# Dynamics of DNA Replication Factories in Living Cells

**Heinrich Leonhardt,* Hans-Peter Rahn,* Peter Weinzierl,‡† Anje Sporbert,* Thomas Cremer,‡ Daniele Zink,‡ and M. Cristina Cardoso***

*Max Delbrück Center for Molecular Medicine, D-13125 Berlin, Germany; and ‡Institute for Anthropology and Human Genetics, LMU, D-80336 Munich, Germany

*Abstract.* DNA replication occurs in microscopically visible complexes at discrete sites (replication foci) in the nucleus. These foci consist of DNA associated with replication machineries, i.e., large protein complexes involved in DNA replication. To study the dynamics of these nuclear replication foci in living cells, we fused proliferating cell nuclear antigen (PCNA), a central component of the replication machinery, with the green fluorescent protein (GFP). Imaging of stable cell lines expressing low levels of GFP-PCNA showed that replication foci are heterogeneous in size and lifetime. Time-lapse studies revealed that replication foci clearly differ from nuclear speckles and coiled bodies as they neither show directional movements, nor do they seem to merge or divide. These four dimensional analyses suggested that replication factories are stably anchored in the nucleus and that changes in the pattern occur through gradual, coordinated, but asynchronous, assembly and disassembly throughout S phase.

Key words: cell cycle • DNA replication foci • green fluorescent protein• nuclear organization • proliferating cell nuclear antigen

## *Introduction*

Over the last decade it has become clear that the mammalian nucleus, despite the absence of intranuclear membranes, is organized into functional domains or foci where nuclear processes like DNA replication, ribosome biogenesis, transcription, and RNA processing take place (Spector, 1993; Xing and Lawrence, 1993; Scheer and Weisenberger, 1994). These compartments have been identified in some cases by phase-contrast microscopy, by in situ hybridization techniques, and more commonly by detection of incorporated nucleotides or associated proteins. Different proteins participating in the same process are organized together in one functional compartment, which is often referred to as functional organization of the nucleus (Leonhardt and Cardoso, 1995). In the case of replication foci, these proteins include not only replication factors, but also other proteins that are not directly involved in DNA replication, such as the cell cycle regulators cyclin A (Cardoso et al., 1993; Sobczak-Thepot et al., 1993) and cdk2 (Cardoso et al., 1993), uracil-DNA glycosylase (UNG2; Otterlei et al., 1999) involved in base excision repair, and also DNA methyltransferase (Dnmt1; Leonhardt et al., 1992).

These replication machines and factories are obviously bound to the DNA they are replicating, but they also seem to be tethered to an underlying framework called the nuclear matrix or skeleton (Tubo and Berezney, 1987; Hozak et al., 1993). The molecular mechanism underlying the cell cycle-dependent association of a growing list of proteins with subnuclear replication foci is mostly unknown. In the case of two of these factors, Dnmt1 and DNA ligase I, specific protein sequences were identified that are separated from the catalytic domains and are necessary and sufficient for association with replication foci (Leonhardt et al., 1992; Cardoso et al., 1997). These targeting sequences seem to position the different factors at the right place at the right time to assemble a processive replication-methylation machinery working like an assembly-line (Cardoso and Leonhardt, 1998).

The existence of replication foci, which had been shown in fixed and stained cells, was recently established in living mammalian cells (Cardoso et al., 1997). It has often been proposed, but not directly shown, that replication foci patterns undergo reproducible changes in individual cells throughout S phase. Furthermore, it is not known how these different patterns form and possibly change during S phase. To address these questions, we set out to establish a cellular system to investigate the dynamics of DNA replication factories in living cells during the cell cycle, making use of translational fusions of replication factors with the green fluorescent protein (GFP)[1]. We chose proliferating

†Dr. Peter Weinzierl died on 13 October 1999.

Address correspondence to M.C. Cardoso, Franz Volhard Clinic, Wiltbergstr. 50, 13125 Berlin, Germany. Tel.: 49-30-9417-2341. Fax: 49-30-9417-2336. E-mail: cardoso@fvk-berlin.de

[1]*Abbreviations used in this paper:* 4D, four-dimensional; BrdU, 5-bromo-2′-deoxyuridine; GFP, green fluorescent protein; PCNA, proliferating cell nuclear antigen.

cell nuclear antigen (PCNA) as a marker for replication factories since it is highly conserved from yeast to mammalian cells, has no known enzymatic activity, but is nevertheless a central and essential factor for DNA replication (Jaskulski et al., 1988; Bauer and Burgers, 1990; Waseem et al., 1992). PCNA was first identified as the processivity factor for DNA polymerase delta (Bravo et al., 1987; Prelich et al., 1987). It forms a homotrimeric ring around the DNA serving as a sliding clamp that tethers the polymerases, as well as many other replication associated factors, to the DNA ensuring high processivity (Wyman and Botchan, 1995; Jonsson and Hubscher, 1997; Kelman and Hurwitz, 1998). Historically, PCNA was the first protein identified at replication foci during S phase (Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1987) and is since widely used as a marker for replication foci.

Unlike coiled bodies (Boudonck et al., 1999; Sleeman and Lamond, 1999) and speckles (Misteli et al., 1997), which have been recently shown to move and merge, the results presented here indicate that replication foci visualized in living cells by GFP-PCNA do not seem to move, merge, or divide, but instead assemble and disassemble in an asynchronous manner throughout S phase.

## Materials and Methods

### GFP-PCNA Expression Plasmid

Expression of the fusion protein is driven by the CMV promoter and the translation signals of the thymidine kinase gene, both provided by the pEVRF expression vector (Matthias et al., 1989). The GFP-PCNAL2 fusion protein contains a SV40 nuclear localization signal at the $NH_2$ terminus followed by an enhanced mutant GFP gene that is fused to the human PCNA by a flexible and hydrophilic linker (GEGQGQGQGPGRGYAYRS). The GFP gene uses a humanized codon usage for better expression in mammalian cells and mutations confering improved spectral properties (enhanced GFP; Clontech) and thermal stability (Siemering et al., 1996).

### Generation of Mammalian Cell Lines Expressing GFP-PCNA Fusion Protein

C2C12 mouse myoblast cells were grown in DME supplemented with 20% FCS. To generate GFP-PCNA expressing cell lines, linearized plasmid DNA coding for the fusion protein GFP-PCNAL2 was cotransfected with the plasmid pSV2neo (at a ratio of 20:1) into C2C12 cells by the lipofectamine method (GIBCO BRL). 24 h after transfection, the GFP expressing cells were sorted by flow cytometry (FACSort; Becton Dickinson), replated at clonal density, and grown for 9 d in media containing 600 μg/ml G418 (Geneticin; GIBCO BRL). GFP-PCNA–expressing cell clones were selected under the fluorescence microscope, expanded, and further subcloned by the limiting dilution method to ensure their clonal nature.

### Western Blot Analysis

For immunoblot analysis, $5 \times 10^5$ COS7 cells, transiently transfected by the DEAE dextran pretreatment method, and the same number of mouse myoblast cells were extracted and immunoblots were performed, both as described before (Cardoso et al., 1997). Blots were probed with the mouse monoclonal anti-PCNA antibody (clone PC10; Dako).

### Flow Cytometry Analysis

To analyze the intracellular DNA content, cells were trypsinized, washed in PBS, and fixed in 100% methanol. Fixed cells were subsequently stained in a solution containing 50 μg/ml propidium iodide, 0.1 mg/ml RNAse, 0.1% NP-40, and 0.1% trisodium citrate. Samples were then washed in PBS and DNA content estimated by measuring propidium iodide fluorescence with a flow cytometer (FACSort; Becton Dickinson) using the 600-nm long pass filter. Data were analyzed using the ModFit LT software (Becton Dickinson).

### Immunofluorescence Analyses

Indirect immunofluorescence stainings were carried out as previously described (Cardoso et al., 1997). The following primary antibodies were used: mouse monoclonal anti-PCNA antibody (clone PC10; Dako), rabbit polyclonal anti-Dnmt1 antiserum (Leonhardt et al., 1992), and affinity-purified rabbit polyclonal anti-DNA ligase I antibody (Cardoso et al., 1997).

C2C12 cells were transfected with plasmid DNA coding for the fusion protein GFP-PCNAL2 by the calcium phosphate precipitation method, and 24 h later pulse-labeled for 20 min with 10 μM 5-bromo-2'-deoxyuridine (BrdU). Cells were then formaldehyde-fixed, followed by acid denaturation of the DNA, and staining with anti-BrdU antibody (Becton Dickinson) as described (Zink et al., 1998). Optical sections were acquired with a Leica TCS four-dimensional (4D) confocal microscope.

### Live Cell Microscopy and Image Analysis

For live cell observation, cells were plated onto 40-mm glass coverslips and allowed to attach overnight. They were then assembled into a FCS2 live cell microscopy chamber (Bioptechs) set to 37°C that was mounted on a Zeiss Axiovert inverted microscope. For most observations, a 63× NA 1.4 Plan apochromat oil immersion objective heated to 37°C and a bandpass fluorescein filter set (excitation 450–490 nm, dichroic 510 nm, emission 515–565 nm; Zeiss) were used. Shorter excitation wavelengths proved to be phototoxic to the cells. Time of exposure per image varied between 0.4–4 s. In most experiments, stacks of 21 images at 0.5-μm Z increments were collected at each time point. Images were acquired with a cooled CCD camera, Sensicam (1,280 × 1,024 pixels; 6.7 μm pixel size) using the Quanticell software (VisiTech) or, in the case of Fig. 3 B, with a MicroMax camera (1,317 × 1,035 pixels, 6.8 μm pixel size) using MetaMorph 3.0 software (Universal Imaging Corp.). Images were assembled and annotated using NIH Image 1.6, Adobe Photoshop 4.0, and Adobe Illustrator 7.0 software on Power MacIntosh computers.

Series of image stacks collected at different time points were first analyzed for movements of the nucleus itself. After correcting for cellular movements and focal drift, we examined replication foci over time for movements, assembly, or disassembly using the neighboring foci and nucleoli as reference points. For digital deconvolution (see Fig. 5, middle) Z axis series of images were loaded into Image-Pro Plus 3.0 software and removal of out-of-focus light was performed with the Microtome IP nearest neighbor algorithm. Replication foci intensity was quantified with the ImageQuant 1.2 software (Molecular Dynamics) and bar graphs were generated with Microsoft Excel 5 software.

## Results

### Generation of Mammalian Cell Lines to Study DNA Replication Factories in Living Cells

The construction of translational fusions with GFP allows in vivo labeling of proteins but often impairs the function of the fusion partner creating dominant negative mutants. To minimize these potential interferences, we took advantage of the known crystal structures of GFP and human PCNA (Gulbis et al., 1996; Ormö et al., 1996) and designed the fusions to allow independent folding and function of both proteins. We fused GFP at the $NH_2$ terminus of human PCNA since this part is freely exposed at the outer surface of the trimeric PCNA ring wrapped around the DNA. Since PCNA interacts with numerous other factors during the cell cycle and in the process of DNA replication, we used long linker sequences that were glycine-rich to ensure flexibility and hydrophilic to increase surface probability in the folding of the fusion protein (Fig. 1 A).

Several fusions of PCNA and GFP with different linkers were constructed and tested in transient transfection assays. All of them could be expressed transiently at high

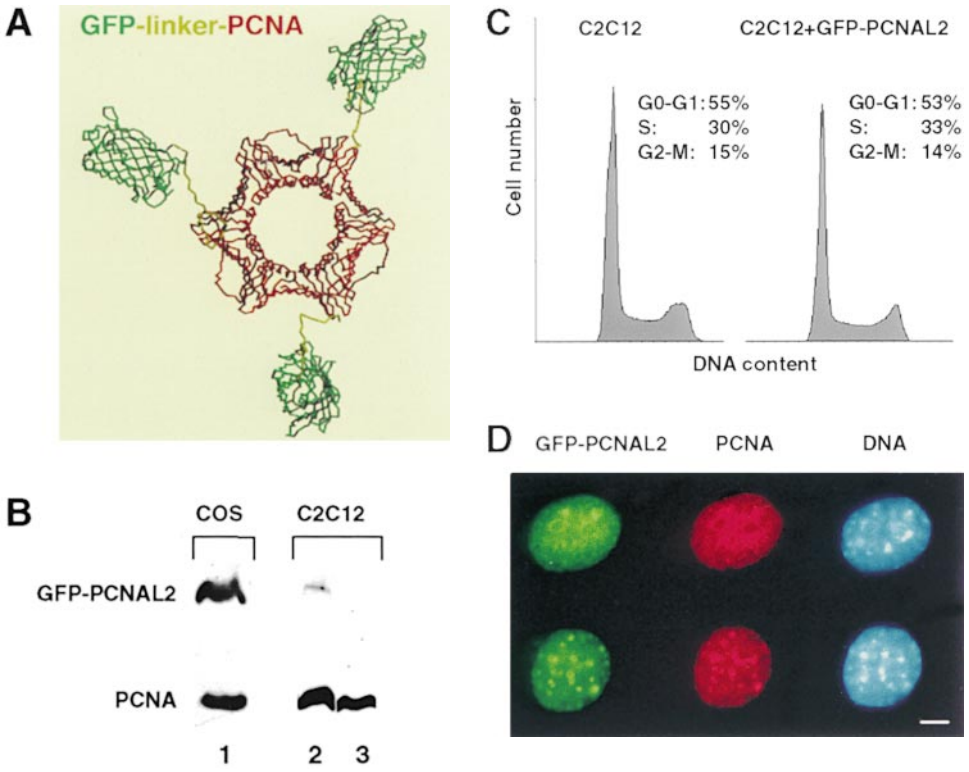


*Figure 1.* Construction of mammalian cell lines expressing GFP-tagged PCNA. A, The putative structure of the GFP-linker–PCNA fusion protein was assembled using published crystal structure information for GFP and PCNA (Gulbis et al., 1996; Ormö et al., 1996). The linker structure is a putative 18 residue polypeptide chain. B, Expression of the GFP–PCNAL2 fusion protein and the endogenous PCNA protein was monitored by Western blot analysis with an mAb against PCNA. Extracts from transiently transfected COS7 cells (lane 1), from one stably transfected C2C12 cell line (lane 2) and from untransfected C2C12 cells (lane 3) were compared. In lanes 2 and 3, extracts from half a million cells were loaded. C, Cell cycle distribution of an asynchronously growing population of C2C12+GFP–PCNAL2 stable cell line was compared with the untransfected parental C2C12 cells by FACS analysis of the DNA content stained with propidium iodide. 100,000 events were collected for each sample and the data analyzed using the ModFit LT software (Becton Dickinson). D, The localization of the autofluorescent GFP–PCNAL2 fusion protein was compared with the endogenous PCNA in the stable cell lines. Notice that the mAb against PCNA also recognizes the fusion protein which, however, amounts to <5% of the endogenous PCNA pool (see lane 2 in B). DNA was stained with Hoechst 33258. Bar, 5 μm.

levels in COS7 cells, but only one of them, GFP-PCNAL2, allowed the establishment of stable cell lines. Using a combination of FACS sorting, antibiotic selection, and screening for GFP-PCNA positive clones with a fluorescence microscope, we were able to establish stably transfected cell lines. From these, four independent clonal cell lines were further characterized and showed indistinguishable properties. The direct comparison in Fig. 1 B shows that the GFP-PCNAL2 fusion protein is expressed only at minute amounts in the stable C2C12 cell lines, which on average corresponds to <5% of the endogenous PCNA. At these low expression levels the fusion protein did not interfere with normal cell cycle progression as evidenced by flow cytometric analyses of the cell cycle distribution of asynchronous populations of stable cell lines in comparison with the original C2C12 cell line (Fig. 1 C).

Since fusion proteins can often form unspecific aggregates and may show aberrant distribution patterns, we analyzed the localization of the GFP-PCNAL2 protein in detail. C2C12 cells expressing GFP-PCNAL2 were fixed and stained for well established components of mammalian replication foci. The fusion protein colocalized with the endogenous PCNA (Fig. 1 D) and, even at these low expression levels, efficiently labeled replication foci. In addition, cells were stained for DNA ligase I and Dnmt1, which were also found to colocalize with GFP-PCNAL2 fusion proteins at nuclear replication foci (Fig. 2 A). Finally, we could show with BrdU-labeling experiments that these nuclear replication foci visualized by GFP-PCNAL2 are in fact functional complexes that are actively synthesizing DNA (Fig. 2 B). These properties of GFP-PCNAL2 can be found throughout S phase as documented by the different patterns shown for each staining.

These results clearly show that the GFP-PCNAL2 fusion protein labels functional and active replication foci in living cells without disrupting normal cell cycle progression.

### *Changes in the Distribution of Replication Factories Throughout the Cell Cycle*

Live cell studies face two major challenges, the establishment of adequate growth conditions on the microscope stage and possible photodamage by the exciting light. Thus, stable C2C12 cell lines expressing the GFP-PCNAL2 fusion protein were grown in a heated live cell microscopy chamber (FCS2 chamber) with an additional objective heater for optimal growth temperature control. Under these experimental conditions, cells were growing and dividing at normal rates for up to three days, indicating normal cell cycle progression. The image acquisition process was optimized to minimize the risk of photodamage and thus allowed us to follow individual cells throughout the cell cycle (Fig. 3 A).

Experiments with synchronized cells suggested an ordered transition of replication foci patterns throughout S phase (Nakamura et al., 1986; Nakayasu and Berezney, 1989; van Dierendonck et al., 1989; Fox et al., 1991; O'Keefe et al., 1992). Since these experiments were based

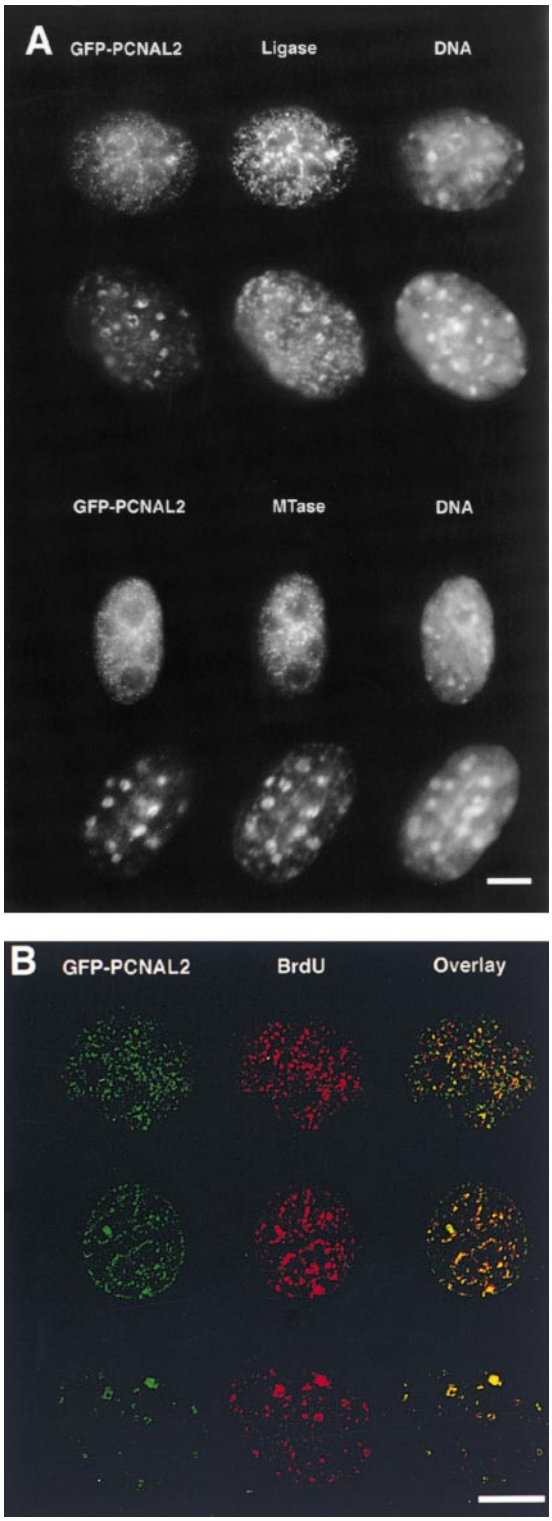


*Figure 2.* The GFP-tagged PCNA labels subnuclear DNA replication factories. A, The subcellular localization of the autofluorescent GFP–PCNAL2 fusion protein is shown in comparison with other known components of mammalian replication factories in two different typical S phase patterns. Stably transfected cells were stained with polyclonal antibodies against DNA ligase I (Ligase) and Dnmt1 (MTase). DNA was stained with Hoechst 33258. Bar, 5 μm. B, C2C12 cells were transfected with the GFP–PCNAL2 construct and 24 h later pulse-labeled with BrdU and fixed. Subnuclear sites of ongoing DNA replication were highlighted by staining with BrdU-specific antibodies (red). Four or five midplane confocal optical sections were stacked and overlays were made using Adobe Photoshop and MetaMorph programs. Yellow color shows the colocalization of the fusion protein (green) at replication foci. Bar, 10 μm.

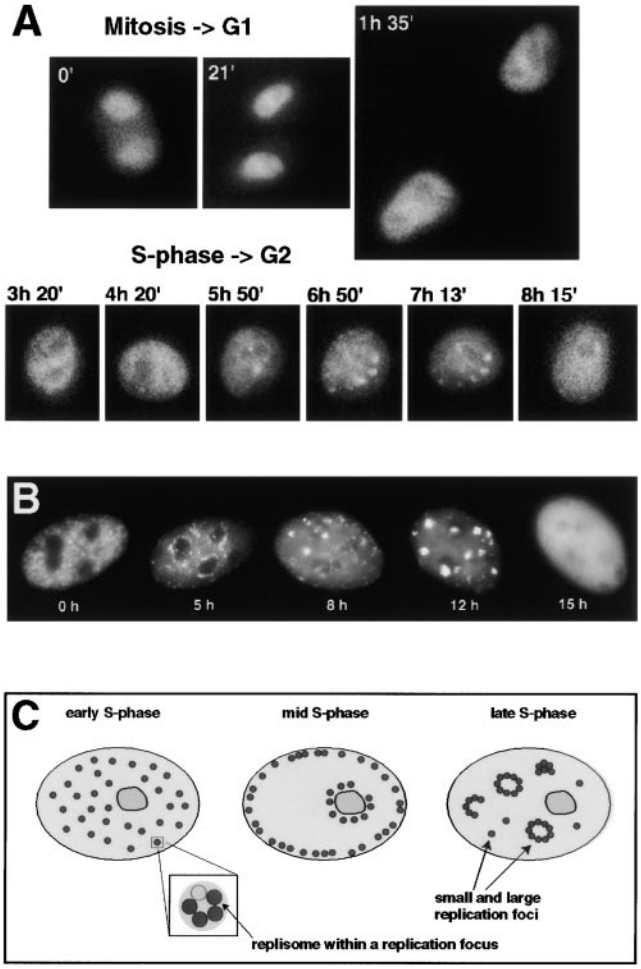


*Figure 3.* Changes in the distribution of replication factories throughout the cell cycle. Stably transfected cells (C2C12+GFP–PCNAL2) were grown in a live-cell microscopy chamber (FCS2 chamber, Bioptechs, Inc.) and monitored for up to 3 d. A, Selected images of a cell finishing mitosis and of one of the daughter cells followed until the next G2 phase are shown. B, A cell was imaged at higher resolution starting in early S phase with a fine punctate pattern of replication foci (time 0 h), succeeded by a perinucleolar pattern (at 5 h) and a characteristic late pattern with few, but large, foci (at 8–12 h). C, Typical early, mid, and late S phase patterns are schematically illustrated.

on isolated snapshots of fixed cells, they do not provide direct evidence for a change of replication patterns during S phase in individual living cells. Moreover, there is some disagreement in the literature concerning the temporal order and number of these patterns.

Time-lapse series following single cells through S phase are shown in Fig. 3. S phases of different duration were recorded and, in Fig. 3, A and B, we show two extreme ex-

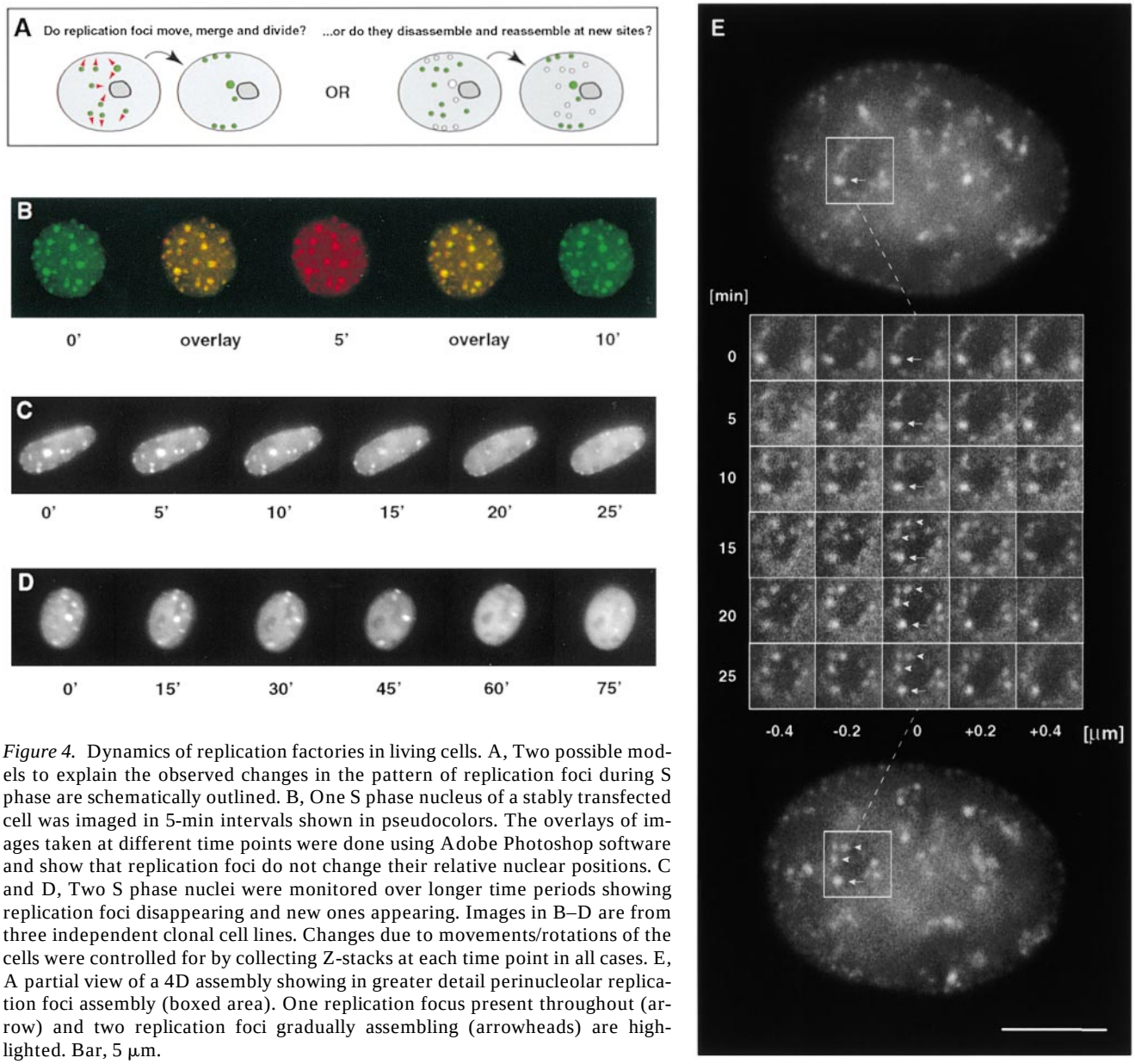


*Figure 4.* Dynamics of replication factories in living cells. A, Two possible models to explain the observed changes in the pattern of replication foci during S phase are schematically outlined. B, One S phase nucleus of a stably transfected cell was imaged in 5-min intervals shown in pseudocolors. The overlays of images taken at different time points were done using Adobe Photoshop software and show that replication foci do not change their relative nuclear positions. C and D, Two S phase nuclei were monitored over longer time periods showing replication foci disappearing and new ones appearing. Images in B–D are from three independent clonal cell lines. Changes due to movements/rotations of the cells were controlled for by collecting Z-stacks at each time point in all cases. E, A partial view of a 4D assembly showing in greater detail perinucleolar replication foci assembly (boxed area). One replication focus present throughout (arrow) and two replication foci gradually assembling (arrowheads) are highlighted. Bar, 5 μm.

amples (5 versus 12 h). It is likely that different culture conditions contribute to these differences. Although the distribution of replication foci undergoes gradual and continuous changes, several characteristic patterns can be distinguished that are schematically illustrated in Fig. 3 C. During early S phase (Fig. 3 B, 0 h) hundreds of small foci are distributed throughout the nucleoplasm with the exception of the nucleoli. During mid S phase replication, foci are concentrated around the nucleoli (Fig. 3 B, 5 h) and in the nuclear periphery. In late S phase (Fig. 3 B, 8 and 12 h) replication foci decrease in number but increase in size, and often take on characteristic ring and horseshoe-like structures. We would like to point out that these characteristic patterns are most obvious in the mid-focal plane of the cell. In other focal planes, especially the early and mid S phase, patterns look different and the subdivisions are less evident. That might also explain the discrepancies in the number and order of these patterns in previous reports (Nakayasu and Berezney, 1989; Fox et al., 1991; O'Keefe et al., 1992). In addition, we have obtained similar results using human cell lines (data not shown). Our results clearly and directly show that replication foci in single cells undergo characteristic and reproducible changes throughout S phase that can thus serve as a marker for early, mid, and late S phase. These easily identifiable patterns are part of a sequence and change continuously throughout S phase.

### *Dynamics of Replication Factories in Living Cells*

The occurrence of different patterns of replication foci during S phase raises the question of how these changes are brought about. The transition from, e.g., an early to a mid S phase pattern could either be accomplished by the

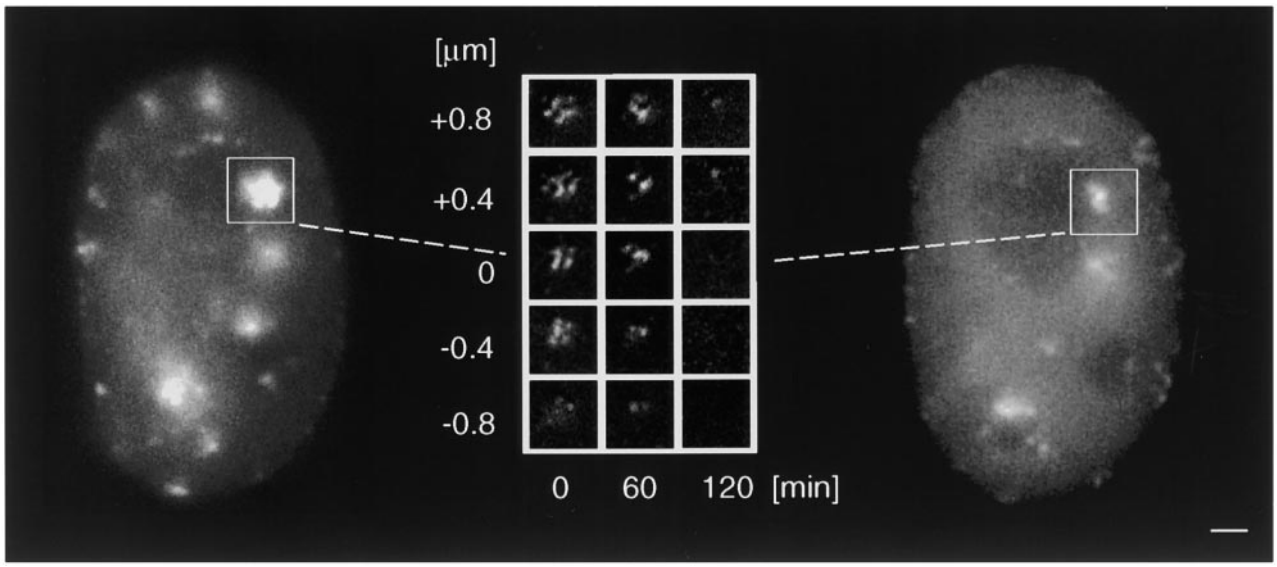


*Figure 5.* Larger replication foci are composed of independent smaller foci. One late S phase nucleus of a stably transfected cell was imaged over 2 h. Together with small size foci (less than 1-μm diam) several large foci are visible at the beginning of the experiment (left side nucleus). 2 h later some new small foci have formed and the large ones did not change their nuclear position, but decreased in size concomitantly with an increase in the disperse nucleoplasmic GFP–PCNAL2 protein (right side nucleus). Deconvolved images from different Z planes (middle) reveal that these large foci are made of clusters of small size foci which form and disassemble independently giving rise to the different shapes and sizes of the large foci over time. The signal (within the white box) seen in the undeconvolved whole nucleus after 2 h (right image) comes from out-of-focus light from the planes above. Bar, 1.5 μm.

movement, merging, and division of replication foci, or by the disassembly of foci and reassembly at new sites (Fig. 4 A). The only two subnuclear compartments examined to date in living cells are speckles and coiled bodies, which showed the first type of dynamics meaning change through directional movement and merging (Misteli et al., 1997; Boudonck et al., 1999; Sleeman and Lamond, 1999). In situ detergent and salt extraction experiments indicated that the components of these structures are insoluble, and it has often been proposed that they are tethered to an underlying nuclear skeleton. Nevertheless, these structures labeled in living cells show large scale directional movements. It is not known whether these structures move along this skeleton or whether they are moved along with the underlying skeleton.

Time-lapse series of replication foci were recorded and analyzed to test whether replication foci show directional movements as coiled bodies and speckles. The overlay of pseudocolored images from subsequent time points failed to detect any substantial movement of replication foci (Fig. 4 B). From a total of 500 foci, only five showed movement which, however, was confined to a limited area (four times their radius). Longer time-lapse series of 25 and 75 min (Fig. 4, C and D) again show that replication foci do not substantially move and that changes in the pattern occur by disappearance of foci and appearance of new ones at different places within the nucleus. Small scale movements around a given nuclear position can occur, but do not lead to changes in the replication patterns. We can rule out that foci just disappear by moving out of the focal plane since we collected, at each time point, complete Z-stacks covering the entire nucleus. An assembly of a subset of images from one 4D analysis is shown in Fig. 4 E. The formation of replication foci around one nucleolus shown in greater detail highlights one focus (arrow) present throughout the time course and two foci (arrowheads) that assembled gradually during the 25-min time period. The focal planes above and below show that these foci did not simply move from another focal plane, but form de novo and their relative positions are also quite stable.

These results are also not specific to one cell clone since these experiments were performed with three different clonal cell lines, and actually the series shown in Fig. 4, B, C, and D, are each done with a different cell line. These complete three-dimensional analyses over time failed to detect any directional movements. To exclude potential irradiation artifacts, we also collected images from single focal planes at shorter time intervals. From a total of 220 foci recorded every two minutes in mid to late S phase for up to 30 min, 20 foci disassembled and 40 new ones were formed.

A closer investigation of large replication foci (>1 μm diam) showed that they are composed of several independent smaller size foci (Fig. 5; see also late S phase schematic illustration Fig. 3 C). The smaller foci are so close together in the nucleus that under normal conditions they are not distinguishable as discrete units (Fig. 5, left and right whole nucleus). Deconvolution of Z axis series of images significantly increases the resolution by removing out-of-focus light and reveals that these smaller foci do not fuse with each other and seem to assemble and disassemble independently of their immediate neighbors (Fig. 5, middle). These different lifetimes generate the different shapes and sizes which can be observed in large foci over time.

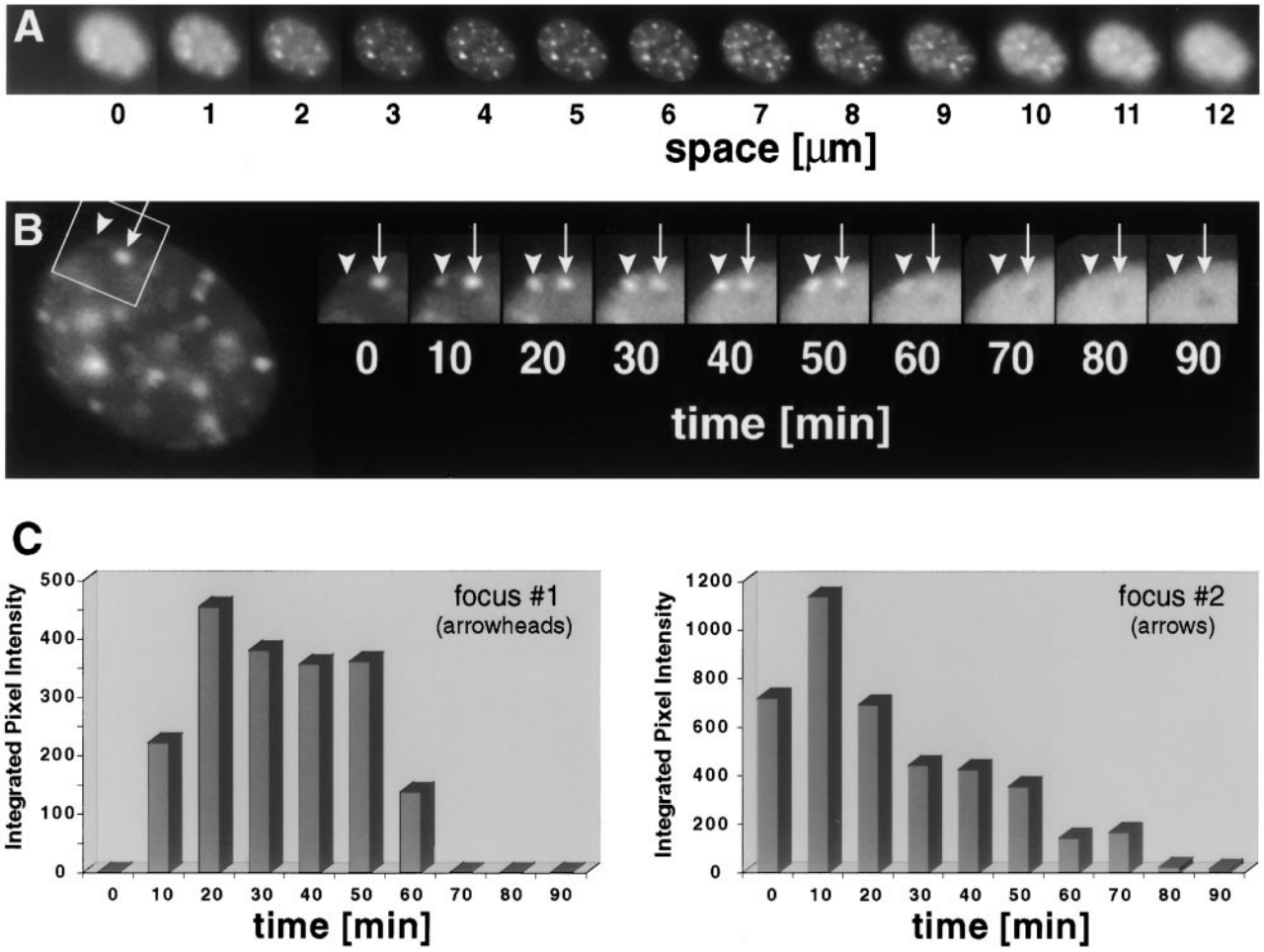


*Figure 6.* 4D analysis of replication factory assembly during S phase. A, Stably transfected cells were grown in a perfusion chamber and images were collected at high speed in different focal planes throughout the nucleus. Images were collected at 0.5-μm Z axis intervals and we show here every other section. B, Stacks of images, as shown in A, were collected in 10-min intervals. Changes occurring at two foci over a period of 90 min are shown at higher magnification. Notice the distance between these two foci remains constant, but the signal intensity changes over time. At the end of this time course, most GFP–PCNAL2-labeled foci disassembled and the corresponding protein is now dispersed throughout the nucleoplasm indicating the cell has entered G2 phase. C, The signal intensities above the nucleoplasmic GFP–PCNAL2 signal for the two foci shown in B were quantified and expressed as arbitrary units of integrated pixel intensities over time.

### *Four-dimensional Analysis of Replication Factory Assembly during S Phase*

Each replication focus is composed of several active replicons (Jackson and Pombo, 1998; see also Fig. 3 C). Thus, the appearance and disappearance of foci might be caused by either simultaneous assembly and disassembly of replication machines at all replicons within a focus or by a sequential process. In addition, all foci within one cell could be all assembled and disassembled in either a synchronous or an asynchronous fashion. To test these two hypotheses, we analyzed the dynamics of replication foci in space and time. Z-stacks focusing throughout the entire nucleus (Fig. 6 A) were collected in ten-minute intervals. Fig. 6 B shows at higher magnification an equatorial section through one S phase nucleus and the dynamics of two foci over a time period of 90 min. This time-lapse series again shows that these two foci do not change their relative nuclear position. A look at this series of images and also the quantification in Fig. 6 C clearly show that focus #1 (marked with an arrowhead) gradually forms and disassembles over about one hour. Interestingly, focus #2 (marked with an arrow) exists before focus #1 is formed.

From these results we conclude that replication machines are assembled and disassembled at the replicons within one focus, not in a synchronous fashion, but rather in a gradual and sequential mode. One possible interpretation of these results would be that all replicons within one focus become activated at a specific time during S phase and that the assembly of replication machines at individual origins within a cluster occurs within a short time, but in a random fashion. If then every replication machine is active for a specific time of ~30–50 min also, the disassembly would occur in an asynchronous fashion as shown in Fig. 6. But clearly, the assembly of replication machines within a cluster has to be coordinated in a time scale of minutes for the cluster to be microscopically visible as a replication focus over about one hour.

4D image analysis of replication factories of small (~0.25 μm), medium (~0.75 μm), and large (~1.25 μm) size from nuclei derived from three different cell clones showed, even within individual cells, a vast heterogeneity not only in their size, but also in their lifetimes. In these mid to late S phase cell nuclei the lifetime of replication factories varied between 30 min and 3 h. In general, there seemed to be a small preference for large foci to have longer lifetimes. However, no strict correlation between size and lifetime was found since larger foci with short lifetimes (~30 min) and small foci with long lifetimes (~3 h) were observed (data not shown). As shown in Fig. 5, large foci are probably composed of smaller foci, and their lifetime could therefore depend on the degree of synchrony of their individual subfoci.

## *Discussion*

DNA replication has been studied with a variety of different techniques, but we are still far from really understanding how the mammalian genome is replicated once, and only once, per cell cycle in a precise and coordinated manner. A lot of work has been done on the DNA side of this process with sophisticated labeling approaches, but little is known about the regulation and dynamics of the replication machinery itself in living cells. Since there has been some confusion about the terminology, we define replication foci as subnuclear sites of ongoing DNA replication, which is not only a morphological, but also a functional definition, as these subnuclear sites can be directly or indirectly labeled by short pulses with fluorescently or antigen-labeled nucleotides, respectively (Fig. 2 B, and data not shown). In addition, several nuclear proteins are found associated with these replication foci and thus serve as markers for these sites (Fig. 2 A). At the molecular level DNA replication is initiated at origins that control the duplication of a flanking stretch of DNA called replicon. Replication proteins and their auxiliary factors involved in bidirectional replication initiated at one origin are organized in complexes, referred to as replisomes or replication machines. Several of these replicons with their attached replisomes are organized in clusters, termed clustersomes or replication factories, and are visible by fluorescence microscopy as subnuclear foci.

The results presented here clearly show that replication foci, unlike coiled bodies and speckles, do not change position within the nucleus suggesting that they are stably anchored (Figs. 4–6). This immobilization could be caused by binding to the DNA being replicated and/or attachment to an underlying nuclear skeleton. Both possibilities are by no means mutually exclusive and chromosome territory organization recently has been shown to rely on association with the nuclear skeleton (Ma et al., 1999). Early biochemical fractionation experiments showed that large replication enzyme complexes are bound to some insoluble nuclear structures (Tubo and Berezney, 1987), but these biochemical studies can obviously not rule out active or passive movements within the nucleus, as exemplified by coiled bodies. Pulse-labeling experiments analyzed by EM showed that the distance between replicated DNA and replication factories increases with time (Hozak et al., 1993), but could not discriminate which of the components moved and/or whether new replication factories assembled in the vicinity. Our live cell studies clearly show that replication machines and factories are immobilized in mammalian nuclei, which implies that the genomic DNA is spooled through these complexes. Obviously, we cannot rule out movements of individual replication machines at the molecular scale that are beyond the resolution of a light microscope. Interestingly, similar results were recently obtained in bacterial cells, i.e., the *Bacillus subtilis* DNA polymerase was found to be not randomly distributed, but rather in fixed intracellular positions, indicating that stationary replication factories also exist in prokaryotes (Lemon and Grossman, 1998).

Studies of fixed and living cells indicated relatively stable nuclear positions of chromosomal loci, subchromosomal foci or domains, and DNA sequences with a particular DNA replication timing (Shelby et al., 1996; Ferreira et al., 1997; Marshall et al., 1997; Zink et al., 1998; Bornfleth et al., 1999; Manders et al., 1999; Sadoni et al., 1999). The activation of replisome clusters at fixed sites within the nucleus and at different times during S phase would then generate characteristic and dynamic patterns of replication foci (Fig. 3). Whether each replication factory indeed replicates one subchromosomal focus remains to be studied. The sequential assembly and disassembly of replication factories shown here is not in contradiction with earlier studies using DNA fiber autoradiography, from which it was concluded that clusters of adjacent replicons are replicated synchronously (Edenberg and Huberman, 1975; Hand, 1978). In fact, the heterogeneity in the size of replication units observed in those studies could at least in part be explained by sequential activation of replication machines within one cluster. A recent reevaluation of these fiber autoradiography data revealed a large heterogeneity of replicon size and asynchrony in origin firing (Berezney et al., 2000).

Furthermore, our results show that replication foci within one nucleus do not appear and disappear in a synchronous fashion. In other words, early, mid, and late replication does not occur in distinct waves, but replication foci are rather continuously assembling and disassembling throughout S phase, leading to a sequence of theoretically infinite number of patterns with some patterns arbitrarily chosen as characteristic landmarks. Furthermore, replicons do not seem to be activated synchronously in waves. The continuous activation of replicons and the assembly of replication machines throughout S phase requires the continuous presence of an activator, which fits with our previous observation that cyclin A and cdk2 are present at early, mid, and late replication foci (Cardoso et al., 1993). This higher-order nuclear organization may thus provide the framework for the efficient and precise coordination and integration of cell cycle regulation and genome duplication.

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