

# Stable chromosomal units determine the spatial and temporal organization of DNA replication

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## Summary

DNA replication occurs in mammalian cells at so-called replication foci occupying defined nuclear sites at specific times during S phase. It is an unresolved problem how this specific spatiotemporal organization of replication foci is determined. Another unresolved question remains as to what extent DNA is redistributed during S phase. To investigate these problems, we visualized the replicating DNA and the replication machinery simultaneously in living HeLa cells. Time-lapse analyses revealed that DNA was not redistributed to other nuclear sites during S phase. Furthermore, the results showed that DNA is organized into stable aggregates equivalent to replication foci. These aggregates, which we call sub-chromosomal foci, stably

maintained their replication timing from S phase to S phase. During S-phase progression, the replication machinery sequentially proceeded through spatially adjacent sets of sub-chromosomal foci. These findings imply that the specific nuclear substructure of chromosomes and the order of their stable subunits determine the spatiotemporal organization of DNA replication.

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Key words: Replication dynamics, Replication foci, Sub-chromosomal foci

## Introduction

In mammalian cell nuclei, DNA replication takes place at so-called replication foci. Here, proteins involved in DNA replication are assembled into microscopically visible functional complexes (Leonhardt et al., 2000b). The proliferating cell nuclear antigen (PCNA) is a central component of these complexes (Wyman and Botchan, 1995). At replication foci, nucleotide analogues like bromodeoxyuridine (Berezney et al., 1995b; Gratzner, 1982) or fluorescently labelled nucleotides (Manders et al., 1999; Pepperkok and Ansorge, 1995; Zink et al., 1998) are incorporated into nascent DNA, which gives rise to typical focal DNA labelling patterns in pulse-labelling experiments. A large percentage of individually labelled DNA foci correspond to replicon clusters rather than to single replicons (Berezney et al., 2000; Jackson and Pombo, 1998). The average DNA content of a replicon cluster organized into a microscopically visible focus is about 1 megabase pair (Mb), although the DNA content of individual foci may vary considerably (Berezney et al., 2000).

It has been observed that focal DNA labelling patterns obtained after pulse-labelling with nucleotide analogues were maintained at subsequent cell cycle stages and cell cycles (Berezney et al., 1995b; Jackson and Pombo, 1998; Ma et al., 1998; Sparvoli et al., 1994; Zink et al., 1999; Zink et al., 1998). Based on this observation, it has been suggested that DNA might be organized into stable aggregates equivalent to replication foci. One way to prove that microscopically visible foci represent such stable DNA aggregates is to show

that individual DNA foci labelled during the first S phase convert into replication foci again at subsequent S phases. Corresponding experiments have been performed with cells synchronized at the G1/S border, which have been pulse-labelled with two different nucleotide analogues at the very beginning of two consecutive S phases (Jackson and Pombo, 1998; Ma et al., 1998). The results reveal a high degree of colocalization at nuclear sites first initiated during S phase (Jackson and Pombo, 1998; Ma et al., 1998). These findings indicate the presence of stable DNA aggregates corresponding to the earliest replicating foci, which stably maintain their replication timing. Nevertheless, it has been difficult to show exact colocalization at the level of individual foci and the question remains whether replication timing is stably maintained for the chromosomal regions replicating later during S phase. Given these uncertainties, the question whether chromosomes are organized into stable subunits equivalent to replication foci, that stably maintain their replication timing, remains controversially discussed.

Another unresolved question with regard to the S phase-related organization of DNA is whether and to what extent DNA is redistributed within the nucleus during S phase. Previous results obtained with fixed HeLa cells suggest that nascent DNA is extruded from replication sites (Hozak et al., 1993). In addition, recent studies addressing replication dynamics in living cells of *Bacillus subtilis* imaged by light microscopy propose that the DNA template moves through a stationary replisome (Lemon and Grossman, 1998; Lemon and Grossman, 2000). In addition, DNA double pulse-labelling

experiments performed with mammalian cells suggested that newly replicated DNA gradually moves away from the site of replication (Manders et al., 1992; Manders et al., 1996). Thus, previous work suggests that DNA is dynamically redistributed to other nuclear sites during S phase. However, the difficulty with DNA double pulse-labelling experiments is that they only visualize the DNA. Therefore, there were difficulties with the interpretation of previous results as it remained unclear how the replication machinery contributed to the dynamics observed in mammalian cells. It was still an open question whether only the DNA moves, whether the replication machinery moves, or whether both move (see Manders et al., 1996). Recent results obtained with living mammalian cells show that the replication machinery is more dynamic than previously thought (Leonhardt et al., 2000a; Sporbert et al., 2002). The results reveal that new GFP-PCNA foci assemble adjacent to previously active replication foci during S-phase progression (Leonhardt et al., 2000a; Sporbert et al., 2002). However, these studies did not address the corresponding DNA dynamics and therefore it remained unclear whether and in which way DNA rearrangements were involved in the processes observed. These difficulties with the interpretation of previous results showed that the question of how the replication machinery and the DNA contribute to S-phase dynamics can only be resolved by investigating both simultaneously.

One of the most striking features regarding the dynamic organization of S phase is that replication foci appear at specific nuclear sites during specific temporal stages of S phase (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992). Therefore, it is also said that foci display a characteristic spatiotemporal organization. One of the most intriguing but unresolved questions is how the formation of the characteristic patterns of replication foci is spatially and temporally coordinated. The finding that the replication machinery disassembles and reassembles at adjacent sites during S-phase progression suggests that rearrangements of the chromatin/chromosome structure at sites adjacent to actively replicating foci might play a role in the spatiotemporal coordination of S-phase progression (Sporbert et al., 2002). However, as the underlying chromosomal structure was unclear it was difficult to determine how it might contribute to the coordination of S phase.

Here, we analysed the interaction between the replication machinery and replicating DNA in living HeLa cells. We aimed to address what kind of dynamic rearrangements DNA shows during S phase, whether chromosomes are organized into stable subunits equivalent to replication foci, which stably maintain their replication timing and how the chromosomal substructure contributes to S-phase coordination. Our analyses revealed local DNA rearrangements at replication sites but no redistribution of DNA to other nuclear regions during S phase. Furthermore, the results demonstrated that chromosomes are organized into stable subunits equivalent to replication foci that stably maintain their replication timing from S phase to S phase. We call these stable chromosomal units sub-chromosomal foci. In addition, the data revealed that during S-phase progression, adjacent and defined sets of sub-chromosomal foci occupying defined nuclear positions became sequentially activated. Thus, during the temporal progression of S phase, the replication machinery followed specific spatial

arrangements of sub-chromosomal foci. These findings imply that the spatiotemporal organization of DNA replication is determined by the specific nuclear order of these stable chromosomal subunits.

## Materials and Methods

### Cell culture and replication labelling

HeLa S6 cells were cultured and microinjected into the nucleus as described (Zink et al., 1998). To observe synthesis of nascent DNA at replication sites, cells were microinjected with a GFP-PCNA expression plasmid at 2 ng/ml in PBS (Leonhardt et al., 2000a) and on the next day with 100  $\mu$ M Cy3-dUTP (Amersham Pharmacia Biotech) before time-lapse microscopy. For triple labelling, cells were first microinjected 4 hours after release from a mimosine block (16 hours, 200  $\mu$ M; Sigma) with a mixture of the GFP-PCNA expression plasmid and Cy3-dUTP and 1.5 hours later with 100  $\mu$ M Cy5-dUTP (Amersham Pharmacia Biotech). Microscopy was performed in this case on the next day. Cells were fixed for 10 minutes at room temperature in 3.7% formaldehyde in PBS.

### Microscopy

Cells were imaged with a Zeiss confocal laser-scanning microscope 410 or 510. For live cell microscopy cells were mounted with conditioned medium supplemented with 1 mM Trolox (Sigma) into a FCS2 chamber (Bioptechs, Inc.) maintained at 37°C. The pixel size of the images was between 70 $\times$ 70 nm and 100 $\times$ 100 nm. The distances between the focal planes were between 300 nm and 700 nm. During time series images were captured every 17-25 minutes. The exposure time was 4.3 seconds/channel and nuclear plane.

### Image analysis

Image analysis was performed with Metamorph software (version 4.5, Universal Imaging) and Image J software (1.29h, public domain, <http://rsb.info.nih.gov/ij>). For visual inspection, regions of interest were selected and projections were made from all focal planes covering objects within the region of interest. After analyzing a projection, the corresponding gallery including focal planes above and below the planes selected for the projection was re-inspected to exclude false interpretations of the observed rearrangements in the three-dimensional (3D) space. For further analysis we concentrated on rearrangements observed in the *x,y* plane, as here the microscopic resolution is optimal and no preferred directions for rearrangements were observed.

For measuring changes in the distances between pairs of DNA foci during and after their replication, the 3D distances between the intensity centres were measured using the confocal image stacks and Image J software. For investigating the appearance of GFP-PCNA foci adjacent to Cy3-labelled foci, distance measurements between GFP-PCNA foci and Cy3-labelled DNA were performed on single light optical sections. For all GFP-labelled foci detected in a single nuclear plane the nearest Cy3-labelled foci were defined by visual inspection (two people performed the analysis independently). The intensity centres of the neighbouring GFP- and Cy3-labelled foci were determined using the Image J software and the distances between the intensity centres of the neighbouring foci were calculated. As the analysis was performed on single light optical sections, the number of neighbouring foci in adjacent focal planes might be underestimated. Therefore, the minimum number of GFP-labelled foci found in close vicinity (less than 1  $\mu$ m) to Cy3-labelled foci was ~94% at 90 minutes after microinjection.

Colocalization analysis was performed on single light optical sections and all focal planes of a given nucleus were evaluated. Individual objects (foci) were defined and counted and the numbers

of objects (e.g. Cy3-labelled foci) colocalizing or not colocalizing with another class of defined objects (e.g. GFP-PCNA foci) were determined using Metamorph software. Only objects consisting of at least four neighbouring pixels (one pixel: 100×100 nm) were counted. For determining the distances between the intensity centres of colocalizing GFP-PCNA and DNA foci, the 3D distances between the intensity centres were measured using the confocal image stacks and Image J software.

#### Mathematical analysis

Calculations were performed with Mathematica 4.2.

## Results

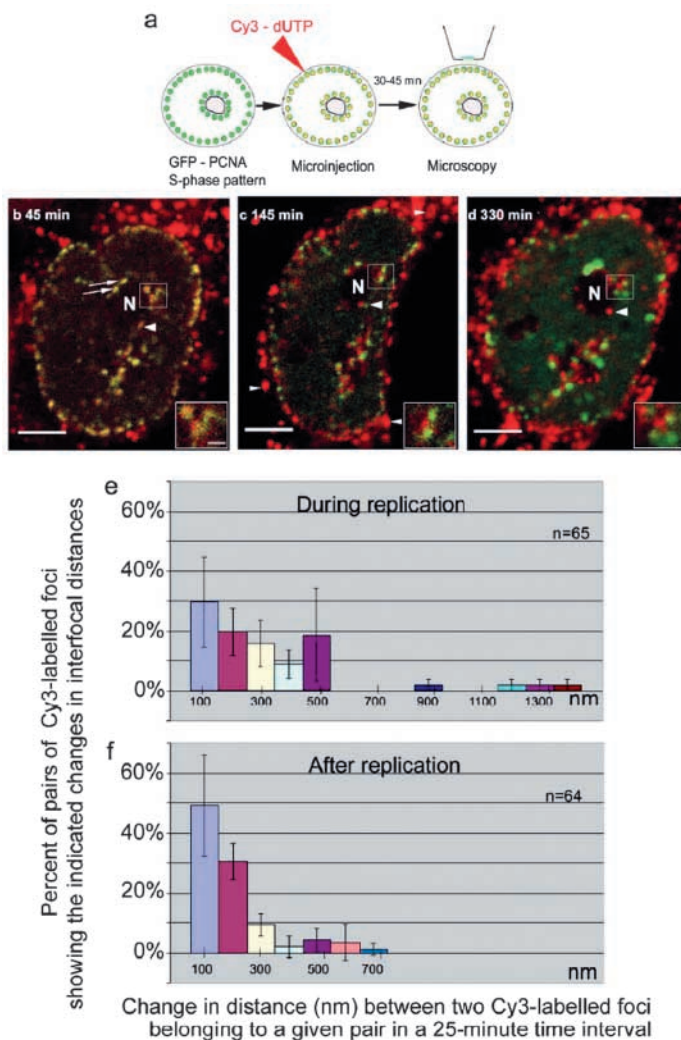
### Synthesis of nascent DNA involves only local dynamics

In order to analyse the dynamics of DNA during S phase, we first investigated the synthesis of nascent DNA in living HeLa S6 cells. Functional complexes of replication, proteins were labelled for live cell microscopy with a GFP-PCNA fusion protein as described (Leonhardt et al., 2000a; Sporbert et al., 2002). HeLa cells displaying typical S-phase patterns of the fusion protein were microinjected with Cy3-dUTP, which is incorporated into nascent DNA during DNA replication (Zink et al., 1998). After microinjection, cells were transferred to the

confocal microscope (for the experimental procedure see Fig. 1a). Owing to the time required to mount the cells and to set the conditions on the microscope, imaging of the time series started between 30 and 45 minutes after microinjection. Confocal imaging was performed in all cases, so that three-dimensional information was available for all nuclei and time points (see Materials and Methods). Each cell was imaged for several hours.

Fig. 1b-d shows a cell nucleus imaged until 330 minutes after microinjection. Forty-five minutes after microinjection this nucleus showed a pattern typical for mid S phase (Leonhardt et al., 2000a; O'Keefe et al., 1992; Sadoni et al., 1999) with many replication foci concentrated at the nuclear and nucleolar peripheries. At this time point all GFP- and Cy3-labelled foci colocalized (yellow in Fig. 1b), indicating the synthesis of nascent DNA at all GFP-labelled foci, but no synthesis of nascent DNA at non GFP-labelled sites. Subsequent time points (Fig. 1c,d) displayed patterns of GFP-PCNA typical of later stages of S phase with progressively fewer and larger foci (Leonhardt et al., 2000a; Sadoni et al., 1999). Synthesis of nascent DNA at all GFP-labelled sites as well as normal S-phase progression through the typical sequence of patterns confirmed that the labelled replication foci were functional and, importantly, displayed a normal spatiotemporal behaviour during imaging.

During S-phase progression, separation of newly synthesized DNA and GFP-labelled foci was observed. Evaluation of the Cy3-labelling patterns revealed that nascent DNA remained stably associated with its sites of synthesis at the nuclear and nucleolar peripheries (Fig. 1b-d). This is consistent with the results of our previous extensive analyses of the dynamic behaviour of replication labelled DNA foci, demonstrating that DNA foci mainly perform slow and locally confined small-scale rearrangements (Bornfleth et al., 1999; Edlmann et al., 2001; Zink et al., 1998). In order to investigate the dynamic behaviour of DNA foci during and after their replication we measured the distances between the two foci of

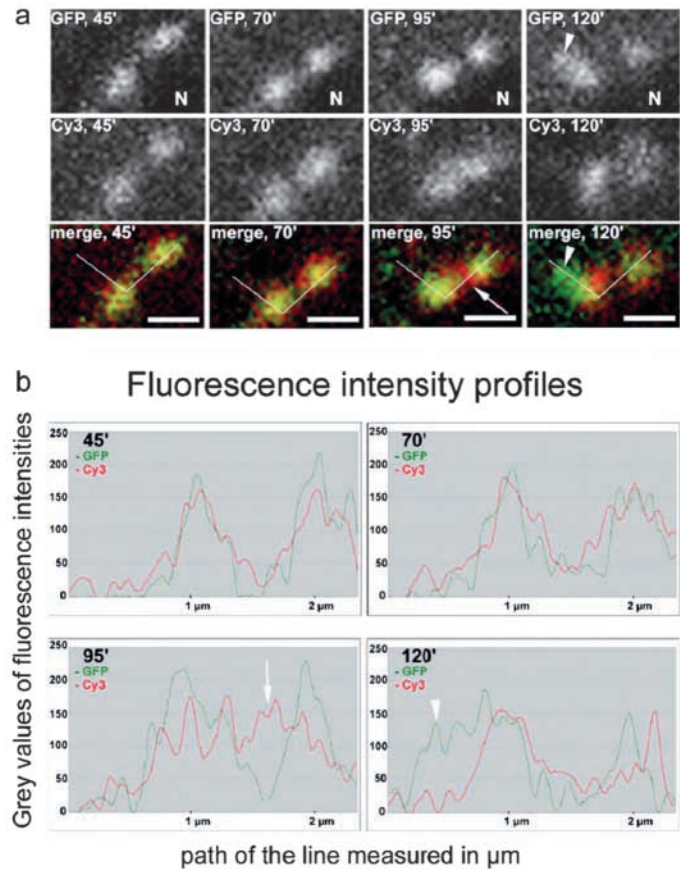


**Fig. 1.** Overall nuclear dynamics during S-phase progression in living HeLa cells. (a) Labelling scheme. (b-d) Time series (time points indicated in minutes after microinjection) of a double-labelled nucleus (green, GFP-PCNA; red, Cy3). Colocalizing Cy3 and GFP fluorescence appears yellow. Each panel displays a projection of four focal planes ( $\Delta z=600$  nm). The GFP-PCNA pattern proceeds from a mid (45 minutes) to a late (330 minutes) S-phase pattern. After initial colocalization of nascent DNA with GFP-PCNA foci (45 minutes), GFP-PCNA foci appear at sites adjacent to nascent DNA at 145 minutes and at increasingly distant sites during S-phase progression (see enlargements of the framed regions in the insets). The large arrowhead indicates a region where GFP-PCNA foci disappeared after DNA synthesis. Two replication sites (small arrows) at the nucleolar (N) periphery are shown enlarged in Fig. 2. Small arrowheads indicate Cy3-labelled cytoplasmic vesicles. Bar, 5  $\mu$ m; inset bar, 1  $\mu$ m. (e,f) The distance between the two foci of a given pair of Cy3-labelled DNA foci was measured at two different time points ( $\Delta t=25$  minutes). The bars indicate the percentages of pairs of foci showing changes in their distances within a given interval (0-100 nm, 101-200 nm etc.) after 25 minutes. (e) To determine DNA dynamics during replication, it was ensured that at both time points both DNA foci of a given pair colocalized with GFP-PCNA. (f) DNA dynamics after replication of the foci were measured in the same nuclei at later time points, when GFP-PCNA foci occupied different nuclear regions. n, number of pairs of foci evaluated.

a given pair of DNA foci at two different time points ( $\Delta t=25$  minutes). To determine the dynamics of DNA foci during their replication, it was ensured that at both time points both DNA foci of a given pair colocalized with GFP-PCNA. DNA dynamics after replication were measured in the same nuclei at later time points, when GFP-PCNA foci occupied different nuclear regions. The results of these measurements are shown in Fig. 1 (e and f). During, as well as after their replication, the relative positional changes of DNA foci did not exceed  $0.5 \mu\text{m}$  in about 95% of cases. A tendency towards greater positional changes was observed during replication. This is consistent with enhanced local chromatin rearrangements, which is not surprising given the fact that twice the amount of DNA has to be organized at a given nuclear region when active replication foci are present (for local DNA rearrangements during replication see also Figs 2 and 3). However, also during replication about 95% of the relative positional changes did not exceed  $0.5 \mu\text{m}$  and even the most extreme positional changes observed did not exceed  $1.4 \mu\text{m}$ . These results are consistent with the visual impression that DNA shows only local rearrangements and is not transferred to other nuclear regions during its replication or after its replication. In addition, these results are consistent with our previous investigations addressing the dynamics of replication labelled DNA foci (Bornfleth et al., 1999; Edlmann et al., 2001; Zink et al., 1998).

Comparison of the Cy3- and GFP-labelling patterns revealed that the separation of nascent DNA and the replication machinery was caused by the appearance of GFP-PCNA foci during S-phase progression at positions adjacent to sites of previous DNA synthesis (Fig. 1b,c). It should be noted that no increasing amounts of labelled nascent DNA were produced during S-phase progression, as the nuclear pool of labelled nucleotides was depleted  $\sim 1.5$  hours after microinjection due to the stable incorporation of labelled nucleotides into cytoplasmic vesicles (see Fig. 1c). A quantitative analysis (see supplementary material) confirmed the visual impression (see Figs 1 and 2) that new GFP-labelled foci first appeared predominantly at distances of less than  $1 \mu\text{m}$  from the previously synthesized DNA. The observed GFP-PCNA dynamics are in accordance with previous results (Leonhardt et al., 2000a; Sporbert et al., 2002). Furthermore, the data showed that during further S-phase progression GFP-PCNA foci appeared at sites progressively more distant from sites of previous DNA synthesis (see insets in Fig. 1b-d) or disappeared completely from regions of previous DNA synthesis (Fig. 1b-d), where the previously synthesized DNA could still be observed. This is consistent with the fact that the numbers of replication foci decrease during S-phase progression.

For a closer evaluation of the dynamic interactions between nascent DNA and GFP-PCNA foci, we analysed individual replication sites associated with the nucleolar periphery. Here, the relative localization of labelled structures could be determined with regard to this nuclear landmark. For the analysis of small-scale dynamics we concentrated on alterations taking place in the  $x,y$  planes, as here the microscopic resolution is optimal and no preferred directions for rearrangements were observed. However, in all cases, the focal planes above and below the analysed foci were inspected (see Fig. 3c) to exclude misinterpretations due to focal shifts



**Fig. 2.** Local dynamics at DNA replication sites in living HeLa cells. (a) Enlargements of two replication sites associated with the nucleolar (N) periphery (labelled with small arrows in Fig. 1) at the indicated time points (minutes after microinjection). GFP and Cy3 fluorescence and the corresponding merged images are shown (green, GFP; red, Cy3). At 95 minutes the nascent DNA synthesized at the two GFP-labelled sites is translocated between these two sites (arrow). At 120 minutes intense GFP fluorescence appears at a site previously devoid of GFP-PCNA (arrowhead), adjacent to a previous site of synthesis where the nascent DNA is still associated with the nucleolar periphery. (b) Fluorescence intensity profiles along the lines indicated on the merged images in a. The  $x$ -axis indicates the path of the line measured in  $\mu\text{m}$  with 0 corresponding to the left end of the line. Arrow and arrowhead point to the same regions as in a. Bars,  $1 \mu\text{m}$ .

and contributions from adjacent foci in other nuclear planes and from movements in the  $z$  direction.

Two replication sites marked with arrows in Fig. 1 are shown in detail in Fig. 2. Until 70 minutes after microinjection, nascent DNA colocalized at these two sites with the GFP-PCNA foci (green), which produced the labelled DNA in this region. However, by 95 minutes after microinjection, a part of the nascent DNA appeared between these two GFP-labelled foci (Fig. 2, arrow). As the data revealed that labelled DNA is not produced at sites not labelled by GFP (Fig. 1, 45 minutes and Fig. 3, 32 minutes) this showed that the nascent DNA previously synthesized at the two GFP-PCNA foci has been located between these foci at 95 minutes. At 120 minutes after microinjection the nascent DNA was reorganized into two well-separated foci (see also line-scans in Fig. 2b) associated

with the nucleolar periphery, where it remained for the rest of the imaging period. The possibility that the labelled DNA observed here has been relocalized from another nuclear plane was excluded by examining the corresponding sections above and below (example shown in Fig. 3c). Together, the results demonstrate again that nascent DNA displayed some rearrangements, which were, however, locally confined within a 1  $\mu\text{m}$  distance (Fig. 2b) and did not lead to a redistribution of nascent DNA to other nuclear sites.

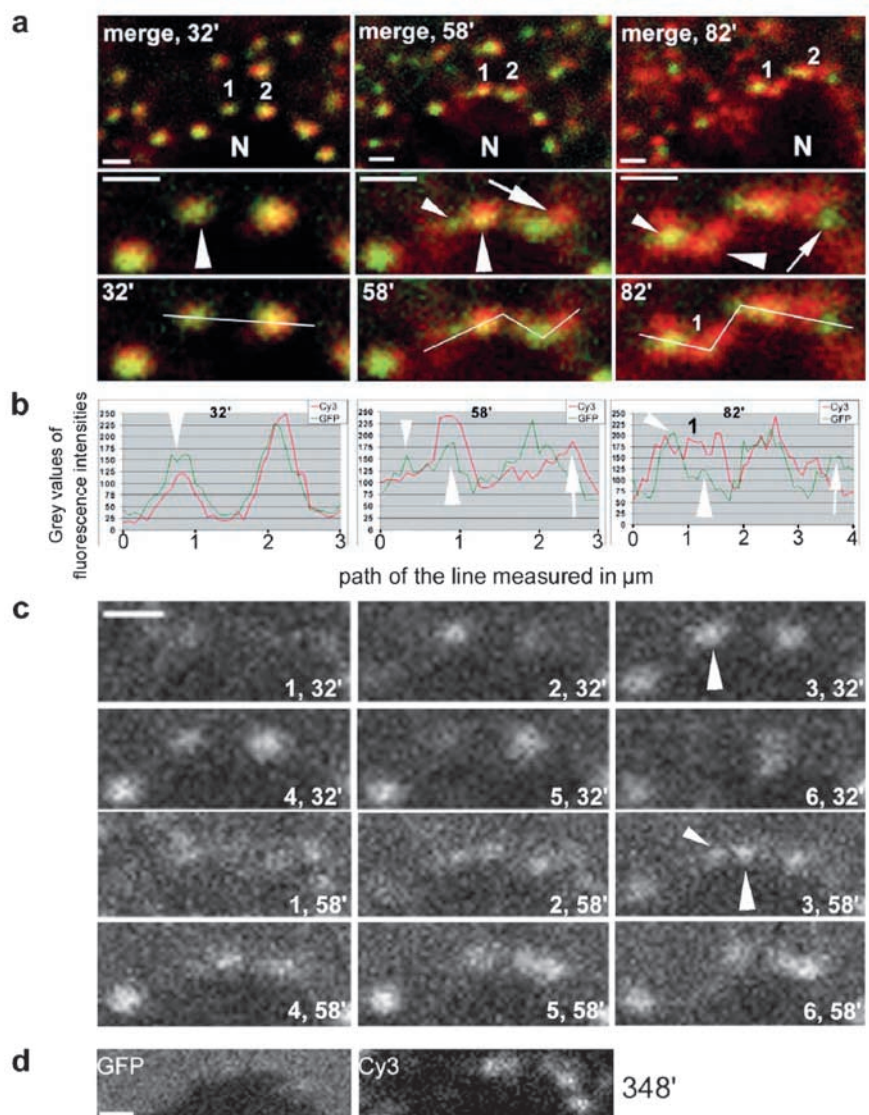
Regarding the dynamics of GFP-PCNA foci, the replication sites shown in detail in Fig. 2 remained separated and associated with the nucleolar periphery until 95 minutes after microinjection. However, at 120 minutes, a GFP-labelled focus appeared in an adjacent region to the lower, left replication site (Fig. 2, arrowhead). The GFP-labelled focus appeared at a distance of less than 1  $\mu\text{m}$  from the previous site of synthesis, but further away from the nucleolar periphery. This region adjacent to the previous site of synthesis was largely devoid of nascent DNA. Previous photobleaching data did show that the appearance of GFP-PCNA at adjacent sites is not because of movement of GFP-PCNA previously present there, but rather

to the new assembly of GFP-PCNA recruited from the nucleoplasmic pool (Sporbert et al., 2002).

Fig. 3 illustrates the activation of adjacent nuclear sites in more detail. Here, GFP- and Cy3-labelled foci colocalized at the nucleolar periphery at the beginning of the imaging period (32 minutes). Around one hour after microinjection (58 minutes) the replication machinery and the nascent DNA separated from each other and new GFP-labelled foci appeared at adjacent sites devoid of nascent DNA (small arrowhead, site 1, 58 minutes). It should be noted that at this transition stage, where adjacent sites became occupied, the GFP-PCNA foci at those sites first active slowly disappeared (Fig. 3a and b, compare 58 minutes and 82 minutes, large arrowheads). In parallel, the new foci at adjacent sites became increasingly intensely labelled and started also to produce nascent DNA (Fig. 3a and b, compare 58 minutes and 82 minutes, small arrowheads).

The nascent DNA also showed some local rearrangements. For example, the nascent DNA at site 2 shown in Fig. 3 was in the beginning (32 minutes) closely associated with the nucleolar periphery, where it colocalized with a corresponding

**Fig. 3.** DNA shows only local rearrangements whereas the replication machinery appears at neighbouring sites. The nucleus was labelled according to the labelling scheme depicted in Fig. 1a. (a) Time series (minutes after microinjection) of a perinucleolar region. The nucleolus appears dark and is labelled N. Projections representing four consecutive light optical sections ( $\Delta z=300$  nm) are depicted for each time point. The merged images of GFP (green) and Cy3 fluorescence (red) are shown with colocalization appearing yellow. The upper three panels represent the whole area whereas enlargements of two regions of nascent DNA synthesis labelled 1 and 2 are displayed in the lower six images. A large arrowhead indicates the first site of synthesis at region 1. A small arrowhead points to a GFP-labelled focus appearing in a closely adjacent region first visible at 58 minutes. Nascent DNA extruded away from the nucleolar border at 58 minutes at site 2 is labelled with a large arrow (the GFP-labelled focus at site 2 remains associated with the nucleolar periphery). At 82 minutes all nascent DNA is again closely associated with the nuclear periphery. In regions at the nucleolar periphery so far devoid of nascent DNA, new GFP-PCNA foci appeared. A small arrow indicates a GFP-labelled focus that appeared at 82 minutes in such a region devoid of nascent DNA. (b) Fluorescence intensity scans along the lines indicated on the images in a (arrowheads and arrows point to the same regions as in a), with 0  $\mu\text{m}$  corresponding to the left end of the line in a. (c) Consecutive light optical sections (confocal raw data,  $\Delta z=300$  nm) from the enlarged region at 32 minutes and at 58 minutes (arrowheads as in a and b). The new GFP-labelled focus appearing at the left of site 1 at 58 minutes did not relocalize to this site from another nuclear plane. (d) Projections of three consecutive light optical sections from the same perinucleolar area at 348 minutes after microinjection. The nucleus progressed normally into G2. During G2, GFP-PCNA is uniformly distributed over the extranucleolar nuclear regions. The nucleolus appears dark and the Cy3-labelled DNA is still associated with the nucleolar periphery. Bars, 1  $\mu\text{m}$ .



GFP-labelled focus. At 58 minutes this DNA previously produced at the nucleolar periphery localized farther away from the nucleolar periphery (large arrow, 58 minutes), whereas the corresponding GFP-PCNA focus remained closely associated with the nucleolus. However, at 82 minutes all nascent DNA was again closely associated with the nucleolar periphery, where it remained during further S-phase progression. Fig. 3d shows the same perinucleolar region at 348 minutes. The uniform distribution of GFP-PCNA in the nucleoplasm indicates that the nucleus was already in G2 at 348 minutes. However, the Cy3-labelled DNA produced earlier at the nucleolar periphery (note that Cy3-dUTP is only incorporated for a restricted time period) was still associated with the nucleolar periphery.

In summary, the analysis of overall nuclear patterns as well as of local dynamics of individual replication sites showed that no large-scale dynamics were involved in the synthesis of nascent DNA. Nascent DNA showed some local rearrangements but remained associated with its sites of synthesis and was not redistributed to other nuclear sites. GFP-PCNA foci appeared first at adjacent sites devoid of nascent DNA during S-phase progression and then at progressively more distant sites. Previously present GFP-foci disappeared in parallel at earlier active sites, where the previously synthesized DNA was left stably positioned. Thus, separation of nascent DNA and the replication machinery during S-phase progression did not involve redistribution of the DNA to other nuclear sites, but resulted from the dynamics of the replication machinery. To confirm that these observations represented the general behaviour of replication foci and nascent DNA, we analysed 20 randomly selected nuclei representing different stages of S phase and containing thousands of replication sites. The analysis of overall nuclear patterns as well as 60 arbitrarily chosen replication sites confirmed the results described above. In particular, the analysis of nuclei from earlier stages of S phase (see supplementary material, Fig. S1) indicated that the results obtained here represent a general feature of S phase and are not specific for a particular stage of S phase or a specific nuclear sub-region.

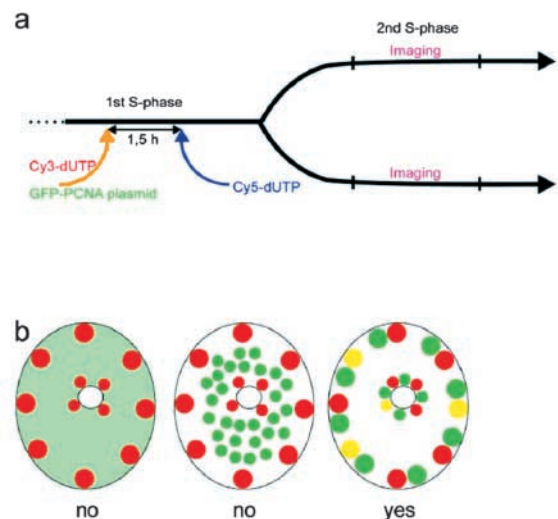
#### Interaction of the replication machinery with DNA foci labelled in the previous S phase

In a complementary set of experiments, we investigated the interaction of the replication machinery with DNA foci labelled in the mother cell. For this purpose HeLa S6 cells were blocked with mimosin at the G1/S transition. This single synchronization step was performed in order to obtain a higher yield of S-phase cells (about 71% after synchronization) during labelling by microinjection. Four hours after release, cells were microinjected with the GFP-PCNA expression plasmid and Cy3-dUTP. Imaging was performed on the next day during the first S phase after labelling (see labelling scheme in Fig. 4a). In some cases, we additionally microinjected Cy5-dUTP 1.5 hours after the microinjection of Cy3-dUTP. Double pulse-labelling with Cy3- and Cy5-dUTP allowed the identification of foci with distinct replication timing in the mother cell. No free Cy3- or Cy5-dUTP is available for incorporation at replication sites in daughter nuclei as demonstrated by (1) the absence of detectable dispersed Cy3- and Cy5-fluorescence in daughter nuclei; (2) the absence of Cy3- or Cy5-fluorescence

at GFP-PCNA foci occupying different nuclear regions than labelled DNA foci in daughter nuclei (see Fig. S2 in supplementary material); and (3) the synthesis of unlabelled sister chromatids in daughter nuclei of Cy3- or Cy5-dUTP labelled mothers (Manders et al., 1999; Sadoni et al., 1999; Zink et al., 1998). It should also be noted that even during the first S phase, the pool of free Cy3-dUTP was rapidly depleted (Fig. 1).

In order to avoid possible artefacts we did not synchronize the cells further. As expected, most of the labelled cells evaluated on the next day were not in S phase at all (GFP-PCNA uniformly distributed) nor at that stage of S phase corresponding to the DNA-labelling pattern (GFP-PCNA foci localizing in nuclear regions other than the labelled DNA) (Fig. 4b, Fig. S2 in supplementary material). As we were interested in the interaction of the replication machinery with labelled DNA foci, we selected for imaging those cells where labelled DNA foci and GFP-PCNA foci occupied similar nuclear regions (Fig. 4b). This configuration was rarely observed owing to the relatively low efficiency of the sophisticated labelling procedure and the short period in the cell cycle during which differently labelled foci occupy similar nuclear regions. However, we were able to identify 15 nuclei where labelled DNA foci and GFP-PCNA foci occupied similar nuclear regions. These nuclei were imaged by confocal microscopy. Among the 15 nuclei were three pairs of sister nuclei.

Fig. 5 shows a time series of a corresponding nucleus, where DNA foci have been labelled in the mother cell and where



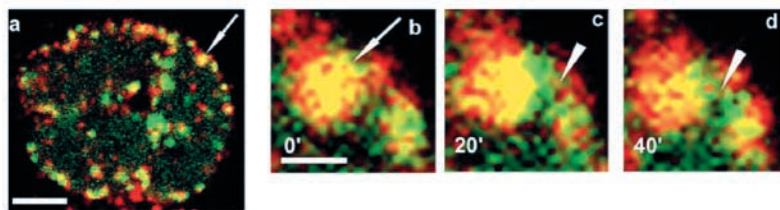
**Fig. 4.** Experimental procedure and selection of daughter cells for imaging. (a) Scheme depicting the experimental procedure. Cells were microinjected during the first S phase with a mixture of the GFP-PCNA expression plasmid and Cy3-dUTP. After 1.5 hours, some cells were microinjected again with Cy5-dUTP. Cells were imaged during the next S phase after cell division. (b) Schematic drawing of nuclei selected for imaging. During the imaging period following cell division, labelled cells were at different cell cycle and S-phase stages. Cells not in S phase at all (left, uniform distribution of GFP-PCNA) or not at that stage of S phase that corresponded to the DNA-labelling pattern (middle, GFP-PCNA localizing in different nuclear regions to the labelled DNA) were not chosen for imaging. Only those cells where GFP-PCNA foci and labelled DNA foci occupied similar nuclear regions were imaged (right).

labelled DNA foci and GFP-PCNA foci occupied similar nuclear regions in the daughter. Although at the beginning of the imaging period GFP-labelled foci colocalized with Cy3-labelled DNA foci, the degree of overlap between initially colocalizing foci subsequently decreased. The time series showed that DNA foci stably occupied their nuclear positions, whereas GFP-PCNA appeared at adjacent nuclear regions during S-phase progression. These findings were consistent with the previous results described here regarding nascent DNA synthesis.

The results obtained so far are summarized in Fig. 6a. DNA foci remain stably positioned, whereas GFP-PCNA foci appear at adjacent sites during S-phase progression. In addition, GFP-PCNA foci transiently colocalize with DNA foci labelled in the mother cell and occupying defined nuclear regions (middle image in Fig. 6a, compare to Fig. 5b). During a transition stage, GFP-PCNA foci disappear at previously active sites, while new GFP-PCNA foci appear at neighbouring sites (compare with Fig. 3a and b, site 1 at 58 and 82 minutes).

#### Labelled DNA foci are equivalent to replication foci and stably maintain their replication timing

Next, we addressed the question whether labelled DNA foci might represent stable aggregates equivalent to replication foci, which stably maintain their replication timing from S phase to S phase. If labelled DNA foci convert into replication foci again at subsequent S phases and stably maintain their replication timing from S phase to S phase, then those foci that simultaneously replicated in the mother cell should become simultaneously occupied again by the replication machinery in the daughter cells. Thus, at the given stage of S phase, a high percentage of Cy3-labelled foci (which replicated simultaneously in the mother cell) should colocalize with GFP-PCNA foci in daughter nuclei. Taking the temporal dynamics of S phase into account (Fig. 6a), one would expect to find a correspondingly high degree of colocalization in only a fraction of daughter nuclei (middle image of Fig. 6a; note that for imaging, nuclei were randomly chosen where Cy3- and GFP-labelled foci occupied similar nuclear regions, without further selection for specific patterns of colocalization).



**Fig. 5.** Spatial dynamics of GFP-PCNA at sites harbouring labelled DNA foci. (a) Light optical section of a nucleus labelled according to the scheme depicted in Fig. 4a at the beginning of the imaging period (red, Cy3 fluorescence; green, GFP fluorescence; yellow, colocalization). The arrow points to a region shown enlarged in b-d. (b-d) Time series (indicated in minutes) of the nuclear sub-region marked with an arrow in a. The arrow in b points to a Cy3-labelled focus that colocalized with a GFP-PCNA focus at the beginning of the imaging period. At later time points (c,d), the GFP fluorescence appeared in an adjacent region (arrowhead) between this and a neighbouring Cy3-labelled focus. Bar in a, 5  $\mu\text{m}$ ; b-d, 1  $\mu\text{m}$ .

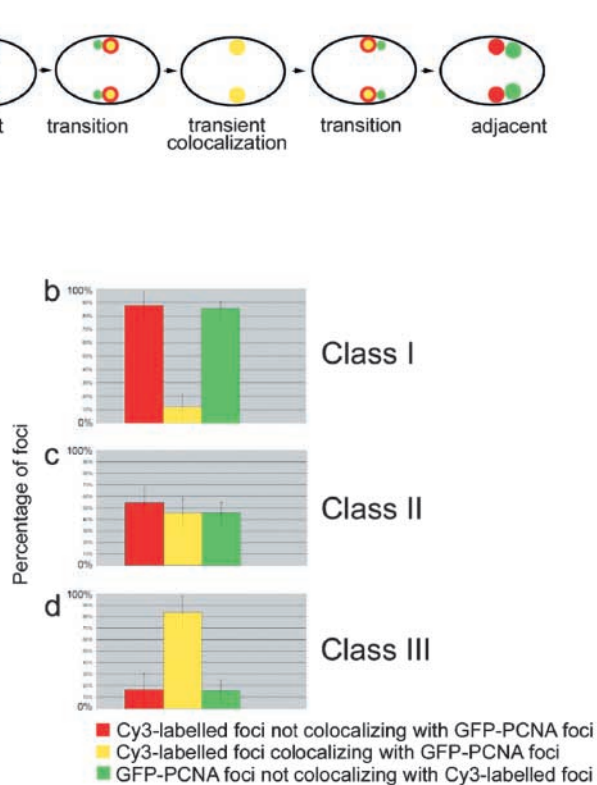
Thus, in order to address the temporal stability of replication foci, we analysed the patterns of colocalization of Cy3- and GFP-PCNA foci in the daughter nuclei. In each individual nucleus, the numbers of Cy3- and GFP-labelled foci were counted and the fractions of colocalizing foci were determined (see Materials and Methods). In the 15 nuclei analysed, a total of 21872 Cy3-labelled foci and 17401 GFP-labelled foci were evaluated.

We observed different degrees of colocalization between Cy3- and GFP-labelled foci in the individual nuclei (Fig. 6b-d): In one third (five) of the nuclei there was a low degree of colocalization (class I nuclei). Here, on average only about 15% of Cy3-labelled foci colocalized with GFP-PCNA foci and vice versa. Another third (five) of the nuclei showed an intermediate degree of colocalization, which was on average about 50% (class II nuclei). The remaining five nuclei displayed a high degree of colocalization. In these nuclei about 85% of individual Cy3- and GFP-labelled foci colocalized (class III nuclei).

Examples of class I and class III nuclei are shown in Fig. 7 (for details of numbers and types of Cy3- and GFP-PCNA patterns see Table S1 in supplementary material). In class I nuclei (Fig. 7a) GFP-PCNA foci occupied sites adjacent to labelled DNA foci, but showed only minor overlap, and a correspondingly low degree of colocalization was observed in this class of nuclei (about 15%, Fig. 6b). By contrast, in class III nuclei GFP-PCNA foci directly occupied labelled DNA foci (Fig. 7b). This finding was consistent with the high degree of colocalization observed in class III nuclei (about 85%, Fig. 6d). The findings that GFP-PCNA frequently occupied sites adjacent to labelled DNA foci (class I), or directly occupied these foci (class III) were in agreement with our previous findings regarding the temporal progression of S phase at sites harbouring labelled DNA foci (compare with Fig. 6a). Furthermore, the data suggested that nuclei showing an intermediate degree of colocalization (class II), represented the transition stage (second and fourth nuclei from the left in Fig. 6a) between the class I and class III patterns. In accordance, the transition between colocalization and the appearance of GFP-PCNA foci at adjacent sites was directly observed in these nuclei (the nucleus shown in Fig. 5 belonged to class II). Together, the results suggest that the dynamic sequence of events shown in Fig. 6a corresponds to the following classes of colocalization: I-II-III-II-I.

The observation that a fraction of nuclei displayed a high degree of colocalization (class III) supported the idea that DNA foci stably maintain their replication timing from S phase to S phase. Given the spatiotemporal dynamics illustrated in Fig. 6a, only a fraction of nuclei should display a high degree of colocalization under the experimental conditions used, even if all DNA foci maintain their replication timing. Alternatively, a fraction of nuclei might show a high degree of colocalization due to random crowding of foci within those nuclear regions occupied during given stages of S phase. In order to examine this possibility, we calculated the probability of observing 85% colocalization if foci randomly occupy those nuclear regions typical for a given stage of S phase (see supplementary material for details of the statistical analysis). As individual

foci were easier to analyse during later stages of S phase, we confined this analysis to nuclei at stages typical of the second half of S phase. The results revealed that the probability of observing 85% colocalization in these nuclei was  $10^{-50}$ ; the observed high degree of colocalization in class III nuclei was therefore not due to random crowding of foci. Visual inspection revealed that in early S-phase nuclei, DNA foci and GFP-PCNA foci colocalized exactly with each other at the level of individual foci (Fig. 7b). These findings also showed that we



**Fig. 6.** Analysis of Cy3 and GFP-PCNA colocalization patterns. (a) A scheme summarizing the spatiotemporal dynamics observed. During the temporal progression of S phase (indicated by black arrows) DNA foci (red) stably occupy their nuclear positions, whereas GFP-PCNA foci appear at adjacent sites. A transition stage is observed during which GFP foci at the previously active sites cease, whereas GFP foci at adjacent new sites increase (compare with Fig. 3a and b, 58 minutes and 82 minutes). Colocalization with labelled DNA (middle) is only observed during a restricted period (compare Figs 2 and 5). This scheme illustrates the behaviour of the majority of replication sites and does not take into account intranuclear heterogeneity, which might occur when the replication programme is not exactly reproduced at all sites. (b-d) Quantitative analysis of colocalization between Cy3 and GFP-PCNA foci in daughter cells. 21872 Cy3-labelled and 17401 GFP-labelled foci were evaluated. Red and yellow bars represent the percentage of Cy3-labelled foci colocalizing (yellow) or not colocalizing (red) with GFP-PCNA labelled foci. Green bars represent the percentage of GFP-labelled foci not colocalizing with Cy3-labelled foci. Individual nuclei display different degrees of colocalization. (b) In class I nuclei on average only about 15% of Cy3-labelled foci colocalize with GFP-labelled foci. (c) In class II nuclei about half of the Cy3-labelled foci colocalize with GFP-PCNA foci. (d) In class III nuclei on average about 85% of Cy3-labelled foci colocalize with GFP-labelled foci.

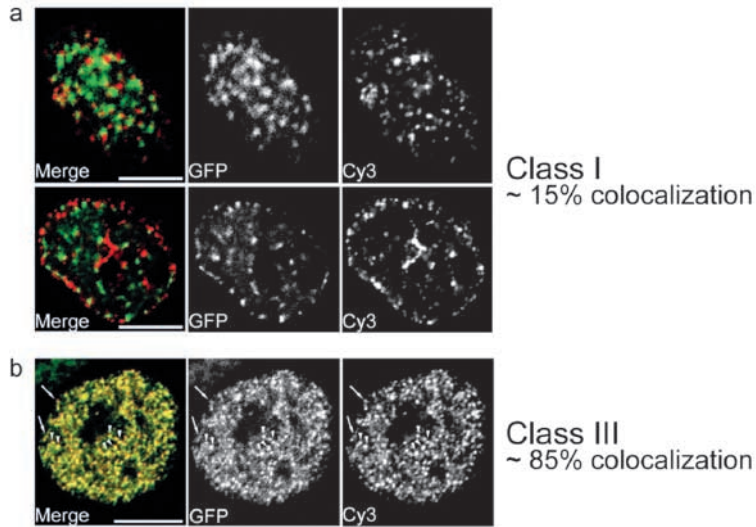
did not observe random patterns of colocalization in early S-phase nuclei, but specific targeting of DNA foci by the replication machinery. It should also be noted that of the relatively low number of 15 nuclei that could be analysed, there were already five nuclei showing a high degree of colocalization. This argues against the possibility that class III nuclei might be rare and exceptional.

It was not clear whether the ~85% colocalization observed in class III nuclei reflected a failure of our image analysis procedure to detect colocalization at all double-labelled sites. Visual re-inspection of the data revealed that strong differences in the Cy3- and GFP-fluorescence intensities at individual sites resulted in a failure to detect colocalization. To address this problem, we analysed colocalization during nascent DNA synthesis (examples shown in Figs 1-3). We observed no separation of Cy3-labelled DNA and the GFP-labelled replication machinery at the first time points of imaging (about 30-45 minutes) after microinjection (Figs 1-3). Visual inspection of the data sets also revealed that all GFP-labelled sites actively produced Cy3-labelled DNA (Figs 1-3), but that no Cy3-labelled DNA was produced at sites not labelled with GFP. Therefore, although the degree of colocalization based on visual inspection was 100%, the degree of colocalization measured by computer analysis at 30-45 minutes after microinjection was only  $83 \pm 16\%$  (for example 80% in the nucleus shown in Figs 1 and 2 at 45 minutes). This corresponds to the observed degree of colocalization in class III nuclei imaged one S phase later. This result suggested that the degree of colocalization in class III daughter nuclei was even higher than 85%.

To investigate the colocalization between Cy3- and GFP-labelled foci further, we measured the 3D distances between the intensity centres of colocalizing foci during the synthesis of nascent DNA (first S phase, examples shown in Figs 1-3) and in class III daughter nuclei. During the first S phase, some offset between the intensity centres of colocalizing DNA and GFP-PCNA foci was observed, ranging between 4 nm and 200 nm ( $n=162$ ). This offset is partially due to chromatic aberrations (Zink et al., 1999). However, the GFP-PCNA foci in particular showed a very dynamic behaviour and displayed changes in shape and fluorescence intensity distributions over time. Thus, they did not always perfectly recapitulate the shape and fluorescence intensity distribution of colocalizing DNA foci, which probably contributed to some of the offset observed. The offset observed for colocalizing foci in class III daughter nuclei was slightly larger. However, in about 80% of the cases ( $n=108$ ) the offset still did not exceed 200 nm. In about 20% of the colocalizing foci, the offset between the intensity centres ranged between 200 nm and 300 nm. Visual re-inspection of the data suggested that this increased offset in a minor fraction was probably caused by a slight asynchrony in the replication timing of these sites, leading to the inclusion of preceding or following temporal stages. In cases where a GFP-PCNA focus fading or appearing at closely adjacent sites cannot be spatially resolved from the colocalizing GFP-PCNA focus (see e.g. Fig. 2, 120 minutes), a shift in the intensity centre will also be observed.

So far, the results indicate that labelled DNA foci are equivalent to replication foci and stably maintained their replication timing from S phase to S phase, with some variations in a minor fraction of foci. The results also





**Fig. 7.** Spatial arrangements of GFP-PCNA and Cy3-labelled foci in class I and class III nuclei. Nuclei were labelled according to the experimental procedure outlined in Fig. 4a. GFP and Cy3 fluorescence and the corresponding merged images (green, GFP; red, Cy3; yellow, colocalization) are shown. (a) Single light optical sections of a class I nucleus. The upper panels show the nuclear periphery whereas the lower panels show an equatorial plane. GFP-PCNA foci and labelled DNA foci localize adjacent to each other. (b) Single light optical sections showing an equatorial plane of a class III nucleus. The nucleus displays a pattern typical for early S phase with many foci distributed throughout the nuclear interior. Colocalization at the level of individual foci becomes evident in regions relatively devoid of labelled foci (arrows). However, colocalization at the level of individual foci can also be observed in more crowded regions (arrowheads). Bars, 10  $\mu$ m.

suggested that a correspondingly high degree of colocalization between labelled DNA and the replication machinery during the following S phase is observed in only a fraction of daughter nuclei because of the changing spatial arrangements of foci during the temporal progression of S phase (Fig. 6a). To confirm that the different patterns of colocalization (Fig. 6) represented different temporal stages, we inspected nuclei that inherited Cy3- and Cy5-labelled DNA foci from their mother (Cy5-dUTP was microinjected 1.5 hours after Cy3-labelling, see labelling scheme in Fig. 4a). Unfortunately, owing to technical problems, it was not possible to address the temporal sequence of foci activation by following directly the progression of S phase in triple-labelled cells. However, 'snapshots' from individual triple-labelled nuclei imaged at single time points representing specific stages of the dynamic process could be obtained. The observed range of patterns further illustrates our findings presented above.

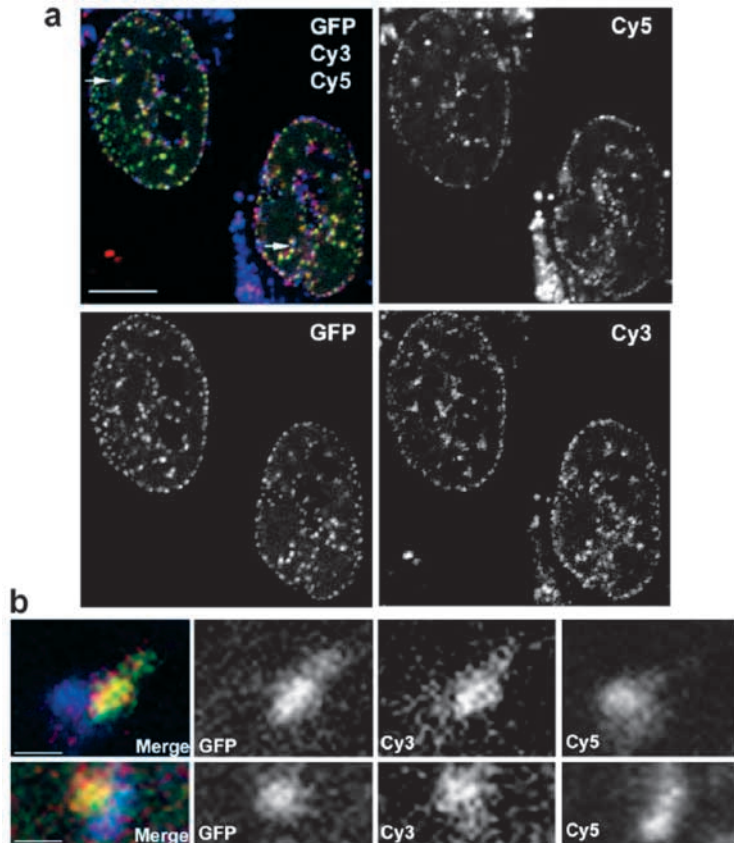
Fig. 8 shows triple-labelled sister nuclei progressing almost synchronously through S phase (similar patterns of GFP-PCNA foci) and displaying similar patterns of DNA foci ('similar' describes here a comparable relative positioning with regard to other nuclear structures like nucleoli or the periphery, but does not mean identical). The observation of similar patterns of DNA foci in the sister cells was in accordance with the finding that the positioning of DNA with a specific replication timing is clonally inherited (Ferreira et al., 1997; Sadoni et al., 1999). The fact that previously synchronized and doubly microinjected cells still showed normal patterns of BrdU incorporation (during the same or during the following S phase; see supplementary material, Fig. S3) demonstrated that also under these relatively stressful conditions DNA synthesis was not detectably compromised.

GFP- and Cy3-labelled foci colocalized to a high degree in the sister nuclei (Fig. 8). Adjacent foci labelled in the mother cell 1.5 hours later with Cy5-dUTP showed only peripheral overlap with GFP/Cy3-labelled replication foci (at higher magnification in Fig. 8b). The distinct interaction of Cy3- and Cy5-labelled foci with the replication machinery in the daughter cells suggested, consistent with the previous results, that the different replication timing of the adjacent sets of foci was stably maintained from S phase to S phase. Colocalization

analysis revealed that the nuclei belonged to class III (high degree of colocalization, compare Figs 6 and 7) with regard to the earlier replicating Cy3-labelled foci. By contrast, they belonged to class I (low degree of colocalization, compare Figs 6 and 7) with regard to the later replicating Cy5-labelled foci. These observations further supported the conclusion that the different patterns of colocalization observed in daughter cells indeed reflected different temporal stages of S-phase progression at sites harbouring labelled DNA foci with a stably maintained replication timing.

The temporal sequence of foci activation is also illustrated by the nuclei shown in Fig. 9. Numbers and patterns of replication foci are characteristic for the different stages of S phase. Therefore, at the stage of S phase in the daughter cells that corresponds to the stage of S phase where the mother cell has been labelled, the numbers and positions of GFP-PCNA foci should correspond to the numbers of labelled DNA foci. This is indeed the case (Fig. 7b and Cy3/GFP-PCNA foci in Fig. 8). However, at earlier stages of S phase in the daughter cells, the numbers of GFP-PCNA foci should be relatively high compared to the numbers of Cy3- and Cy5-labelled DNA foci, and they should also occupy more interior positions if they represent a pattern typical for the first half of S phase. Conversely, at more advanced stages of S phase in the daughter cells there should be relatively few GFP-PCNA foci compared to the numbers of Cy3- or Cy5-labelled DNA foci, respectively, as the numbers of replication foci decrease during S-phase progression (Leonhardt et al., 2000a; Nakayasu and Berezney, 1989) (see also Fig. 1).

The former situation (daughter at an earlier stage of S phase than the mother during labelling) can be observed in the nucleus displayed in Fig. 9a and b, as indicated by the relatively numerous GFP-PCNA foci occupying more interior positions. In this situation, GFP-PCNA foci occupied sites adjacent to Cy3- but not to Cy5-labelled foci. By contrast, the relatively low numbers of GFP-PCNA foci compared to the numbers of labelled DNA foci identify the nucleus shown in Fig. 9c and d as being at a later stage of S phase than the mother cell during labelling. About half of the GFP-PCNA foci colocalized with labelled DNA foci, whereas the other half did not. This pattern of colocalization identified the nucleus as a



**Fig. 8.** Spatiotemporal interaction of the replication machinery with adjacent sets of DNA foci. (a) The panels show light optical sections from the equatorial planes of two sister nuclei labelled and imaged according to the experimental procedure outlined in Fig. 4a. Arrows point to regions with labelled foci shown enlarged in b. The panels show single GFP, Cy3 and Cy5 fluorescence with merged image as indicated (green, GFP; red, Cy3; blue, Cy5; red and green appears yellow; red and blue appears violet; a merge of all three colours appears white). The number of GFP foci corresponds to the number of Cy3-labelled foci. (b) GFP-labelled foci colocalize with Cy3 but not with adjacent Cy5-labelled foci. Bar in a, 10  $\mu\text{m}$ ; b, 1  $\mu\text{m}$ .

class II nucleus (transition stage, compare with Fig. 6a) at the transition to a later stage of S phase. Those labelled DNA foci, which were still occupied by the replication machinery, were the Cy5-labelled foci (enlarged in the upper panels of Fig. 9d, the relatively small size of the GFP-PCNA focus probably indicated ceasing of this focus). In addition, GFP-PCNA foci occupied sites adjacent to the Cy5-labelled foci (lower panels in Fig. 9d). Together, the range of patterns observed in triple-labelled nuclei suggests the following sequence of foci activation: sites adjacent to Cy3-labelled foci; Cy3-labelled foci; Cy5-labelled foci; sites adjacent to Cy5-labelled foci. These observations, suggesting sequential activation of neighbouring foci with stably maintained replication timing, support our conclusions based on the results obtained with double-labelled cells.

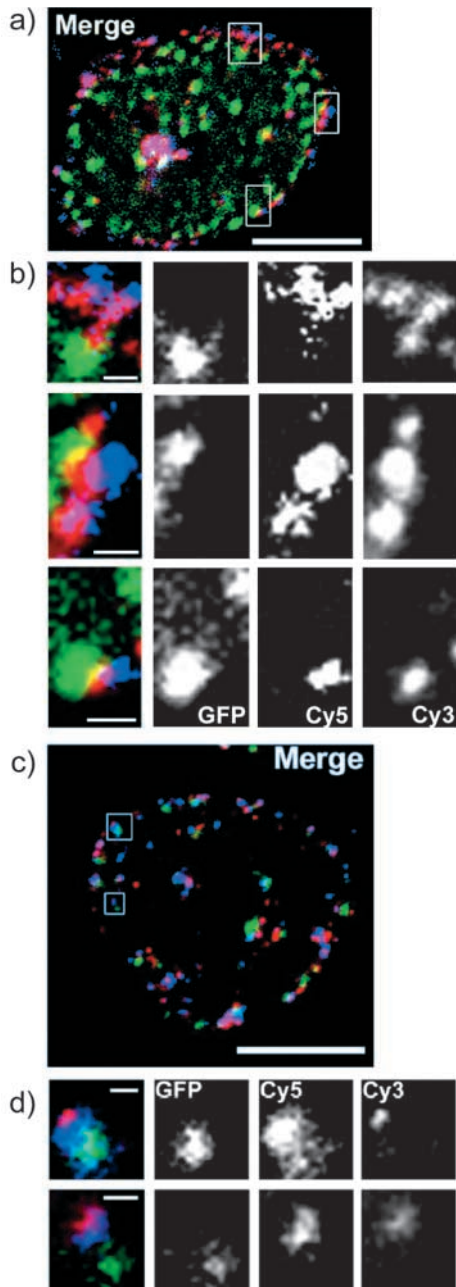
## Discussion

Our study shows for the first time the dynamic interaction of the replication machinery with DNA in living eukaryotic cells. Our results showed that during S-phase progression, functional complexes of replication proteins appear at sites adjacent to sites of previous DNA synthesis. These findings are in accordance with earlier results obtained with fixed cells by double pulse-labelling of nascent DNA (Ma et al., 1998; Manders et al., 1992; Manders et al., 1996), as well as recent results of time-lapse photobleaching experiments of replication proteins (Sporbert et al., 2002). However, until now, the DNA dynamics at replication sites remained an unresolved issue (see Introduction). The results of this study revealed that DNA

shows some local rearrangements but is not redistributed to other nuclear sites during S phase. In accordance with a study investigating the replication of chorion genes of *Drosophila melanogaster* (Calvi and Spradling, 2001), our results do not support extreme interpretations of a model proposing that DNA is spooled through fixed replication factories (Cook, 1999; Hozak et al., 1993). However, spooling of DNA within the limits of individual foci would be compatible with our data.

Several lines of evidence suggest that individual replication foci correspond to single replicon clusters harbouring ~5-10 synchronously firing replicons and comprising on average about 1 Mb of DNA (Berezney et al., 2000; Berezney et al., 1995b; Jackson and Pombo, 1998). It has been suggested that DNA present at individual replication foci during S phase might remain stably aggregated giving rise to stable chromosome subunits equivalent to replication foci (Berezney et al., 1995b; Jackson and Pombo, 1998; Ma et al., 1998; Sparvoli et al., 1994; Zink et al., 1999; Zink et al., 1998). Central to these studies is the observation that after S phase, pulse labelling patterns and numbers of labelled foci were maintained during subsequent cell cycles and cell cycle stages. Similar foci were also observed on mitotic chromosomes (Jackson and Pombo, 1998; Sparvoli et al., 1994) and double pulse-labelling experiments indeed suggest a close relationship between replication-labelled foci observed in the nucleus and the characteristic structural organization of mitotic chromosomes into bands and sub-bands (Zink et al., 1999). This suggests that chromosomes are organized into stable units, equivalent to replication foci during S phase and to chromosomal bands and sub-bands during mitosis, which we called sub-chromosomal foci (Zink et al., 1999; Zink et al., 1998).

To address the stability of replication-labelled DNA foci, double pulse-labelling experiments with highly synchronized cells have been performed that were imaged after fixation (Jackson and Pombo, 1998; Ma et al., 1998). As these studies addressed only those sites initiated first during S phase and as it was difficult to demonstrate exact colocalization at the level of individual foci, the issue of whether chromosomes are organized into stable units equivalent to replication foci remained controversial. In the present study, we were able to demonstrate colocalization at early as well as at later stages of S phase at the level of individual foci. Furthermore, our data did show that those foci, which replicated synchronously in the mother cell, converted again synchronously together into

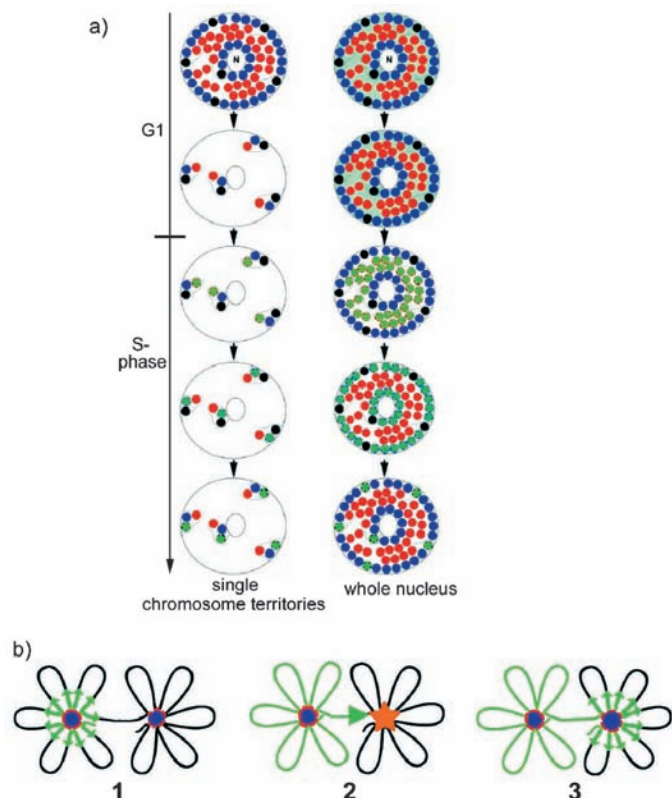


**Fig. 9.** Spatial arrangements of GFP-PCNA foci and labelled DNA foci in triple-labelled daughter cells. Equatorial planes of two nuclei labelled and imaged according to the experimental procedure outlined in Fig. 4a are shown. GFP, Cy3 and Cy5 fluorescence and the merged images are shown as indicated (green, GFP; red, Cy3; blue, Cy5; red and green appears yellow; red and blue appears violet; green and blue gives rise to cyan; and a merge of all three colours appears white). (a,b) A daughter nucleus at an earlier stage of S phase during labelling than the mother cell, indicated by relatively numerous GFP-PCNA foci occupying interior positions. The boxed regions are shown enlarged in b. GFP-PCNA foci are adjacent to Cy3-labelled but not to Cy5-labelled DNA foci. (c,d) A nucleus at the transition to a later stage of S-phase than the mother cell during labelling, indicated by the relatively low number of GFP-foci compared to the number of Cy3- or Cy5-labelled foci. The framed regions are shown enlarged in d. (d) GFP-PCNA foci colocalize either with Cy5-labelled foci (upper panels) or sites adjacent to Cy5-labelled foci (lower panels). Bars in a and c, 10  $\mu$ m; in b, 1  $\mu$ m; in d, 0.5  $\mu$ m.

replication foci in the daughter cells at the corresponding stage of S phase. In addition, DNA with different replication timing in the mother cell was organized into distinct sets of foci within daughter cells showing distinct patterns of colocalization with GFP-PCNA foci. These results strongly support the hypothesis that chromosomes are organized into stable structural/functional units equivalent to replication foci, that stably maintain their replication timing from S phase to S phase. It should be noted that the temporal resolution of experiments addressing the temporal stability was in the range of a pulse-length, which is  $\sim$ 1-1.5 hours. Currently, the substructure of these sub-chromosomal foci is not known, nor is it known whether they maintain a similar sub-focal organization at different cell cycle stages. A model has been proposed predicting that replicon clusters are arranged during replication in a series of loops attached to the nuclear matrix (Berezney et al., 1995b). Folding of the DNA into 120 kb loops and a rosette-like arrangement of these loops into sub-compartments in the megabase pair size range is also consistent with the focal patterns observed in the microscope after replicational pulse-labelling (Münkel et al., 1999).

Our results revealed that sets of sub-chromosomal foci, which replicated together in the mother cell, became occupied again in the daughter cells by the replication machinery at the appropriate time point of S phase. Furthermore, the data showed that adjacent sites became sequentially occupied by the replication machinery during S phase progression. The results also suggested that adjacent sites were occupied by different sets of sub-chromosomal foci with a distinct but stably maintained replication timing. Finally, the results suggested that these adjacent sets of sub-chromosomal foci were sequentially activated during S-phase progression and that this is conserved from S phase to S phase. It has been shown previously by different groups that the spatial positioning of DNA with a defined replication timing is established during early G1 (Dimitrova and Gilbert, 1999; Ferreira et al., 1997; Sadoni et al., 1999). Thus, the replication machinery follows during S-phase-specific spatial arrangements of sub-chromosomal foci, which have been established earlier during the cell cycle (Fig. 10a). These findings imply that the nuclear order of sub-chromosomal foci determines the spatiotemporal patterns of replication foci observed during S phase.

Currently, the mechanism leading to sequential activation of neighbouring sites is not known (see also Sporbert et al., 2002). A study investigating activity and replication timing of yeast origins suggested that 'neighbouring, secondary origins can be activated only after the incoming replication forks initiated from the earlier origins reaches close to the former origins' (Yamashita et al., 1997). Furthermore, a study addressing replication of the IgH locus of murine erythroleukemia cells showed that a single replication fork was responsible for the replication of about 400 kb from this locus (Ermakova et al., 1999). This fork starts at a cluster of early-firing origins and ends at a cluster of late-firing origins. It is tempting to speculate that the incoming fork triggers activation of the late-firing origins. Based on the results and ideas outlined above we developed the model shown in Fig. 10b. In this model, the DNA remains stationary, organized into sub-chromosomal foci, whereas the replication machinery is dynamic (see also Calvi and Spradling, 2001). An incoming replication fork from an earlier replicating focus triggers activation of a later replicating



**Fig. 10.** Models of DNA replication. (a) The replication machinery follows an established path of sub-chromosomal foci during the temporal progression of S phase. The scheme depicts a cell nucleus harbouring a nucleolus (N) and DNA organized into sub-chromosomal foci. The spatial organization of sub-chromosomal foci with a defined replication timing (red, early; blue, mid; black, late) is established during G1 and is stably maintained during interphase (see Sadoni et al., 1999). Individual chromosome territories organized into sub-chromosomal foci are shown on the left. For simplicity, only a limited number of individual territories are shown, each harbouring only a few sub-chromosomal foci (it is estimated that sub-chromosomal foci harbour a DNA content of about 1 Mb). During S-phase progression, the replication machinery (green stars indicate replication foci, GFP-PCNA is uniformly distributed during G1) follows the established pattern of sub-chromosomal foci and neighbouring foci become sequentially occupied. At the level of the whole nucleus (on the right), this results in the typical spatiotemporal patterns of replication foci described in the literature (see Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Sadoni et al., 1999). (b) Substructure of replication foci and activation of neighbouring foci. We propose that replication foci and sub-chromosomal foci correspond to replicon clusters, organized into loops. Furthermore, we propose that the bases of these loops correspond, at least during S phase, to the origins of replication (red), attached to a central structure (blue). Neighbouring foci are connected by one half of a bi-directional replicon. (1) Replication of an earlier replicating focus (left) started, while the neighbouring focus (right) is still inactive. Duplicated DNA is shown in green and the direction of movement of the fork is indicated by green arrows. Unduplicated DNA is shown in black. (2) Replication of the earlier replicating focus finishes and the incoming fork from the earlier replicating focus activates the later replicating neighbouring focus. Synchronous activation (orange star) of the origins of the corresponding replicon cluster might be facilitated by their spatial clustering. (3) Replication of the later replicating focus started.

neighbouring focus. Within a focus, DNA is possibly organized into loops, with the origins clustered at a central structure, which may correspond to the nuclear matrix (see also Berezney et al., 1995a; Munkel et al., 1999). Clustering of DNA loops at a central structure might help to trigger the spatial organization of foci within the nucleus. Assembly of origins belonging to a focus at a central point might help to trigger their synchronous activation by an incoming fork. According to this model, only neighbouring foci from the same chromosome should be sequentially activated. This prediction can be tested by combining replicational pulse-labelling with in-situ hybridization experiments.

Our results are in agreement with previous studies indicating a close relationship between the nuclear positioning of chromosomal loci and the coordination of S-phase events (Dimitrova and Gilbert, 1999; Gilbert, 2001; Heun et al., 2001). Currently it is unresolved whether the nuclear positioning of chromosomal loci determines their replication timing, or whether chromosomal units with a defined replication timing adopt specific nuclear positions. In order to unravel this problem, it will be important to find out what determines the nuclear positioning of chromosomal loci.

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