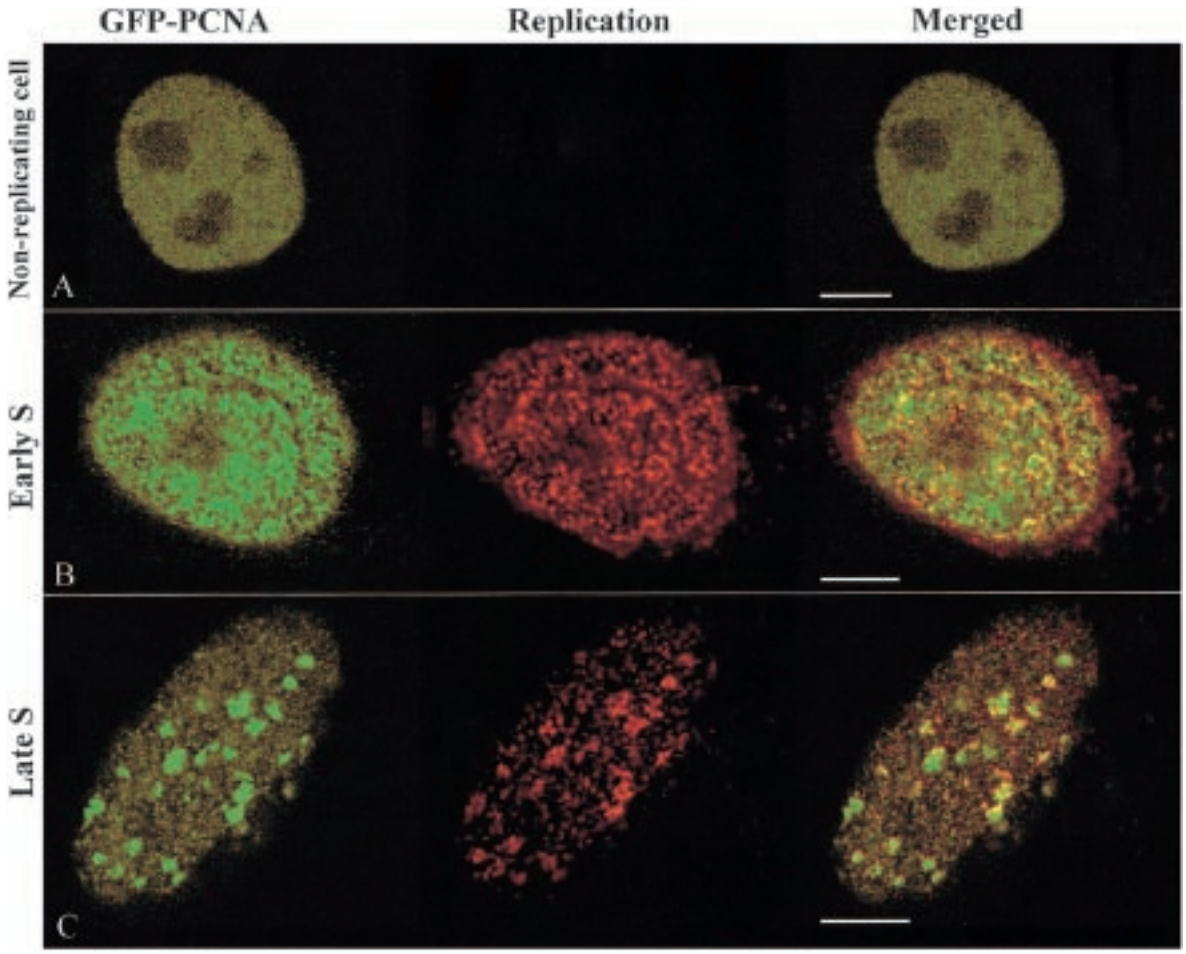

Fig. 3. Association of GFP-PCNA with DNA replication sites. The association of GFP-PCNA with DNA replication sites was examined in a 3T6 mouse fibroblast cell line (7-4) stably tranfected with GFP-PCNA. Cells were grown on cover slips for approximately 12 h after induction with 100 ng/ml of doxycycline and labeled with digoxigenin-dUTP. Patterns of replication sites in early (**B**), and late S (**C**), shown in the middle panels were compared to those of GFP-PCNA localization in the left panels with merging of the two channels in the right panels. GFP-PCNA in nonreplicating cells (**A**) is found nearly exclusively in the nucleus in a diffuse pattern. Bars, 5 μm.

viceversa. Thus there could be significant regions of PCNA and replication site labeling that are colocalized but do not show a yellow color due to a large imbalance in the signal intensity of the two labels at a particular area. This limitation of colocalization based on yellow color is apparent in our results by comparing the individual channels of PCNA and replication site labeling before merging. Often the degree of yellow color is much less than would be expected (see Figs. 1C and 3C). We overcome this difficulty by using parameters in our image analysis which segment "yellow" even when only a relatively low intensity of green fluorescence is found at the same pixel site as higher intensity red fluorescence and viceversa.

Dynamic Translocation of GFP-PCNA During S-Phase of Living Cells

With the confidence that GFP-PCNA expressed over a 12-hour period significantly colocalizes with replication sites throughout the S-phase in these stably transfected cells (Fig. 3), we have carried out real time studies of GFP-PCNA localized in living cells. Coverslips containing 3T6 cells, stably transfected with GFP-PCNA, were put in a microscopy chamber for live cells that maintained temperature at 37°C. Characteristic early, mid- and late-S type patterns of GFP-PCNA were readily identified (Fig. 5A–C). A series of optical sections of individual nuclei were then collected over time

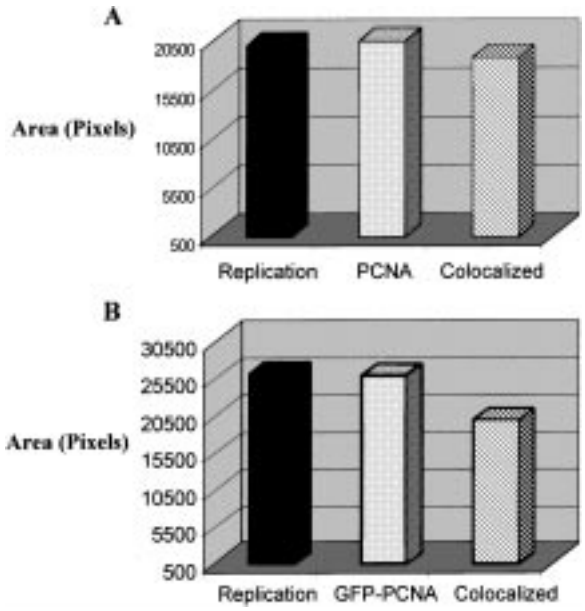

Fig. 4. Extent of colocalization between endogenous PCNA, GFP-PCNA and replication sites during early S-phase. Three color segmentation was performed on the confocal images to determine the extent of colocalization between endogenous PCNA (**A**) or GFP-PCNA (**B**) and replication sites. The area occupied by the segments was determined based on the number of pixels enclosed within the segments. Each area represents the average of at least 12 determinants.

with the confocal microscope. Cells expressing the fusion construct underwent characteristic changes in the localization of the protein. Depending on when in the S-phase a cell was imaged, the patterns changed with time to those characteristic of later S-phases. Figure 5D–F, shows a nucleus imaged over a period of 6 h. During the initial stages the nucleus contains small punctate foci that appear throughout the nucleus and are characteristic of early S replication sites (Fig. 5D). These then change to larger granular sites characteristic of mid and late S replication patterns (Fig. 5E,F). Similarly, Figure 5G–I tracks a nucleus over a 4 hour period showing a GFP-PCNA pattern characteristic of mid S-phase replication sites (Fig. 5G) going to late S-phase (Fig. 5H) and further onto the non-replicating state of G_2 phase (Fig. 5I). Our results are consistent with a dynamic translocation of PCNA to active sites of DNA replication throughout the S-phase of living cells and provide a new approach for directly studying the targeting of replicative factors to replication sites in living cells.

DISCUSSION

We have studied in mammalian cells the spatial and temporal dynamics of PCNA, an essential factor of the DNA replication machinery. Our results demonstrate that a detergent-resistant form of PCNA associates at sites of replication during all stages of S-phase. These findings are consistent with other studies that have demonstrated that the detergent-resistant form of the protein occurs only during the S-phase [Bravo and Macdonald-Bravo, 1987]. The targeting of PCNA to replication sites may be related to a preferential phosphorylation of the protein by association with cyclins and cyclin-dependent kinases [Prosperi et al., 1994]. Since the kinases associate with PCNA in both the bound and unbound forms, phosphorylation could be the signal that triggers the association of the protein with DNA at sites of replication. Alternatively, phosphorylation could occur following binding of the protein to DNA, prior to or during the elongation phase of DNA synthesis.

Following termination of replication at a site, we find that PCNA dissociates and targets to other sites of active replication. Temporally, this occurs within a window of 2 h or less in the early S-phase. These results are consistent with previous reports demonstrating that the time taken for completion of replication at a site is less than 1 h [Manders et al., 1992, 1996; Jackson and Pombo, 1998; Ma et al., 1998]. Our observations on the targeting of PCNA to active sites of replication are supported by similar results observed in CHO cells [Dimitrova et al., 1999]. The mechanisms by which the protein dissociates and then re-associates with sites of ongoing replication, however, remain to be resolved.

Upon induction, cells expressing a stable GFP-PCNA fusion construct showed dynamic spatial rearrangements that mimic expression of endogenous PCNA observed in fixed cells. The chimeric protein also targeted to sites of replication throughout the S-phase, indicating that the fusion of GFP to PCNA did not inhibit its targeting to DNA replication sites. While this paper was in preparation, Leonhardt et al. [2000] also demonstrated the colocalization of GFP-PCNA to active sites of DNA replication. Similar results were observed in studies that used GFP fusion constructs to other replication proteins, including DNA ligase and DNA

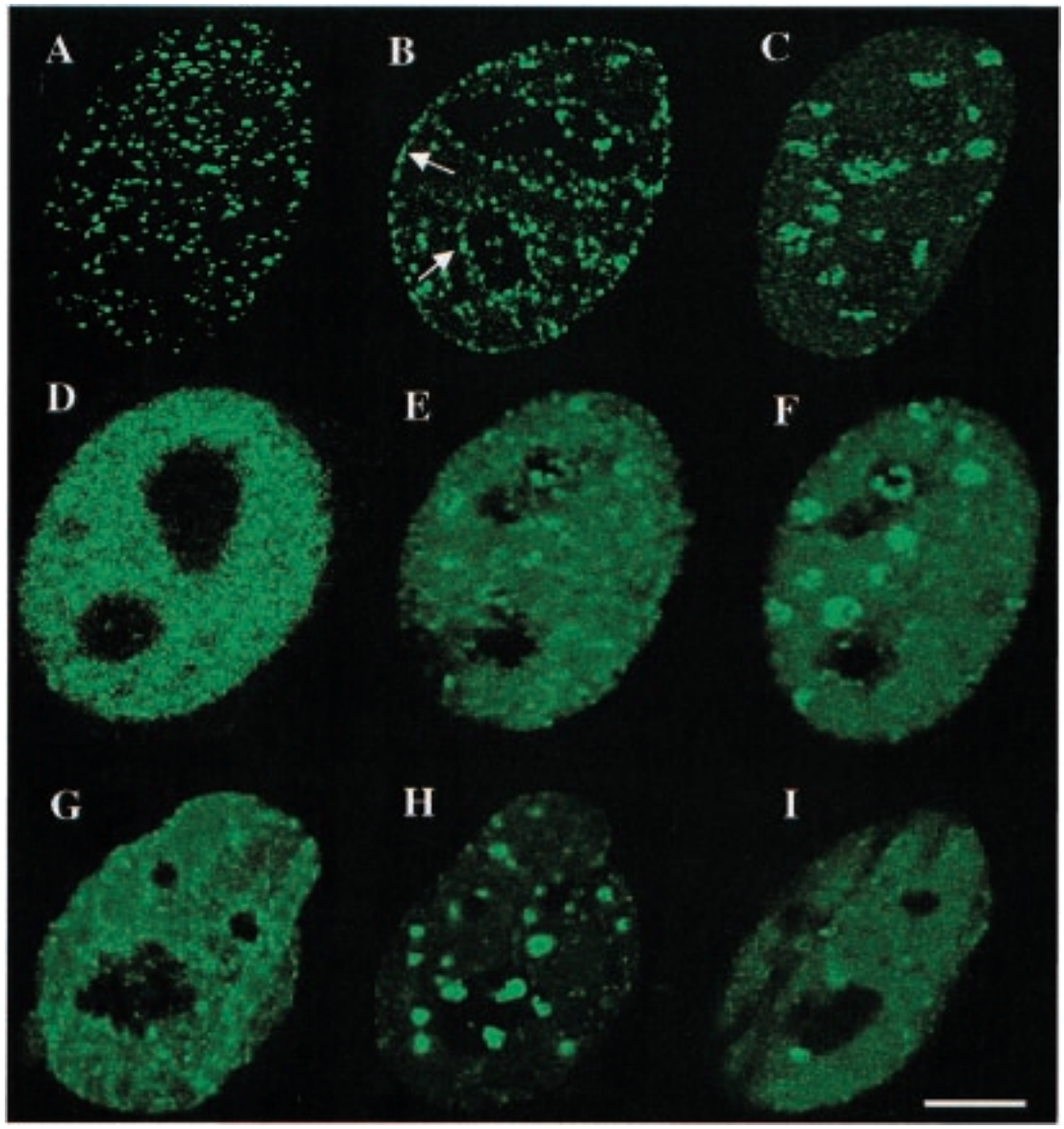

Fig. 5. Dynamic changes in PCNA localization during S-phase in living cells correlates with the characteristic S-phase dependent patterns of replication. Using the stable transfectant cell line (7-4) we tracked the pattern of GFP-PCNA in living cells pretreated, with 100 ng/ml of doxycycline for 12 h. Cells displaying early-S (**A**), mid-S (**B**) and late S-like (**C**) replication patterns of the GFP-PCNA were readily identified. Individual cells were then tracked by collecting a series of confocal sections approximately every hour. Row (**D**–**F**) shows GFP-PCNA going from early S to late S patterns at 2-hour intervals. Row (**G**–**I**) shows a transition from mid-S to late-S to G_2 patterns at 2-hour intervals. Arrows in (B) point to GFP-PCNA located at the perinucleolar (bottom) and perinuclear (top) borders, respectively. Bars, 5 μm.

methyltransferase [Leonhardt et al., 1992; Cardoso et al., 1997].

There is growing evidence that functions in the nucleus such as DNA replication, transcription, and RNA splicing are spatially and temporally segregated, with factors and enzymes involved in these processes targeting to their respective sites at presumably high concentrations [Spector, 1993; Berezney et al., 1995; Nickerson et al., 1995; Stein et al., 1995, 1999; van Driel et al., 1995; Berezney and Wei, 1998; Wei et al., 1999]. During the cell cycle this may be achieved by a myriad of genetic and biochemical signals that target these factors to the various compartments within the nucleus [Leonhardt and Cardoso, 1995; Zeng et al., 1997, 1998]. Using the replication protein PCNA as a model, we have shown that this

factor undergoes dynamic spatial rearrangements during the cell cycle that result in its targeting to sites of replication. Ongoing investigations in our laboratory are currently focused on the dynamic changes occurring at replication sites during the S-phase and the involvement of nuclear architecture in these processes.

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REFERENCES

Baserga R. 1991. Growth regulation of the PCNA gene. J Cell Sci 98:433–436.

Berezney R, Mortillaro MJ, Ma H, Wei X, Samarabandu J. 1995. The nuclear matrix: a structural milieu for nuclear genomic function. Int Rev Cytol 162A:1–65.

Berezney R, Wei X. 1998. The new paradigm: integrating genomic function and nuclear architecture. J Cell Biochem Suppl 30–31:238–242.

Bravo R. 1986. Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. Exp Cell Res 163:287–293.

Bravo R, Celis JE. 1980. A search for differential polypeptide synthesis throughout the cell cycle of Hela cells. J Cell Biol 84:795–802.

Bravo R, Fey SJ, Bellatin J, Larsen PM, Celis JE. 1982. Identification of a nuclear polypeptide ("cyclin") whose relative proportion is sensitive to changes in the rate of cell proliferation and to transformation. Progress in Clinical & Biological Research, 85(Pt A): 235–248.

Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. 1987. Cyclin/PCNA is the auxillary protein of DNA polymerase-δ. Nature 326:515–517.

Bravo R, Macdonald-Bravo H. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. J Cell Biol 105:1549–1554.

Cai J, Uhlmann F, Gibbs E, Flores-Rozas H, Lee CG, Phillips B, Finkelstein J, Yao N, O'Donnell M, Hurwitz J. 1996. Reconstitution of human replication factor C from its five subunits in baculovirus-infected insect cells. Proc Natl Acad Sci USA 93(23):12896–12901.

Cardoso CM, Joseph C, Rahn HP, Reusch R, Nadal-Ginard B, Leonhardt H. 1997. Mapping and use of a sequence that directs DNA ligase I to sites of DNA replication in vivo. J Cell Biol 139:579–587.

Cook PR. 1999. The organization of replication and transcription. Science 284:1790–1795.

Dimitrova DS, Todorov IT, Melendy T, Gilbert DM. 1999. Mcm2, but not RPA is a component of the mammalian early G1-phase prereplication complex. J Cell Biol 146:709–722.

Dutta A, Bell SP. 1997. Initiation of DNA replication in eukaryotic cells. Ann Rev Dev Biol 13:293–332.

Fox MH, Arndt-Jovin DJ, Baumann PH, Robert-Nicoud M. 1991. Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in mouse fibroblasts. J Cell Sci 99:247–300.

Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promotors. Proc Natl Acad Sci USA 89:5547–5551.

Gossen M, Freundlieb S, Bender G, Muller G, Hillen, Bujard H. 1995. Transcriptional activation by tetracyclines in mammalian cells. Science 268:1766–1769.

Humbert C, Santisteban MS, Usson M, Robert-Nicold R. 1992. Intranuclear co-location of newly replicated DNA and PCNA by simultaneous immunofluorescent labelling and confocal microscopy in MCF-7 cells. J Cell Sci 103: 97–103.

Jackson DA, Pombo A. 1998. Replication clusters are stable units of chromosome structure: evidence that nuclear organization contributes to the efficient activation and propagation of S-phase in human cells. J Cell Biol 140: 1285–1295.

Jaskulski D, DeRiel JK, Mercer WE, Calabretta B, Baserga R. 1988. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. Science 240: 1544–1546.

Kill IR, Bridger JM, Campbell KHS, Maldonado-Codina G, Hutchison CJ. 1991. The timing of the formation and usage of replicase clusters in S-phase nuclei of human diploid fibroblasts. J Cell Sci 100:869–876.

Lee SH, Kwong AD, Pan ZQ, Hurwitz J. 1991. Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase delta. J Biol Chem 266:594–602.

Leonhardt H, Cardoso MC. 1995. Targeting and association of proteins with functional domains in the nucleus: the insoluble solution. Int Rev Cytol 162B:303–335.

Leonhardt H, Page AW, Weier HU, Bestor TH. 1992. A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 71: 865–873.

Leonhardt H, Rahn H-P, Weinzierl P, Sporbert A, Cremer T, Zink D, Cardosa MC. 2000. Dynamics of DNA replication factories in living cells. J Cell Biol 149: 271–279.

Ma H, Samarabandu J, Devdhar RS, Acharya R, Cheng P-C, Meng C, Berezney R. 1998. Spatial and temporal dynamics of DNA replication sites in mammalian cells. J Cell Biol 143:1415–1425.

Madsen P, Celis JE. 1985. S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. A role for cyclin in DNA replication? FEBS Lett 193:5–11.

Manders EM, Stap J, Brakenhoff GJ, van Driel R, Aten JA. 1992. Dynamics of three-dimensional replication pat-

terns during the S-phase, analysed by double labeling of DNA and confocal microscopy. J Cell Sci 103:857–862.
Manders EM, Stap J, Strackee J, van Driel R, Aten JA. 1996. Dynamic behavior of DNA replication domains. Exp Cell Res 226:328–335.
Mathews MB, Bernstein RM, Franza R, Garrels JE. 1984. Identity of the proliferating cell nuclear antigen and cyclin. Nature 309:374–376.
Mazzotti G, Rizzoli R, Galanzi A, Papa S, Vitale M, Falconi M, Neri LM, Zini N, Maraldi NM. 1990. High-resolution detection of newly synthesized DNA by anti-bromodeoxyuridine antibodies identifies specific chromatin domains. J Histochem Cytochem 38:13–22.
Miyashi K, Fritzler MJ, Tan EM. 1978. An autoantibody to a nuclear antigen in proliferating cells. J Immunol 121: 2228–2234.
Nakamura H, Morita T, Sato C. 1986. Structural organization of replicon domains during DNA synthetic phase in the mammalian nucleus. Exp Cell Res 165:291–297.
Nakayasu H, Berezney R. 1989. Mapping replicational sites in the eukaryotic cell nucleus. J Cell Biol 108:1–11.
Nickerson JA, Blencowe BJ, Penman S. 1995. The architectural organization of nuclear metabolism. Int Rev Cytol 162A:67–124.
Prelich G, Kostura M, Marshak DR, Mathews MB, Stillman B. 1987a. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature 326:471–475.
Prelich G, Stillman B. 1988. Coordinated leading and lagging stand synthesis during SV40 DNA replication in vitro requires PCNA. Cell 53:117–126.
Prelich G, Tan CK, Kostura M, Mathews MB, So AG, Downey KM, Stillman B. 1987b. Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxillary protein. Nature 326:517–520.
Prosperi E, Scovassi IA, Stivala LA, Bianchi L. 1994. Proliferating cell nuclear antigen bound to DNA synthesis sites: phosphorylation and association with cyclin D1 and cyclin A. Exp Cell Res 215:257–262.
Samarabandu J, Ma H, Acharya R, Cheng P-C, Berezney R. 1995. Image analysis techniques for visualizing the spatial organization of DNA replication sites in the mammalian cell nucleus using multi-channel confocal microscopy. SPIE 2434:370–375.
Spector DL. 1993. Macromolecular domains within the cell nucleus. Ann Rev of Cell Biol 9:265–315.
Stein GS, van Wiljnen AJ, Stein J, Lian JB, Montecino M. 1995. Contributions of nuclear architecture to transcriptional control. Int Rev Cytol 162A:251–278.
Stein GS, van Wijnen AJ, Stein JL, Lian JB, McNeil S, Pockwinse SM. 1999. Transcriptional control within the three-dimensional context of nuclear architecture: requirements for boundaries and direction. J Cell Biochem Suppl 32–33:24–31.
Tan CK, Castillo C, So AG, Downey KM. 1986. An auxillary protein for DNA polymerase-d from foetal calf thymus. J Biol Chem 261:12310–12316.
Tsurimoto T, Stillman B. 1990. Functions of replication factor C and proliferating-cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4. Proc Natl Acad Sci USA 87:1023–1027.
Tsurimoto T, Stillman B. 1991. Replication factors required for SV40 DNA replication in vitro. I. DNA structure-specific recognition of a primer–template junction by eukaryotic DNA polymerases and their accessory proteins. J Biol Chem 266:1950–1960.
Tye BK. 1999. Mcm proteins in DNA replication. Annu Rev Biochem 68:649–686.
van Dierendonck JH, Keyzer R, van de Velde CJH, Cornelisse CJ. 1989. Subdivision of S-phase by analysis of nuclear 5-bromodeoxyuridine staining patterns. Cytometry 10:143–150.
van Driel R, Wansink DG, Steensel BV, Grande MA, Schul W, de Jong L. 1995. Nuclear domains and the nuclear matrix. Int Rev Cytol 162A:151–190.
Waga S, Stillman B. 1998. The DNA replication fork in eukaryotic cells. Annu Rev Biochem 67:721–751.
Wei X, Samarabandu J, Devdhar RS, Siegel AJ, Acharya R, Berezney R. 1998. Segregation of transcription and replication sites into higher order domains. Science 281: 1502–1505.
Wei X, Somanathan S, Samarabandu J, Berezney R. 1999. Three-dimensional visualization of transcription sites and their association with splicing factor-rich nuclear speckles. J Cell Biol 146:543–558.
Zeng C, McNeil S, Pockwinse S, Nickerson J, Shopland L, Lawrence JB, Penman S, Hiebert S, Lian JB, van Wijnen AJ, Stein JL, Stein GS. 1998. Intranuclear targeting of AML/CBFalpha regulatory factors to nuclear matrix-associated transcriptional domains. Proc Natl Acad Sci USA 95:1585–1589.
Zeng C, van Wijnen AJ, Stein JL, Meyers S, Sun W, Shopland L, Lawrence JB, Penman S, Lian JB, Stein GS, Hiebert SW. 1997. Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. Proc Natl Acad Sci USA 94:6746–6751.
Zuber M, Tan EM, Ryoji M. 1989. Involvement of proliferating cell nuclear antigen (cyclin) in DNA replication in living cells. Mol Cell Biol 9:57–66.