Histochem Cell Biol (2006) 125: 91–102
DOI 10.1007/s00418-005-0063-3

ORIGINAL PAPER

Stefanie Weidtkamp-Peters · Hans-Peter Rahn
M. Cristina Cardoso · Peter Hemmerich

Replication of centromeric heterochromatin in mouse fibroblasts takes place in early, middle, and late S phase

Accepted: 16 August 2005 / Published online: 18 October 2005
© Springer-Verlag 2005

Abstract The replication of eukaryotic chromosomes takes place throughout S phase, but little is known how this process is organized in space and time. Early and late replicating chromosomal domains appear to localize to distinct spatial compartments of the nucleus where DNA synthesis can take place at defined times during S phase. In general, transcriptionally active chromatin replicates early in S phase whereas transcriptionally inactive chromatin replicates later. Here we provide evidence for significant deviation from this dogma in mouse NIH3T3 cells. While the bulk pericentromeric heterochromatin replicates exclusively during mid to late S phase, centromeric DNA domains associated with constitutive kinetochore proteins are replicated throughout all stages of S phase. On an average, 12 ± 4% of centromeres replicate in early S phase. Early replication of a subset of centromeres was also detected in living C2C12 murine cells. Thus, in contrast to expectation, late replication is not an obligatory feature of centromeric heterochromatin in murine cells and it does not determine their 'heterochromatic state'.

Keywords Heterochromatin · Centromers · Replication · Cell cycle · Chromocenters

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00418-005-0063-3.

S. Weidtkamp-Peters · P. Hemmerich (✉)
Institute of Molecular Biotechnology, Beutenbergstr. 11, 07745 Jena, Germany
E-mail: phemmer@imb-jena.de
Tel.: +49-3641-656262
Fax: +49-3641-656310

H.-P. Rahn · M. C. Cardoso
Max Delbrück Centrum, 13125 Berlin, Germany

Introduction

The eukaryotic nucleus is responsible for the storage, propagation, maintenance and expression of the genetic material it contains. Therefore, a highly organized machinery is required for these processes to take place in an extremely condensed nuclear environment with protein concentrations ranging between 50 and 400 μg/μl. DNA in the form of chromatin is organized in distinct chromosome territories composed of transcriptionally active (euchromatin) and inactive (heterochromatin) domains (Cremer and Cremer 2001). In addition, several domains or compartments have been identified that seem to form in response to the requirement for specific biochemical activities in subnuclear microenvironments (Spector 1993). Morphologically well characterized nuclear compartments include the nucleolus, the splicing factors compartments (speckles), and a family of small nuclear bodies with diverse functions (Hemmerich and Diekmann 2005). Active DNA and RNA metabolism such as transcription and replication occurs within numerous focal sites throughout the nuclear volume, sometimes referred to as 'factories' (Guillot et al. 2005; Lemaitre and Méchali 2005).

In mammalian cells DNA replication occurs at microscopically visible sites in the nucleus, ranging in size between 0.3 and 0.8 μm (Nakamura et al. 1986). Immunofluorescence microscopy of incorporated biotin-tagged dUTP or 5-bromodeoxyuridine (BrdU) label such sites of ongoing DNA synthesis and allow the identification of distinct patterns of localization of replication sites that occur in a specific order and at different times during S phase (Nakayasu and Berezney 1989; van Dierendonck et al. 1989; Nakayasu and Berezney 1989; Fox et al. 1991; Kill et al. 1991; Neri et al. 1992; Sparvoli et al. 1994; Hozak et al. 1994). Immunogold labelling of incorporated BrdU in combination with electron microscopy has confirmed these focal patterns (Raska et al. 1989; Mazzotti et al. 1990; Philimonenko et al. 2004; Koberna et al. 2005).

In these foci, the DNA replication machinery simultaneously initiates and elongates the replication forks of adjacent replicons. As a consequence, many replication factors such as the proliferating cell nuclear antigen (PCNA), DNA ligase I and DNA methyltransferase I also accumulate at replication factories (Celis and Celis 1985; Bravo and MacDonald-Bravo 1987; Leonhardt et al. 1992; Somanathan et al. 2001; Cardoso et al. 1997). PCNA forms a homotrimer around the DNA at the replication fork and stabilizes DNA polymerase δ on the template DNA, thereby increasing its processivity (Diffley and Labib 2002). Live cell microscopy employing GFP-tagged PCNA has revealed that during S phase replication foci assemble de novo adjacent to earlier ones, possibly indicating a domino effect involving local changes in chromatin structure and accessibility (Leonhardt et al. 2000; Sporbert et al. 2002).

Genome duplication in S phase begins at several hundred discrete foci in the internal, euchromatic region of the nucleus, excluding the nucleoli and nuclear periphery region (pattern 1). In pattern 2, replication continues throughout the euchromatic region, but it is also observed in the perinucleolar and nuclear periphery regions. Pattern 3 at mid S phase is characterized by decreased euchromatic foci in the interior and size-increased replication foci at the nuclear and nucleolar periphery. During mid to late S phase most euchromatic foci finish replication and DNA synthesis occurs at pericentric heterochromatin (pattern 4). These larger foci have been decomposed into smaller units with an average diameter of 120 nm by electron microscopy (Koberna et al. 2005). At the end of S phase, small numbers of large replication domains are observed in both the interior and periphery of the nuclei (pattern 5). These five successive patterns of DNA replication have consistently been found in many mammalian cell lines (O'Keefe et al. 1992; Manders et al. 1992; Ma et al. 1998; Jackson and Pombo 1998; Dimitrova and Gilbert 1999; Leonhardt et al. 2000; Dimitrova and Berezney 2002), and although they are commonly accepted, the distribution of replication sites and the temporal order of their appearance can differ in some cell types (Wu et al. 2005). This dynamic replication patterning seems to be an evolutionary, conserved, and fundamental feature of higher order chromatin arrangements since it is also present in the early metazoan polyp Hydra (Alexandrova et al. 2003).

Early and late replicating chromosomal domains appear to localize to distinct spatial compartments of the nucleus where DNA synthesis can take place at defined times during S phase. In general, transcriptionally active (eu)chromatin replicates early in S phase whereas transcriptionally inactive (hetero)chromatin replicates later (Lima-de-Faria and Jaworska 1968; Goldman et al. 1984; Schubeler et al. 2002). Consistent with this observation, centromere DNA which, in higher eukaryotes, is generally composed of repetitive heterochromatic sequences was in most studies found to replicate during mid to late S phase (Bostock and Prescott 1971; Dooley and Ozer 1977; Goldman et al. 1984; Hatton et al. 1988; Ten-Hagen et al. 1990). The late replication timing of centromeres has been proposed to play a role in centromere function and transcriptional control (Csink and Henikoff 1998; Gilbert 2002).

However, significant deviations from this paradigm rule have also been reported. In the fission yeast, *Schizosaccharomyces pombe*, active chromosomal origins of replication have been identified within centromeric DNA (Kim and Huberman 2001). More importantly, while the heterochromatic telomeres of *S. pombe* replicate in late S phase, the heterochromatic centromeres and silent mating cassettes replicate very early in S phase (Kim et al. 2003). A genome-wide replication timing analysis in *Drosophila* has shown that β-heterochromatin located adjacent to the centromeric α-heterochromatin replicates early (Schubeler et al. 2002), and Ahmad and Henikoff (2002) even suggested that centromeres in *Drosophila Kc* cells replicate independently of the pericentromeric heterochromatin as isolated domains in early S phase. These observations demonstrated that late replication is not an obligatory feature of heterochromatin (Kim et al. 2003).

We therefore set out to investigate in detail the replication timing of centromeres in mouse cells. Using specific probes we show that while bulk pericentric heterochromatin replicates during mid to late S phase, kinetochore-associated heterochromatin domains replicate in early, mid, and late stages of S phase.

Materials and methods

Cell culture and transfection

Mouse fibroblast cells NIH3T3 (ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (DMEM, SIGMA-Aldrich, Germany) supplemented with 10% NCS (GIBCO, Karlsruhe, Germany) and human HEp-2 cells (HeLa derivative; ATCC) were grown as recommended in the presence of 10% FCS (SIGMA-Aldrich, Germany) in a 7.5% CO_2 atmosphere at 37°C. Mouse C2C12 cells were cultured in DMEM with 20% FCS and transfected by the calcium phosphate-DNA co-precipitation method as described (Cardoso et al. 1997). For immunolabeling, cells were plated and grown until subconfluency on glass coverslips (15 mm diameter) in six-well-plates. NIH-3T3 cells are 3% hyperploid. Hyperploid cells were not considered in statistical evaluations.

Plasmids

DsRed1-tagged human DNA Ligase I (Easwaran et al. 2005) and GFP-tagged human CENP-B DNA binding domain (Shelby et al. 1996) have been described earlier.

Antibodies

The following primary antibodies have been used for indirect immunofluorescence analyses:

human anti-centromere autoantibody sera against centromeres (Wieland et al. 2004), mouse monoclonal anti-PCNA antibody (clone PC10, Santa Cruz Biotechnologies), mouse monoclonal anti-BrdU antibody (BD Pharmingen; Heidelberg, Germany), rat monoclonal anti BrdU antibody (Harlan Seralab, Borchen, Germany).

Immunofluorescence

Cells grown on coverslips were washed with PBS and fixed for 10 min in 3.7% formaldehyde at room temperature or for 10 min in icecold methanol followed by an incubation in aceton for 3 min. Formaldehyde-fixed cells were permeabilized for 3 min in 0.25% Triton X-100. Immunofluorescence was performed as described previously (Kiesslich et al. 2002). For dual immunofluorescence staining, primary antibodies from different sources (mouse, human or rat) were used simultaneously and detected with species-specific secondary antibodies linked to fluorescein or rhodamine (Jackson Immunosearch, West Grove, PA, USA). DNA was counterstained with ToPro3 (Molecular Probes, Eugene, USA).

DNA replication labeling and immunofluorescence

The thymidine analogue 5-bromo-2′-deoxyuridine (BrdU, BD Bioscience Pharmingen) was added to asynchronously growing cells to a final concentration of 10 μM for 10 min at 37°C. After fixation and permeabilzation of the cells immunofluorescence staining was performed as described above. The cells were then fixed again with 4% formaldehyde for 10 min and permeabilized in 0.1% Triton X-100 for 5 min before denaturation of DNA with 2 M HCl for 30 min. BrdU was detected using an anti-BrdU antibody.

Microscopy

Immunofluorescence microscopy was performed with an LSM 510 META microscope (Carl Zeiss, Jena, Germany) equipped with a 20 mW argon ion laser, a helium neon laser with a 543 nm line, and a helium neon laser with a 633 nm line using a 63×/1.4 oil immersion Plan-Apochromat objective. Fluorescein, Rhodamine and ToPro3 dyes were excited by laser light at 488, 543, or 633 nm wavelengths, respectively. To avoid bleed-through effects in triple staining experiments, each dye was scanned independently in a multitracking mode. For high resolution optical sectioning, z-intervals were set in such a way that each voxel has identical dimensions in the x-, y-, and z-directions (hyperfine sectioning mode of the LSM 510). 3D projections of nuclei were reconstructed from these data sets using LSM software release 3.2. A rendering software 3DVisArt (Carl Zeiss) was used to reconstruct single heterochromatincenters.

Results

Centromeric and pericentromeric chromatin are adjacent but distinct subnuclear entities

To analyze the replication of centromeres throughout S phase and in 3D, several established fixation/permeabilization procedures were initially tested. At the level of DNA topology, the best architectural preservation was obtained using methanol/acetone fixation where nuclei measured 18.4 ± 5.2 μm in diameter (x, y-axis) and 7.5 ± 1.3 μm ($n = 50$) in z-direction (Fig. 1a), identical to the dimensions of DNA-stained NIH3T3 nuclei in formaldehyde-fixed and living cells (data not shown). For this high resolution optical sectioning, z-intervals were set in such a way that each voxel has identical dimensions in x-, y-, and z-directions (see material and methods). Methanol-fixed 3T3 fibroblast nuclei displayed the characteristic DNA-dense chromocenters comprising clustered pericentromeric heterochromatin of the acrocentric mouse chromosomes (see movie 1, suppl. data). The chromocenters varied in size and were distributed throughout the nuclear volume without preferential localization around nucleoli or the nuclear membrane (Fig. 1a, movie 1, and data not shown). In contrast, nuclei processed for immunofluorescence including formaldehyde fixation and an acid denaturation step yielded in flat 3T3 nuclei where the chromocenters, though similar in size and shape, appeared to be collapsed on the nuclear surface facing the coverslide (Fig. 1b). Acid-denaturation is required after BrdU incorporation assays to provide antibody access for the detection of the BrdU epitope. Methanol fixation without any acid-denaturation was therefore selected and used in subsequent immunofluorescence analyses, unless noted otherwise.

A combination of chromocenter (DNA) staining and kinetochore protein staining using anti-centromere autoimmune (ACA) sera recognizing CENP-A, CENP-B and CENP-C (Wieland et al. 2004) in the 3D-preserved methanol-fixed nuclei allowed us to assess the spatial relationship between pericentromeric (chromocenters) and centromeric heterochromatin (ACA signals) (Fig. 1c–e). This classification is based on the recent demonstration that mouse major satellite repeats fully colocalize with and thus represent the pericentromeric heterochromatin in DNA FISH analyses in many mouse cell lines, including NIH3T3 fibroblasts (Guenatri et al. 2004). The same study also showed that minor satellite repeat DNA is fully coincident with the ACA antibody signals. High-resolution confocal microscopy combined with 3D rendering reconstruction revealed that pericentromeric and centromeric chromatin are juxtaposed but segregated domains (Fig. 1, c1–e2). The centromeric

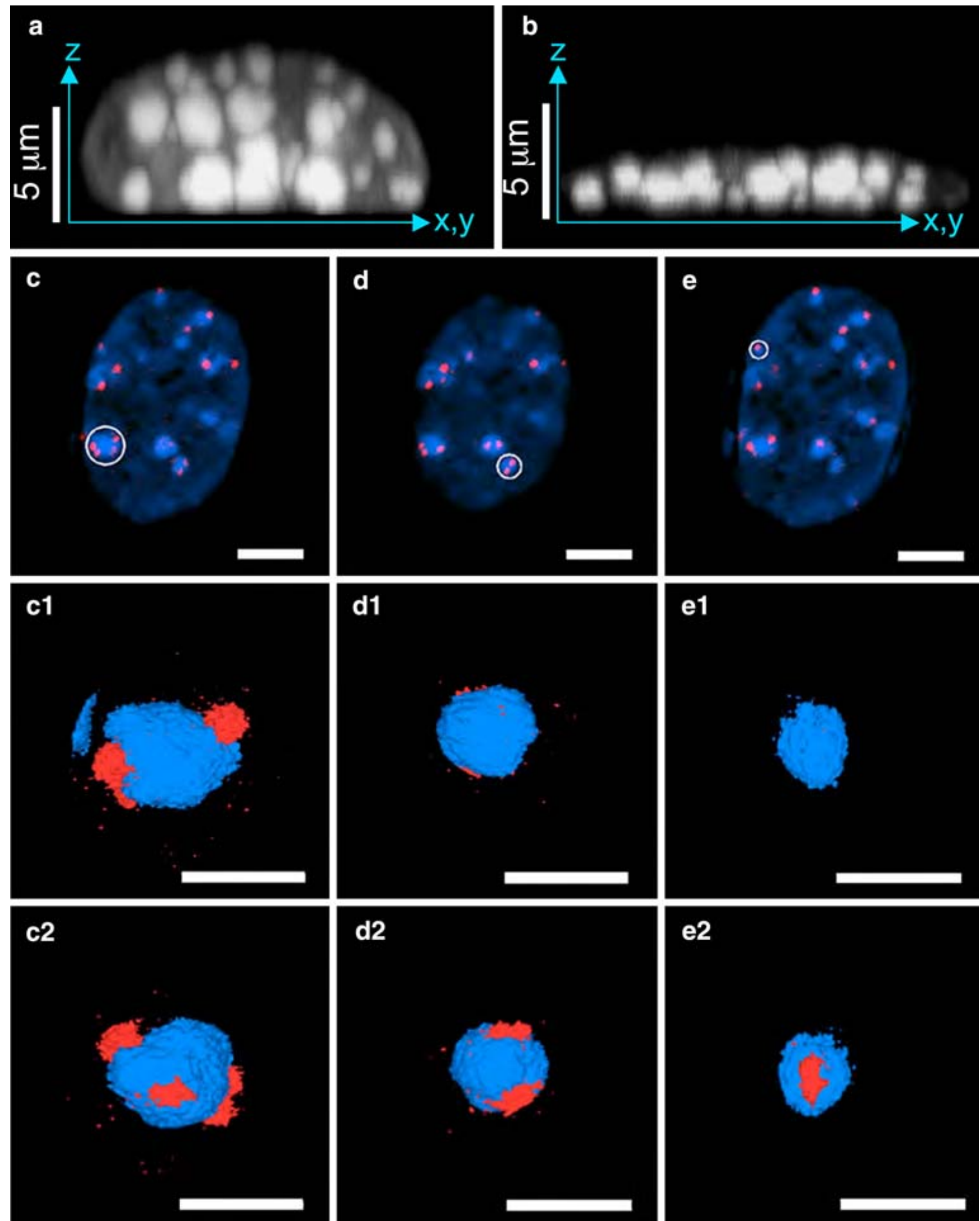

Fig. 1 Chromocenter and centromere organization in interphase mouse cells. NIH3T3 cells were fixed with Methanol/Aceton (**a**) or, after BrdU incorporation, with formaldehyde including an acid-denaturation step (**b**), and processed for immunofluorescence. High resolution confocal scans were performed on single nuclei. Images represent side views of the DNA signals (ToPro3) that were used to reconstruct the chromatin architecture. Three-dimensional reconstruction (**c**–**e**) and surface rendering (**c1**–**e2**) were performed on NIH 3T3 cells co-labelled with ToPro3 (*blue*) and anti-centromere antibodies (*red*). The region of computer rendering containing one chromocenter each is indicated by *white circles*. Reconstructed models of these chromocenters are shown as front views (**c1, d1, e1**) and rear views (**c2, d2, e2**). *Bars*, 5 µm in **c**–**e**, and 2 µm in **c1**–**e2**

domains appear as oval or spherical structures while the pericentromeric domains always have spherical morphology. Qualitatively, the size of the chromocenters correlated with the number of juxtaposed CEN domains: large, medium and small sized chromocenters were associated with 4-5, 2-3, and 1 CEN domain, respectively (Fig. 1, c1–e1). In more than one hundred cells analyzed we found (1) that centromeric domains never did occur in the interior of chromocenters, (2) that there were no centromeres without an associated chromocenter and (3) that there was no chromocenter not associated with at least one centromere. Taken together, these results demonstrate that DNA domains associated with centromere proteins A, B and C (which we will refer to as CEN domains, Sullivan and Karpen 2004) are located as individual entities on the surface of the chromocenters (which we will refer to as periCEN domains).

A subset of centromeres are replicated in early S-phase

In order to analyze the timing of centromere DNA replication in mouse NIH 3T3 cells, we co-labelled sites of ongoing DNA synthesis and centromeres. Labelling of DNA synthesis sites was done with antibodies against proliferating cell nuclear antigen (PCNA) because PCNA fully colocalizes with sites of nascent DNA as revealed by BrdU incorporation during all stages of DNA replication (Leonhardt et al. 2000; Somanathan et al. 2001; Dimitrova and Berezney 2002). In addition, PCNA labeling does not require acid denaturation and therfore preservation of nuclear architecture is significantly improved (Fig. 1a). The spatial patterns of DNA synthesis in mammalian cells are unique to specific times during S phase and can be determined without cell synchronization (Wu et al. 2005). We analyzed cells in

the absence of synchronization drugs to avoid possible perturbation or distortion of cell cycle progression which frequently occurs in mammalian cell lines (Wu et al. 2005). As expected, we could identify the five patterns of DNA replication based on PCNA staining in NIH3T3 cells (Fig. 2a). Similar to other mammalian cell lines (O'Keefe et al. 1992; Dimitrova and Gilbert 1999; Berezney 2002), early S phase cells displayed replication sites as numerous foci distributed throughout the nucleoplasm, excluding nucleoli (Fig. 2a, pattern S1). In early to mid S phase cells, replication foci are still scattered throughout the nucleoplasm but in addition begin to decorate the nucleolar and nuclear periphery (Fig. 2a, pattern S2). Mid S phase cells are characterized by clustering of replication foci around nucleoli, chromocenters and the nuclear rim (Fig. 2a, pattern S3). In mid to late S phase cells (Fig. 2a, pattern S4), sites of DNA replication have become larger in size, fewer in number, and appear predominantly as horseshoe-shaped accumulations at the heterochromatic chromocenters (see also Fig. 5c). The last pattern, characteristic of late S phase cells, displays as few large accumulations of replication sites (Fig. 2a, pattern S5). The classification of these patterns is consistent with previous results on DNA replication sites in mammalian cells (see introduction). Furthermore, this pattern sequence was also observed in synchronized NIH3T3 (data not shown).

The colocalization between CEN domains and replication sites at the different S phase stages was than assessed by confocal microscopy of NIH3T3 cells fluorescently labelled with antibodies against PCNA and centromere proteins (ACA sera) (Fig. 2b–f). Figure 2b–f shows mid confocal sections of one cell nucleus each of the five different S phase patterns with at least one centromere colocalized with the replication machinery. Centromere replication was defined as red (CEN) and green (PCNA) signals with complete or almost complete overlap. Little overlapping or touching signals were scored negative. Positive colocalization events were confirmed by inspection of the adjacent confocal sections in *z*-direction as previously described (Kiesslich et al. 2002), and by deconvolution analysis (data not shown). To confirm replication of centromeres in early S phase, replication sites were also detected after BrdU incorporation in NIH3T3. As seen with PCNA, we also found BrdU foci colocalizing with centromeres in early S phase cells (Fig. 2g). A quantitative evaluation demonstrated that the majority of centromeres are replicated in mid to late S phases (Fig. 3, patterns S3 and S4), consistent with findings in many other mammalian cell lines (O'Keefe et al. 1992; Shelby et al. 2000). However, this analysis also revealed, that a small but significant number of centromeres were already replicated in early (S1, S2) stages of S phase (Fig. 3). On an average, one to two centromeres in each S1 phase cell and four to five centromeres in each S2 phase cell were found to colocalize with replication sites. Taken together, ca. 12 ± 4 % of all centromeres detected in S1 and S2 cells replicated during these early stages of S phase in NIH 3T3 cells. A similar frequency was found in colocalization analyses after BrdU incorporation, thus validating the results obtained with PCNA labelling of replication sites (data not shown). The early replicating CEN domains were found to be randomly distributed throughout the nuclear volume indicating no direct correlation between early replication and nuclear positioning. About two centromeres per nucleus were also found to replicate in very late S phase cells (Fig. 3). We conclude that NIH3T3 cells replicate their centromeric DNA throughout all stages of S phase and that centromere DNA replication is not restricted to only mid and late S phase stages.

To extend these results further, we performed time lapse confocal microscopy analysis of C2C12 mouse myoblast cells co-expressing DNA ligase I fused to DsRed1 and an GFP-CENP-B fusion protein. DNA ligase I, like PCNA, can be used to track dynamics of replication factories in living cells (Cardoso et al. 1997; Easwaran et al. 2005). The GFP-CENP-B fusion protein was shown by immunofluorescence to fully colocalize with signals produced by our ACA serum demonstrating that GFP-CENP-B signals would represent the CEN domains in living cells (data not shown). Confocal stacks of C2C12 cells expressing low levels of DsRed-DNA ligase I and GFP-CENP-B were taken every 10 min for up to 10 h covering the complete S phase. Figure 4 shows one confocal section each of a nucleus at 30 min intervals progressing through the entire S phase. The characteristic pattern of replication factory dynamics over time can be easily appreciated. Although visual inspection of a time-lapse video of this confocal section revealed small changes in the nuclear shape over the course of S phase (movie 2, suppl. data), individual centromeres remained positionally stable indicating no major translocations of this chromosome domain during S phase (Fig. 4). The frequency of colocalization between DNA ligase I and CENP-B during S phase in living cells reflected that of the quantitative analysis in fixed cells (Fig. 3). The degree of colocalization peaked between 210 and 300 min when the cell passed from mid to late S phase. Most importantly, two of the centromeres replicated during the first hour of S phase (Fig. 4, 0–60 min), corroborating the previous finding of early replicating centromere DNA.

Replication of pericentromeric DNA is restricted to mid and late S phase

It is generally accepted that replication in late S phase is a hallmark of constitutive heterochromatin. Therefore, it was interesting to compare the relative timing of replication of CEN and periCEN heterochromatin in NIH3T3 cells. Double immunofluorescence labelling of chromocenters and PCNA allowed us to precisely analyse the spatial relationship between pericentromeric DNA and replication factories by confocal microscopy (Fig. 5a–c). As can be seen in Fig. 5b, chromocenters

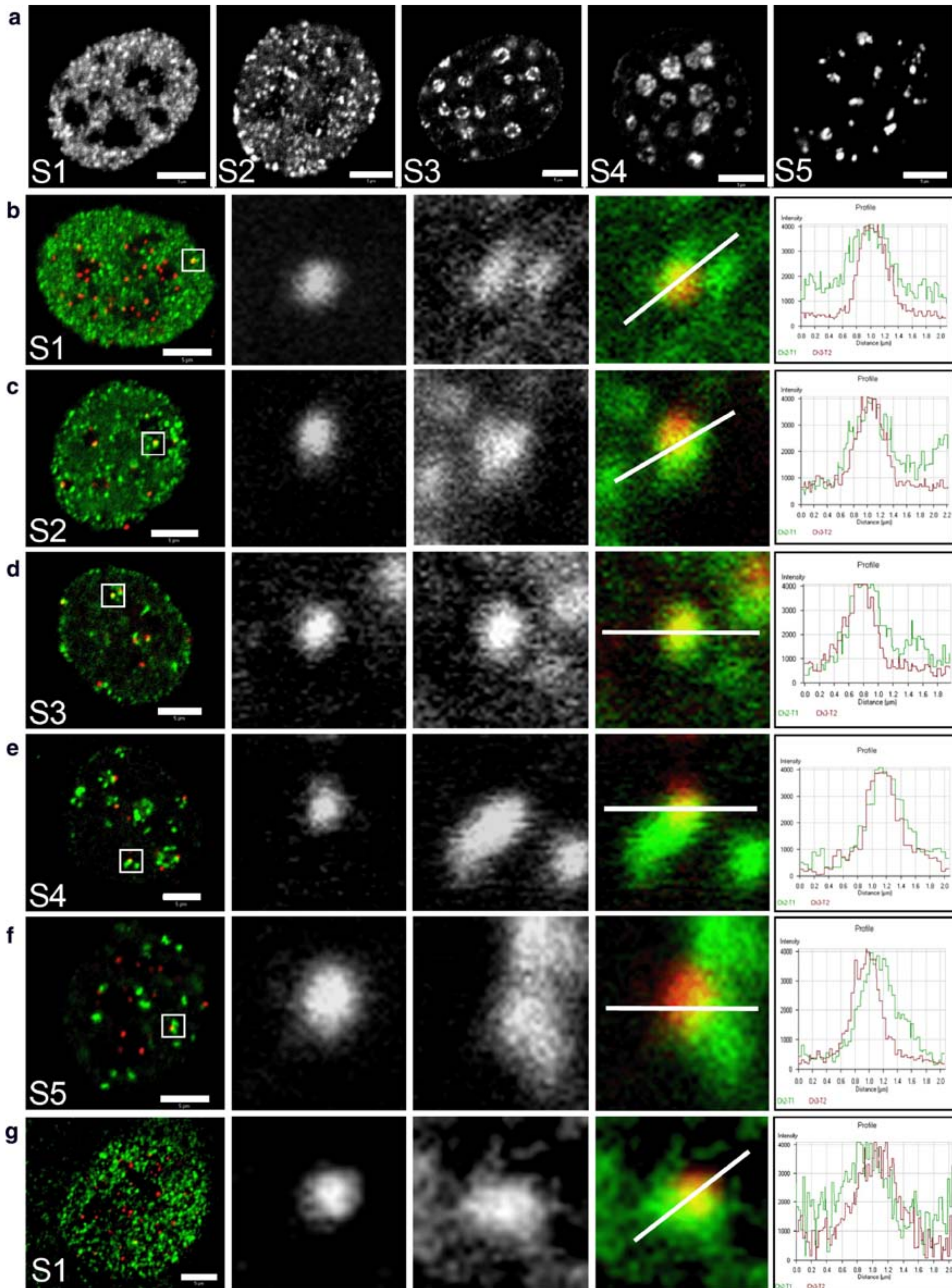

Fig. 2 Spatial relationship between centromeres and replication foci in NIH 3T3 cells. **a** Asynchronous cell cultures were stained with anti-PCNA antibodies. Shown are single confocal mid-nucleus sections of each of the replication labelling patterns. S1: early S phase, S2: early to mid S phase, S3: mid S phase, S4: mid to late S phase, S5: late S phase. **b**–**f** Asynchronous growing NIH3T3 cells were processed for immunofluorescence to visualize replication sites (*green*, PCNA signal) and centromeres (*red*, ACA signal), and analyzed by confocal microscopy. Representative mid-nucleus sections from nuclei at different stages of S phase (S1–S5) are shown on the left side of each image set. Regions within these overlays were selected as indicated by *squares* and shown as magnified images, split into monochrome channels for each color and merged. Line scans were recorded along the *white lines* shown in the merged images and displayed on the right side of each image set. **g** DNA replication sites in an early S phase NIH3T3 cell were detected after BrdU incorporation (*green*) and colocalized with centromeres (*red*)

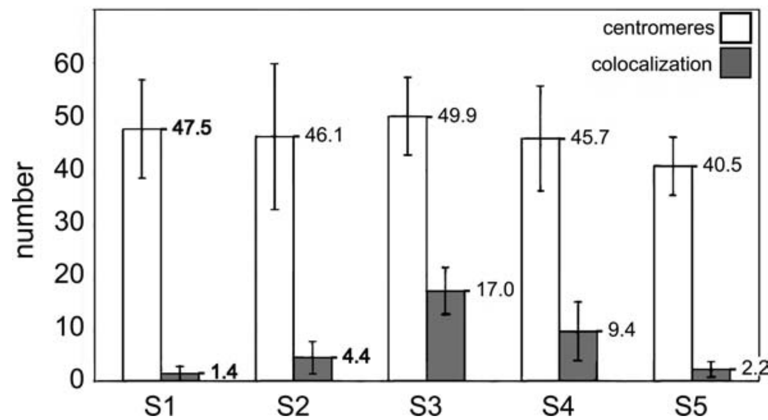

Fig. 3 Replication timing of centromeres in NIH3T3 cells. Asynchronous cultures of NIH3T3 cells were immunolabelled and analyzed as described for Fig. 2. For each of the five replication patterns (S1–S5), 10 nuclei were scanned by confocal microscopy at high resolution in 3D. The number of centromeres (*white columns*) and colocalization events between centromeres and replication foci (*grey columns*) were determined. Colocalizing signals were defined by complete or strong overlapping. Little overlap or touching signals were scored negative. Numbers indicate mean values ± sd

were not associated with replication factories in early S phase stages. This was true for more than 500 chromocenters examined in cells that displayed the S1 or S2 pattern. In contrast, all chromocenters ($n > 500$) of cells at S3 and S4 stage partially colocalized with replication sites (Fig. 5b, S3, S4). In very late S phase cells the degree of association was markedly decreased (Fig. 5b, S5).

We further analyzed the topology of replication factories at chromocenters. Three-dimensional *z*-stacks of replicating chromocenters revealed an assembly of several replication factories at the chromocenter domain (Fig. 5c, d). Similar to the centromeres, the number of chromocenters did not vary significantly when the cells replicate their genome (Fig. 5e). The average number of chromocenters remained constant at around 25 ± 4. This indicates that neither centromeric nor pericentromeric DNA from sister chromatids segregate into microscopically distinct domains during duplication.

Taken together, these data indicate that while centromere DNA replication can be detected during all stages of S phase (Fig. 2), pericentromeric DNA replication is restricted to a window between mid and late S phase. This suggests asynchronous replication timing of these different DNA domains and that a low but significant number of centromeres in NIH3T3 cells may be replicated as isolated domains in early S phase.

Discussion

The high degree of functional organization within the eukaryotic cell nucleus is reflected by the clustering and defined topology of specific chromosomal domains in the 3D space of the nucleus (Cremer and Cremer 2003; Hemmerich and Diekmann 2005). Based on cytological analyses, a global differentiation of the genome was introduced with the description of euchromatin and heterochromatin (Heitz 1928). Heterochromatin corresponds to regions of the genome that remain intensely stained throughout the cell cycle and that is transcriptionally inactive (Dillon 2004). Nevertheless, heterochromatin has essential functions in nuclear architecture, chromosome segregation, and gene silencing (Wallrath 1998; Gasser 2001; Grewal and Moazed 2003). Constitutive heterochromatin at centromeres provides an architectural framework for kinetochore function, ensures a high density of cohesins at centromeric regions, and might also act as a trigger for kinetochore assembly (Pidoux and Allshire 2005). Failure in the function of these elements can lead to genomic instability, with often catastrophic consequences in humans such as miscarriage, congenital birth defects or cancer (Vig et al. 1989; von Mikecz and Hemmerich 2005).

In mammalian interphase cells, pericentromeric heterochromatin assembles into chromocenters by ectopic conjugation between different chromosomes in a cell type-specific manner (Hsu 1975; Haaf and Schmid 1989; Manuelidis 1984; Alcobia et al. 2000). Particularly in mouse cells, the chromocenters formed from centromeres of acrocentric chromosomes are easy to visualize by light microscopy using DNA dyes (Hubert and Bourgeois 1986). Mouse chromocenters consist of large arrays of tandem major satellite repeats which match perfectly with the chromocenter structure in DNA-FISH analysis in murine cell lines (Guenatri et al. 2004). A second class of mouse satellite DNA termed minor satellites form individual entities on the surface of the chromocenters and these domains are specifically associated with the constitutive centromere proteins CENP-A, -B, and -C (Guenatri et al. 2004). Consistent with these observations, our study shows that these CEN domains are associated with, yet are spatially discernible from the spherical periCEN domains (chromocenters) allowing us to determine in detail the replication timing of both domains.

In agreement with the previous studies on centromere replication timing (Ten-Hagen et al. 1990; O'Keefe et al. 1992; Haaf and Ward 1994; Shelby et al.

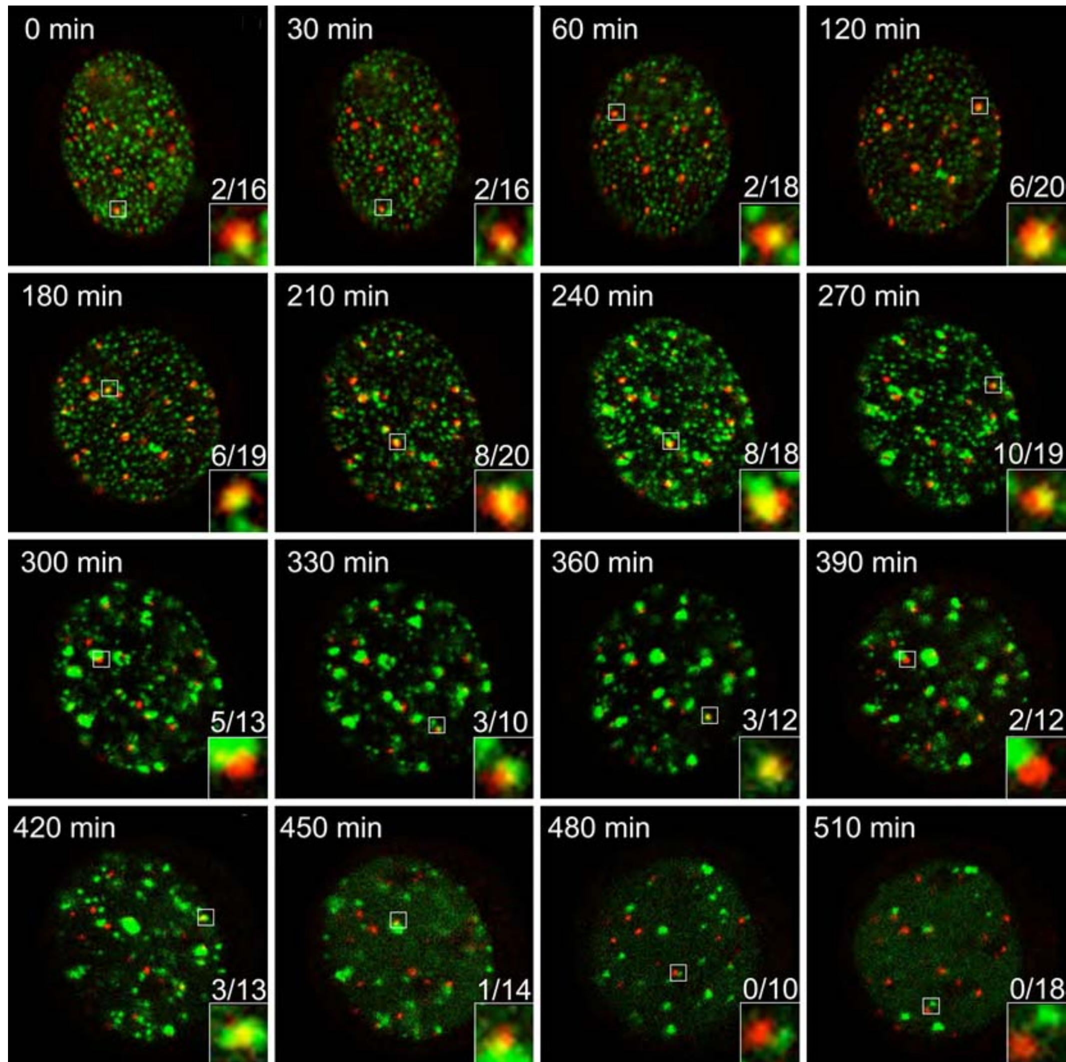

Fig. 4 Dynamics of centromere replication in living cells. C2C12 mouse myoblasts expressing DsRead1-tagged DNA ligase I (*green*) and GFP-tagged CENP-B (*red*) were analyzed during S phase by time-lapse confocal microscopy. Pseudocolors were chosen to be consistent with other fixed cell analysis. Images of the same confocal section in 30 min intervals are shown. Replicating centromeres were selected from each image and displayed as magnified views in the bottom right corner of each image. The number of replicating centromeres and total number of visible centromeres of each image is also shown. The varying total number of centromeres over time can be attributed to small-scale positional movements of the centromeres in and out of the confocal section. Full time lapse is shown in supplementary data, movie 2

2000; Guenatri et al. 2004) we find that the centromeric and pericentromeric domains replicate asynchronously and that the latter are replicated predominantly in mid S phase. However, in disagreement with previous reports, we find that a subset ($12 \pm 4\%$) of centromeres is replicated in early S phase. The unexpected detection of early centromere replication may be attributable to the usage of different cell lines, by improved visualization methods, and by our quantitative approach that was not biased towards late replication of centromeres.

Significant deviations from the paradigm of late centromere replication have been reported. Early replication of centromeric DNA has been demonstrated in *Saccharomyces cerevisiae* (McCaroll and Fangman 1988), in *Schizosaccharomyces pombe* for the outer (Kim and Huberman 2001) and inner (Kim et al. 2003) centromeric heterochromatin, in bovine satellite I DNA within centromeric heterochromatin (Matsumoto and Gerbi 1982), in centromeric heterochromatin of some mouse chromosomes (Vig and Broccoli 1988; Vig et al. 1993; Hollo 1996), in centromeric heterochromatin of *Vicia faba* (Fuchs et al. 1998), and in *Drosophila* (Ahmad and Henikoff 2001, 2002; Sullivan and Karpen 2001). A very interesting finding in this respect is that two murine wild-type embryonic stem cell lines, HM1 and TT2, replicate their pericentric heterochromatin at measurably different times, HM1 late and TT2 early in S phase (Wu et al. 2005). Finally, late replication is not a requirement for centromere function since functional

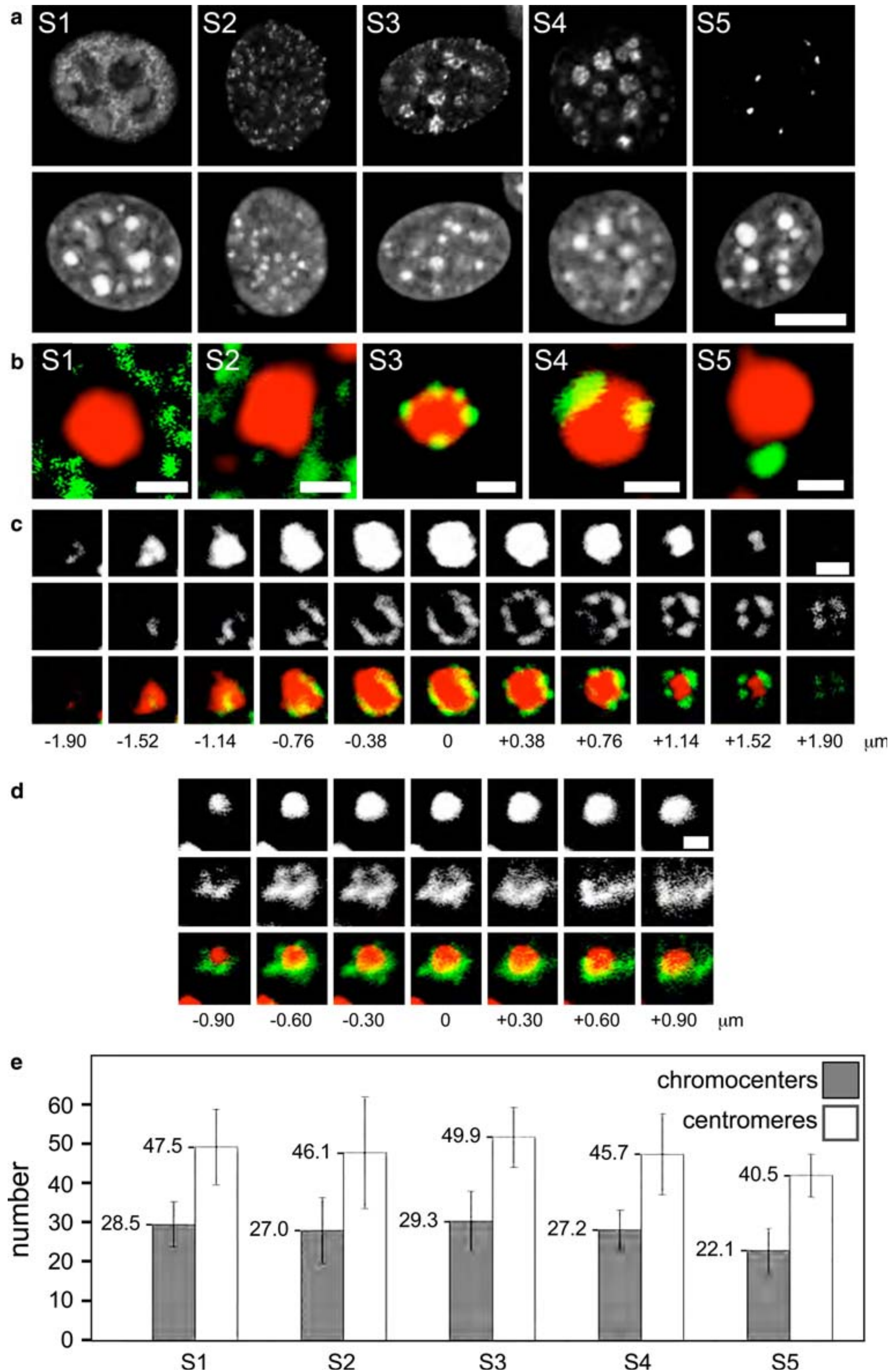

Fig. 5 Replication timing and topology of chromocenters in NIH3T3 cells. **a** Asynchronous cultures of NIH3T3 were PCNA (*upper panel*) and ToPro3 (*bottom panel*) labelled to detect replication sites and DNA, respectively. Mid-confocal nucleus sections of both fluorescence signals are shown for one representative cell each at the different stages of S phase. **b** Representative chromocenters (*red*) co-labelled with replication sites (*green*) were selected from all stages of S phase and are shown as magnified views. Each channel is shown as individual images in monochrome (row 1, and 2) as well as superimposed in color (third row). **c**, **d** High-resolution confocal analysis of chromocenters (*red*) in mid S phase co-labelled with PCNA (*green*, **c**) and BrdU (*green*, **d**). Shown are adjacent confocal sections as single channels and the respective merged images. *Numbers* indicate the *z*-step distance of the confocal sectioning between adjacent image scans. **e** Quantitation of the number of centromeres and chromocenters at each phase (S1–S5) of S phase. *Numbers* indicate mean values from data derived from 20 cells each of the different S phase stages. Standard deviations are shown as *error bars* on the columns. *Bars*: 10 µm in **a**, 1 µm in **b**, **c**, **d**

centromeres on artificial chromosomes can replicate early along with the entire chromosome (Grimes et al. 2004).

There are in addition some examples of other heterochromatin domains replicated in early S phase: Drosophila β-heterochromatin which is located adjacent to the pericentric α-heterochromatin (Schubeler et al. 2002), some human telomeres (Wright et al. 1999), and some X-inactivated autosomal genes after heterochromatin spreading (Sharp et al. 2001). In agreement with our notion that replication of centromeric heterochromatin in mammalian cells can occur at all stages of S phase, it has been demonstrated in *S. pombe* that heterochromatin replication is not restricted to a particular stage of S phase: while the heterochromatic telomeres of *S. pombe* replicate in late S phase, the heterochromatic centromeres and the silent mating cassettes replicate very early in S phase (Kim et al. 2003). That the dogmatic relationship between late replication and heterochromatin is not absolute is also underscored by the observation that some inactive heterochromatic genes replicate early in *Drosophila* (Schubeler et al. 2002).

The results of this study together with the above mentioned examples from other organisms suggest that functions mediated by constitutive heterochromatin, as well as its formation and maintenance do not necessarily require late replication. While in *S. pombe* (Kim et al. 2003) and probably in *Drosophila* as well (Ahmad and Henikoff 2001), centromeres can be replicated as isolated domains in early S phase, mammalian cells appear to replicate their centromere DNA in all phases of the S phase with a peak in mid to late S phase. This suggests that timing of centromere replication is not only species and cell type specific but may also be chromosome-specific. We are currently addressing this question by analyzing the centromere replication timing of individual human chromosomes. Importantly, our data indicate that a centromeric 'state' does not depend on replication in late S phase. This is also underscored by the fact that deposition of the centromere marker CENP-A (a histone H3 variant) takes place during G2 uncoupled from the centromere replication in HeLa cells (Shelby et al. 2000).

The question arises, why in NIH3T3 cells and likely in other mammalian cell lines also, the pericentric heterochromatin follows the dogma of mid to late replication (Fig. 5; Guenatri et al. 2004), while the CEN domain does not. Both domains exhibit different structural and functional features. Major satellites (pericentromeric chromocenters) appear less accessible than minor satellites (centromeres), as revealed by Mnase digestion (Guenatri et al. 2004). Gilbert and Allan (2001) found that chromatin fibers derived from the centromeric domain of NIH3T3 chromosomes exist in a different condensation status when compared to pericentromeric chromatin fibers. The chromocenters are also enriched in trimethylated Lysin 9-Histone 3 (triMe-K9H3) nucleosomes, a feature of inert constitutive heterochromatin (Peters et al. 2003; Rice et al. 2003) accompanied by recruitment of HP1α, while the CENP-associated minor satellites contain significantly less triMe-K9H3 and no HP1α (Guenatri et al. 2004). In addition, high resolution mapping of epigenetic marks revealed that the centromeric chromatin of human and *Drosophila* chromosomes is markedly distinct from both euchromatin and flanking heterochromatin: In CENP-A associated centromere regions Histone H3 is neither Lys9-trimethylated nor Lys9-dimethylated (Sullivan and Karpen 2004). This lack of hyper-methylated Lys9-H3 in centromere DNA may explain the absence of HP1, and may make this chromatin more accessible for the replication machinery. Unfortunatelly, this report did not test for the presence of mono-methylated H3 in CEN domains, because chromatin with this mark replicates very early in S phase (Wu et al. 2005). Wu et al. (2005) also showed that CHO cells replicated a similar fraction of triMe-K9H3 chromatin at all times of S phase, including early S, adding more evidence for early heterochromatin replication in mammalian cells.

The replication timing process seems to involve three components: the epigentic marking of origins as early or late; the use of cell cycle-regulated protein factors to activate origins at the proper times in S phase; and a system of checkpoint genes that ensure replication timing according to schedule (Goren and Cedar 2003). More detailed analyses of these components during centromere replication may shed light on the mechanisms underlying early centromere replication. Currently, it is not easy to reconcile early centromere replication with some of the replication 'rules' that have been uncovered during the last decades. Probably some of these rules may be common but not universal (Kim et al. 2003).

Acknowledgement We appreciate very much the helpful discussions with Stephan Diekmann and are grateful to Xenia Reich who eye-selected the mid-nucleus confocal sections from time-lapse analysis and assembled them into a movie. H.-P. Rahn was supported by the European Union (ESF Program). This work was supported by the Deutsche Forschungsgemeinschaft (grant HE 2484/3-1).

References

Ahmad K, Henikoff S (2001) Centromeres are specialized replication domains in heterochromatin. J Cell Biol 153:101–109

Ahmad K, Henikoff S (2002) Histone H3 variants specify modes of chromatin assembly. Proc Natl Acad Sci USA Early Edition:1–8

Alcobia I, Dilao R, Parreira L (2000) Spatial associations of centromeres in the nuclei of hematopoietic cells: evidence for cell-type-specific organizational patterns. Blood 95:1608–1615

Bostock CJ, Prescott DM (1971) Shift in buoyant density of DNA during the synthetic period and its relation to euchromatin and heterochromatin in mammalian cells. J Mol Biol 60:151–162

Alexandrova O, Solovei I, Cremer T, David CN (2003) Replication labeling patterns and chromosome territories typical of mammalian nuclei are conserved in the early metazoan Hydra. Chromosoma 112:190–200

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

Calza RE, Eckhardt LA, Delgiudice T, Schildkraut CL (1984) Changes in gene position are accompanied by a change in time of replication. Cell 36:689–696

Celis JE, Celis A (1985) Cell cycle-dependent variations in the distribution of the nuclear protein cyclin proliferating cell nuclear antigen in cultured cells: subdivision of S phase. Proc Natl Acad Sci U S A 82:3262–3266

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

Cremer T, Cremer C (2001) Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nat Rev Genet 2:292–301

Csink AK, Henikoff S (1998) Something from nothing: the evolution and utility of satellite repeats. Trends Genet 14:200–204

Diffley JF, Labib K (2002) The chromosome replication cycle. J Cell Sci 115:869–872

Dillon N (2004) Heterochromatin structure and function. Biol Cell 96:631–637

Dimitrova DS, Gilbert DM (1999) The spatial position and replication timing of chromosomal domains are both established in early G1 phase. Mol Cell 4:983–993

Dimitrova DS, Berezney R (2002) The spatio-temporal organization of DNA replication sites is identical in primary, immortalized and transformed mammalian cells. J Cell Sci 115:4037–4051

Dooley DC, Ozer HL (1977) Replication kinetics of three DNA sequence families in synchronized mouse cells. J Cell Physiol 90:337–350

Easwaran HP, Leonhardt H, Cardoso MC (2005) Cell cycle markers for live cell analyses. Cell Cycle 4:453–455

Fox MH, Arndt-Jovin DJ, Jovin TM, Baumann PH, Robert-Nicoud M (1991) Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in mouse fibroblasts. J Cell Sci 99:247–253

Fuchs J, Strehl S, Brandes A, Schweizer D, Schubert I (1998) Molecular-cytogenetic characterization of the *Vicia faba* genome- heterochromatin differentiation, replication patterns and sequence localization. Chrom Res 6:219–230

Gasser SM (2001) Positions of potential: nuclear organization and gene expression. Cell 104:639–642

Gilbert N, Allan J (2001) Distinctive higher-order chromatin structure at mammalian centromeres. Proc Natl Acad Sci USA 98:11949–11954

Goldman MA, Holmquist GP, Gray MC, Caston LA, Nag A (1984) Replication timing of genes and middle repetitive sequences. Science 224:686–692

Goren A, Cedar H (2003) Replicating by the clock. Nat Rev Mol Cell Biol 4:25–32

Gotta M, Laroche T, Formenton A, Maillet L, Scherthan H, Gasser SM (1996) The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J Cell Biol 134:1349–1363

Grewal SI, Moazed D (2003) Heterochromatin and epigenetic control of gene expression. Science 301:798–802

Grimes BR, Babcock J, Rudd MK, Chadwick B, Willard HF (2004) Assembly and characterization of heterochromatin and euchromatin on human artificial chromosomes. Genome Biol 5:R89

Guenatri M, Bailly D, Maison C, Almouzni G, (2004) Mouse centric and pericentric satellite repeats form distinct functional heterochromatin. J Cell Biol 166:493–505

Guillot PV, Sonya M, Pombo A (2005) The organization of transcription in the nucleus of mammalian cells. In: Hemmerich P, Diekmann S (eds) Visions of the cell nucleus. American Scientific Publishers, Stevenson Ranch, pp 95–100

Haaf T, Schmid M (1989) Centromeric association and non-random distribution of centromeres in human tumour cells. Hum Genet 81:137–143

Haaf T, Ward DC (1994) Structural analysis of alpha-satellite and centromere proteins using extended chromatin and chromosomes. Hum Mol Genet 3:697–709

Hatton KS, Dhar V, Brown EH, Iqbal MA, Stuart S, Didamo VT, Schildkraut CL (1988) Replication program of active and inactive multigene families in mammalian cells. Mol Cell Biol 8:2149–2158

Heitz E (1928) Das Heterochromatin der Moose. I Jahrb Wiss Bot 69:762–818

Hemmerich S, Diekmann S (eds) (2005) Visions of the cell nucleus. American Scientific Publishers, Stevenson Ranch

Hollo G, Kereso J, Praznovszky T, Cserpan I, Fodor K, Katona R, Csonka E, Fatyol K, Szeles A, Szalay AA, Hadlaczky G (1996) Evidence for a megareplicon covering megabases of centromeric chromosome segments. Chromosome Res 4:240–247

Hozak P, Jackson DA, Cook PR (1994) Replication factories and nuclear bodies: the ultrastructural characterization of replication sites during the cell cycle. J Cell Sci 107:2191–2202

Hubert J, Bourgeois CA (1986) The nuclear skeleton and the spatial arrangement of chromosomes in the interphase nucleus of vertebrate somatic cells. Hum Genet 74:1–15

Hsu TC (1975) A possible function of constitutive heterochromatin: the bodyguard hypothesis. Genetics 79:137–150

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

Jin Q, Trelles-Sticken E, Scherthan H, Loidl J (1998) Yeast nuclei display prominent centromere clustering that is reduced in nondividing cells and in meiotic prophase. J Cell Biol 141:21–29

Kiesslich A, von Mikecz A, Hemmerich P (2002) Cell cycle-dependent association of PML bodies with sites of active transcription in nuclei of mammalian cells. J Struct Biol 140:1671–1679

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

Kim SM, Huberman JA (2001) Regulation of replication timing in fission yeast. EMBO J 20:6115–6126

Kim SM, Dubey DD, Huberman JA (2003) Early-replicating heterochromatin. Genes Dev 17:330–335

Koberna K, Ligasova A, Malinsky J, Pliss A, Siegel AJ, Cvackova Z, Fidlerova H, Masata M, Fialova M, Raska I, Berezney R (2005) Electron microscopy of DNA replication in 3-D: evidence for similar-sized replication foci throughout S-phase. J Cell Biochem 94:126–138

Leonhardt H, Page AW, Weier HU, Bestor TH (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. Cell 27:865–873

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

Lemaitre J-M, Méchali M (2005) Organisation and Dynamics of the Cell Nucleus for DNA Replication. In: Hemmerich P, Diekmann S (eds) Visions of the cell nucleus. American Scientific Publishers, Stevenson Ranch, pp 37–51

Lima-de-Faria A, Nilsson B, Cave D, Puga A, Jaworska H (1968) Tritium labelling and cytochemistry of extra DNA in *Acheta*. Chromosoma 25:1–20

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

Manders EM, Stap J, Brakenhoff GJ, van Driel R, Aten JA (1992) Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. J Cell Sci 103:857–862

Manuelidis L (1984) Different central nervous system cell types display distinct and nonrandom arrangements of satellite DNA sequences. Proc Natl Acad Sci U S A 81:3123–3127

Matsumoto LH, Gerbi SA (1982) Early initiation of bovine satellite I DNA replication. Exp Cell Res 140:47–54

Mazzotti G, Rizzoli R, Galanzi A, Papa S, Vitale M, Falconi M, Neri LM, Zini N, Maraldi NM (1990) High-resolution detection of newly synthesized DNA by anti-bromodeoxyuridine

antibodies identifies specific chromatin domains. J Histochem Cytochem 38:13–22
McCaroll RM, Fangman WL (1988) Time of replication of yeast centromeres and telomeres. Cell 54:505–513
Nakamura H, Morita T, Sato C (1986) Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. Exp Cell Res165:291–297
Nakayasu H, Berezney R (1989) Mapping replicational sites in the eucaryotic cell nucleus. J Cell Biol 108:1–11
Neri LM, Mazzotti G, Capitani S, Maraldi NM, Cinti C, Baldini N, Rana R, Martelli AM (1992) Nuclear matrix-bound replicational sites detected in situ by 5-bromodeoxyuridine. Histochemistry 98:19–32
O'Keefe RT, Henderson SC, Spector D (1992) Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific α-satellite DNA sequences. J Cell Biol 116:1095–1110
Peters AH, Kubicek S, Mechtler K, O'Sullivan RJ, Derijck AA, Perez-Burgos L, Kohlmaier A, Opravil S, Tachibana M, Shinkai Y, Martens JH, Jenuwein T (2003) Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol Cell 12:1577–1589
Philimonenko AA, Jackson DA, Hodny Z, Janacek J, Cook PR, Hozak P (2004) Dynamics of DNA replication: an ultrastructural study. J Struct Biol 148:279–289
Pidoux AL, Allshire RC (2005) The role of heterochromatin in centromere function. Philos Trans R Soc Lond B Biol Sci 360:569–579
Raska I, Koberna K, Jarnik M, Petrasovicova V, Bednar J, Raska K Jr, Bravo R (1989) Ultrastructural immunolocalization of cyclin/PCNA in synchronized 3T3 cells. Exp Cell Res 184:81–89
Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, Shinkai Y, Allis CD (2003) Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol Cell 12:1591–1598
Schubeler D, Scalzo D, Kooperberg C, van Steensel B, Delrow J, Groudine M (2002) Genome-wide DNA replication profile for Drosophila melanogaster: a link between transcription and replication timing. Nat Genet 32:438–442
Sharp A, Robinson DO, Jacobs P (2001) Absence of correlation between late-replication and spreading of X inactivation in an X; autosome translocation. Hum Genet 109:295–302
Shelby RD, Hahn KM, Sullivan KF (1996) Dynamic elastic behavior of alpha-satellite DNA domains visualized in situ in living human cells. J Cell Biol 135:545–557
Shelby RD, Monire K, Sullivan KF (2000) Chromatin assembly at kinetochores is uncoupled from DNA replication. J Cell Biol 151:1113–1118
Somanathan S, Suchyna TM, Siegel AJ, Berezney R (2001) Targeting of PCNA to sites of DNA replication in the mammalian cell nucleus. J Cell Biochem 81:56–67
Sparvoli E, Levi M, Rossi E (1994) Replicon clusters may form structurally stable complexes of chromatin and chromosomes. J Cell Sci 107:3097–3103
Spector DL (1993) Macromolecular domains within the cell nucleus. Annu Rev Cell Biol 9:265–315
Sporbert A, Gahl A, Ankerhold R, Leonhardt H, Cardoso MC (2002) DNA polymerase clamp shows little turnover at established replication sites but sequential de novo assembly at adjacent origin clusters. Mol Cell 10:1355–1365
Sullivan BA, Karpen G (2001) Centromere identity in *Drosophila* is not determined in vivo by replication timing. J Cell Biol 154:683–690
Sullivan BA, Karpen GH (2004) Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. Nat Struct Mol Biol 11:1076–1083
Ten-Hagen KG, Gilbert DM, Willard HF, Cohen SN (1990) Replication timing of DNA sequences associated with human centromeres and telomeres. Mol Cell Biol 10:6348–6355
Wallrath LL (1998) Unfolding the mysteries of heterochromatin. Curr Opin Genet Dev 8(2):147–153
Westermann KA, Leboulch P (1996) Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination. Proc Natl Acad Sci USA 93:8971–8976
van Dierendonck JH, Keyzer R, van de Velde CJ, Cornelisse CJ (1989) Subdivision of S-phase by analysis of nuclear 5-bromodeoxyuridine staining patterns. Cytometry 10:143–150
Vig BK, Broccoli D (1988) Sequence of centromere separation: differential replication of pericentric heterochromatin in multicentric chromosomes. Chromosoma 96:311–317
Vig BK, Paweletz N, Schroeter D (1993) The centromere-kinetochore complex in cancer. Canc J 6:243–252
von Mikecz A, Hemmerich P (2005) Subnuclear pathology. In: Hemmerich P, Diekmann S (eds) Visions of the cell nucleus. American Scientific Publishers, Stevenson Ranch, pp 184–203
Wieland G, Orthaus S, Ohndorf S, Diekmann S, Hemmerich P (2004) Functional complementation of human centromere protein A (CENP-A) by Cse4p from *Saccharomyces cerevisiae*. Mol Cell Biol 24:6620–6630
Wright WE, Tesmer VM, Liao ML, Shay JW (1999) Normal human telomeres are not late replicating. Exp Cell Res 251:492–499
Wu R, Terry AV, Singh PB, Gilbert DM (2005) Differential subnuclear localization and replication timing of histone H3 lysine 9 methylation states. Mol Biol Cell 16:2872–2881