Experimental Cell Research **247,** 176–188 (1999)
Article ID excr.1998.4311, available online at http://www.idealibrary.com on IDEAL®

Organization of Early and Late Replicating DNA in Human Chromosome Territories

Daniele Zink,*[,1,2] Harald Bornfleth,†[,‡,1] Astrid Visser,§ Christoph Cremer,† and Thomas Cremer[¶]

*Institut für Anthropologie und Humangenetik, LMU München, Goethestrasse 31, D-80336 München, Germany; †Institut für Angewandte Physik, Universität Heidelberg, Albert-Überle-Strasse 3-5, D-69120 Heidelberg, Germany; ‡Interdisziplinäres Zentrum für wissenschaftliches Rechnen, Universität Heidelberg, Im Neuenheimer Feld 368, D-69120 Heidelberg, Germany; §Department of Radiobiology, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands; and ¶Institut für Anthropologie und Humangenetik, LMU München, Richard-Wagner-Strasse 10/I, D-80333 München, Germany

It has been suggested that DNA organized into replication foci during S-phase remains stably aggregated in non-S-phase cells and that these stable aggregates provide fundamental units of nuclear or chromosome architecture [C. Meng and R. Berezney (1991) *J. Cell Biol.* 115, 95a; E. Sparvoli *et al.* (1994) *J. Cell Sci.* 107, 3097–3103; D. A. Jackson and A. Pombo (1998) *J. Cell Biol.* 140, 1285–1295; D. Zink *et al.* (1998) *Hum. Genet.* 112, 241–251]. To test this hypothesis, early and late replicating DNA of human diploid fibroblasts was labeled specifically by incorporating two different thymidine analogs [J. Aten (1992) *Histochem. J.* 24, 251–259; A. E. Visser (1998) *Exp. Cell Res.* 243, 398–407], during distinct time segments of S-phase. On mitotic chromosomes the amount and spatial distribution of early and late replicating DNA corresponded to R/G-banding patterns. After labeling cells were grown for several cell cycles. During this growth period individual replication labeled chromosomes were distributed into an environment of unlabeled chromosomes. The nuclear territories of chromosomes 13 and 15 were identified by additional chromosome painting. The distribution of early and late replicating DNA was analyzed for both chromosomes in quiescent (G_0) cells or at G_1. Early and late replicating DNA occupied distinct foci within chromosome territories, displaying a median overlap of only 5–10%. There was no difference in this regard between G_1 and G_0 cells. Chromosome 13 and 15 territories displayed a similar structural rearrangement in G_1 cells compared to G_0 cells resulting in the compaction of the territories. The findings demonstrate that early and late replicating foci are maintained during subsequent cell cycles as distinctly separated units of chromosome organization. These findings are compatible with the hypothesis that DNA organized into replicon clusters remains stably aggregated in non-S-phase cells. © 1999 Academic Press

Key Words: **chromosome territory; replication foci; replication labeling; subchromosomal foci; chromosome architecture.**

INTRODUCTION

It has been suggested that higher order structural organization of DNA within the cell nucleus and within individual chromosome territories plays a crucial role in nuclear function [7–13]. Distinct models regarding DNA organization within chromosome territories have been developed [8, 11–15]. However, unequivocal experimental evidence for a particular organizational principle was lacking and experimental approaches involving the FISH technique, the most widely used technique for studying nuclear DNA and chromosome organization, resulted in the development of different and partially contradictory models [8, 11–14]. Several studies on replication labeling now strongly suggest that the organization of replication might be closely related to the functional architecture of nuclear DNA and chromosome territories [1–4, 16, 17].

The process of replication is temporally and spatially ordered in S-phase nuclei of mammalian and plant cells. Replicating DNA as well as proteins essential for replication are organized within so-called replication foci or replication granules [2, 16, 18, 19]. Several hundred replication foci with diameters ranging between approximately 0.3 and 0.8 μm are present at most time points during S-phase. The foci are not randomly distributed over the nucleus but are organized into patterns characteristic for different temporal S-phase stages [2, 20–22].

On the molecular level it is known that groups of adjacent replicons (with most single replicons displaying a length between 50 and 300 kb), so-called replicon

[1] The first two authors contributed equally to this work.

[2] To whom reprint requests should be addressed. Fax: ++49-89/5996-618. E-mail: dani.zink@lrz.uni-muenchen.de.

0014-4827/99 $30.00

clusters, are synchronously activated during S-phase [23, 24]. The replication of DNA organized into one replicon cluster is completed within approximately 1 h [25, 26]. Different groups of replicon clusters are subsequently activated during S-phase.

It has been suggested that single replication foci correspond to individual replicon clusters that display in combination with replication factors a defined three-dimensional organization [3, 16, 20]. It was estimated that each replication focus contains about five replicons [3]. A crucial role for the nuclear matrix (skeleton) in organization and positioning of replication foci has been suggested [3, 16, 20, 27].

Different studies suggest that DNA organized into distinct replication foci during S-phase remains stably aggregated in non-S-phase nuclei and during mitosis [1–4, 16, 17]. Chromosome territories of fixed, as well as of living, cells display replication labeled foci that are maintained at least for several cell cycles [2–4, 17]. The foci observed in living cell interphase chromosome territories were referred to as subchromosomal foci [4] and are most likely identical to the foci observed in fixed cells. Similar replication labeled foci were also noted on mitotic chromosomes and arranged in band-like patterns [2, 3]. From these findings it was concluded that stable DNA aggregates corresponding to replication foci might be fundamental units of chromosome structure [2–4].

To find further evidence for the hypothesis that chromosomes are composed of stable DNA aggregates that correspond to S-phase replication foci we extended our analysis of replication labeled chromosome foci. We have previously demonstrated that DNA replicating at different time segments during S-phase occupies distinct foci within chromosome territories that are stably maintained for at least 2 weeks within *in vitro* cultured fibroblasts [4]. Here, we show that these foci of early and late replicating DNA correspond to G-light (R-band) and G-dark band patterns on mitotic chromosomes. These findings support a close structural relationship between the organization of mitotic chromosomes and chromosome territories. An extensive quantitative analysis of distinctly replication labeled foci detected after several cell cycles revealed that there was only a minor overlap of approximately 5 to 10% between the different types of foci. The analyses focused on chromosome 13 and chromosome 15 territories in G_1 and quiescent cells. The degree of separation between foci replicating at different time points was maintained at these two different functional states although a reorganization of the chromosome territories was observed. These results support the hypothesis that stable foci corresponding to replication foci during S-phase are fundamental units of chromosome structure.

MATERIALS AND METHODS

Cell culture. Diploid human female oral cavity fibroblasts (GF 032, kindly provided by P. Tomakidi, Kopfklinik Heidelberg) were cultured at 37°C in an atmosphere of 5% CO_2 with Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, antibiotics (penicillin, 100 μg/ml, and streptomycin, 100 μg/ml), and 10% FCS. Fibroblasts at G_1 were obtained by a shake off of the mitotic cells which were then seeded on coverslips and fixed (10 min PBS, 3.7% formaldehyde) after 6 h. During this period nearly 100% of the seeded cells divided. G_0 cells were obtained by maintaining the cultures on coverslips confluent for 5 days and by keeping the confluent cultures in medium supplemented with only 0.05% FCS during the last 2 days before fixation (10 min PBS, 3.7% formaldehyde). As a control for reproducible replication banding of mitotic chromosomes we used another strain of human diploid fibroblasts (Hv, kindly provided by J. Murken, LMU München).

Control of interphase stage selection. To confirm that the cells fixed after the mitotic shake off were still in G_1, 10 μM BrdU was added to the medium of the seeded cells. The immunodetection of thymidine analogs demonstrated that no BrdU was incorporated during the 6h period before fixation. If BrdU had been incorporated, nuclei should have become completely labeled by immunostaining instead of displaying segregated chromosomes since the anti-IdU and anti-CldU antibodies also react with BrdU [5].

To check whether the cells kept at confluency and serum starvation were quiescent they were stained with a FITC conjugated monoclonal antibody against Ki-67 (DAKO). For this purpose the cells were washed twice in PBS and then fixed in PBS/3.7% formaldehyde for 10 min. Subsequently they were washed in PBS and permeabilized for 20 min in PBS/0.5% Triton X-100. After blocking the cells in PBS/4% BSA for 60 min the antibody was applied for 120 min in the same solution (1/100). The cells were washed four times for 10 min in PBS/0.1% Triton X-100 and once in PBS. Subsequently they were counterstained with DAPI and embedded in Vectashield (Vector) for microscopy. Using a microscope equipped for epifluorescence (Leica) and the appropriate filter sets for FITC and DAPI detection no FITC specific fluorescence was observed in the cells kept at confluency and serum starvation. The absence of detectable Ki-67 antigen confirms the quiescent state of the cells [28]. In contrast, the cells prepared at G_1 all exhibited FITC specific fluorescence as should be expected for permanently cycling cells [28]. The coverslips for Ki-67 staining were prepared in parallel with those for replication label detection.

Replication labeling and segregation. Replication labeling was performed essentially as described in [5, 6] with two important modifications. A different time schedule was applied (2 h IdU pulse labeling/4 h chase/2 h CldU pulse labeling) and a growth period (6–11 cell cycles) followed the second pulse. This growth period in the absence of thymidine analogs was performed in order to segregate the pulse labeled chromosomes to daughter cells. After segregation the cells were fixed at either the G_1 or the G_0 stage (see above) or at mitosis in order to prepare metaphase spreads.

Preparation of metaphase spreads and detection of replication label. Metaphase spreads were prepared according to standard procedures [29]. After denaturation (70% FA, 0.6 × SSC, 75°C, 5 min) the metaphase spreads were washed once in cold 2 × SSC and two times in PBS. Blocking and immunodetection were done essentially as described for interphase nuclei.

Pretreatment of interphase nuclei for simultaneous immunodetection and CISS hybridization. The pretreatment and CISS hybridization procedures were performed as described in [4] except that in addition to the 15q microdissection probe [30], a 13q microdissection probe [30] was also used.

Detection of replication label and hybridized DNA probes. The detection procedure was essentially performed as described in [4].

The following reagents were used: avidin-Cy3 (Dianova) and a biotinylated goat anti-avidin antibody (Dianova) for detection of biotinylated sequences. The replication labeling was detected with the following antibodies: rat anti-BrdU (recognizes BrdU and CldU [5], Seralab) and mouse anti-BrdU (recognizes BrdU and IdU [5], Becton and Dickinson). As secondary antibodies we used either FITC conjugated goat anti-rat (Dianova) with Cy5 conjugated goat anti-mouse antibodies (Dianova) or, for the detection with reversed labeling scheme, Cy5 conjugated goat anti-rat (Dianova) and FITC conjugated goat anti-mouse (Dianova) antibodies.

Light microscopy. Epifluorescence microscopy was performed with a Zeiss Axiophot microscope. Images were taken on color slide film (Kodak Ektarchrome 400) or sampled with a CCD camera (Applied Imaging). Images were transferred to a Power Macintosh computer (color slides were scanned with the Agfa StudioScan IIsi scanner) and analyzed using standard software tools (NIH Image 1.5.4 and Adobe Photoshop 3.05). Series of light-optical sections were recorded with a three-channel Leica TCS 4D confocal laser scanning microscope as described in [31]. Latex microspheres were used in order to determine the microscopic point spread function (PSF).

Determination of chromatic aberrations. In order to determine the chromatic shift between FITC and Cy3, 3D image stacks of polychromatic microspheres with a diameter of 526 nm (Polysciences, Inc.) with excitation by the 488- and 568-nm lines of the ArKr laser were recorded [32]. The spheres were embedded in 90% Vectashield and 10% water. The lateral and axial shifts between the centers of mass were calculated. The lateral shift was found to be negligible ($<$10 nm), whereas the shift in axial direction emerged as 225 $\pm$ 35 nm. Measurement of the axial shift by recording image stacks of a mirror in the reflection mode and calculating the mass centers of the 3D images yielded a similar result. The measured axial chromatic aberration was corrected for during image analysis. Only a small axial shift of 8 $\pm$ 28 nm was found between the Cy3 and the Cy5 channels using the mirror mode.

Segmentation of replication labeled foci. The model-based segmentation algorithm used to extract the parameters of the replication labeled foci ("spots") is described in detail elsewhere [32, 33]. Briefly, segmentation of replication labeled foci was performed in three steps. First, after application of a noise reduction filter (a $3 \times 3 \times 3$ median kernel) and global background subtraction, local maxima were detected using a $5 \times 5 \times 5$ octaedrical top-hat filter, and spot centers were assigned to them. The filter compared the maximum of intensity inside a small inner region comprising 7 voxels to that of a surrounding outer region and assigned a spot center if the inner maximum had a higher intensity value. Second, an iterative algorithm determined individual segmentation thresholds for each focus. In each iteration loop, a spot was allowed to grow until the intensity value dropped below a spot-specific value given as a function of its volume or until a neighboring spot was touched. In a third step, intensity contributions that stemmed from neighboring spots were subtracted to ensure a precise positioning for each spot. Each fluorochrome channel was segmented independently.

The focus-specific threshold value was determined from a function that assigns a threshold value in relation to the volume that a focus had acquired during the growth process. This function relating focus volume to threshold value (threshold-volume function) was determined by modeling images of randomly distributed foci of varying size. The model images were convoluted with the measured 3D microscopic PSF for FITC and Cy5, respectively, noise was added, and the threshold value necessary to regain the original focus volume after segmentation was calculated. Two threshold-volume functions emerged: one for FITC and one for Cy5. The growth process converged after 5–10 iterations. For all modeled images, the deviation from the original volume was $<$11% after segmentation. Centroids and volumes of the early and late replicating foci calculated by the algorithm were used for further statistical analysis.

Overlap. After segmentation of the images, the overlap between early replicating and late replicating foci was calculated. Two images were relevant for the calculation: The first image contained the signals from early replicating chromatin, the second image those from late replicating chromatin. First, the chromatic shift was corrected. In the experiments described here, the chromatic shift between the Cy5 and FITC channels was nearly exactly one voxel in the *z* direction. Thus, it was sufficient to shift the FITC-image by one voxel in the *z* direction. For early replicating chromatin, the overlap was defined by the number of colocalizing voxels (i.e., voxels segmented in both images), divided by the number of voxels found for early-replicating chromatin. For late replicating chromatin, the overlap was similarly found by the number of colocalizing voxels, divided by the number of voxels found for late replicating chromatin. This definition follows [34]. The two values could differ if more late replicating chromatin than early replicating chromatin was observed, or vice versa. The mean overlap for a territory was found as the mean of the two values.

Statistical 3D analysis of focus distance distributions. For each early replicating focus that was found in a chromosome territory, the distances between the intensity center of mass of this focus and all other early replicating foci in the territory were calculated. The median distance found provided a stable estimator of the average distance of this domain to other domains of the same type. Taking again the median of all these median distances gave a parameter describing the overall compaction of the territory. This value described the average distance of an average domain inside the territory to the other domains. It gave a single measurement for each territory. The different values measured for the territories were thus obtained in a statistically independent way. The compaction of territories was compared between cells in the G_1-phase and the G_0-phase by means of a Kolmogorov–Smirnov test. The same analysis was performed for late replicating foci.

Visualization of the segmented 3D images. For a pseudo-3D visualization of the segmented 3D images, a software program based on the graphics library OpenGL was developed. The rectangular voxels were intentionally kept in the visualization to give a true representation of experimental data. Structures below the level indicated by these voxels cannot be resolved by the confocal microscope.

RESULTS

Differential Staining of Early and Late Replicating DNA with Iododesoxyuridine and Chlorodesoxyuridine

In order to visualize DNA replicating at clearly separated time points during one S-phase, the DNA of exponentially growing human female diploid fibroblasts (GF 032) was labeled with two different thymidine analogs, iododesoxyuridine (IdU) and chlorodesoxyuridine (CldU) [5, 6]. The DNA was first pulse labeled for 2 h with IdU. Following a chase of 4 h the DNA was pulse labeled for 2 h with CldU (Fig. 1a). Thus the whole labeling scheme comprised 8 h. Since the S-phase of GF 032 cells is approximately 10 h long (data not shown), early and late replicating DNA was labeled during the same S-phase in those cell nuclei which showed a substantial incorporation of both modified nucleotides.

After labeling, the cells were grown for several cell cycles (Fig. 1b). This step is essential to achieve the

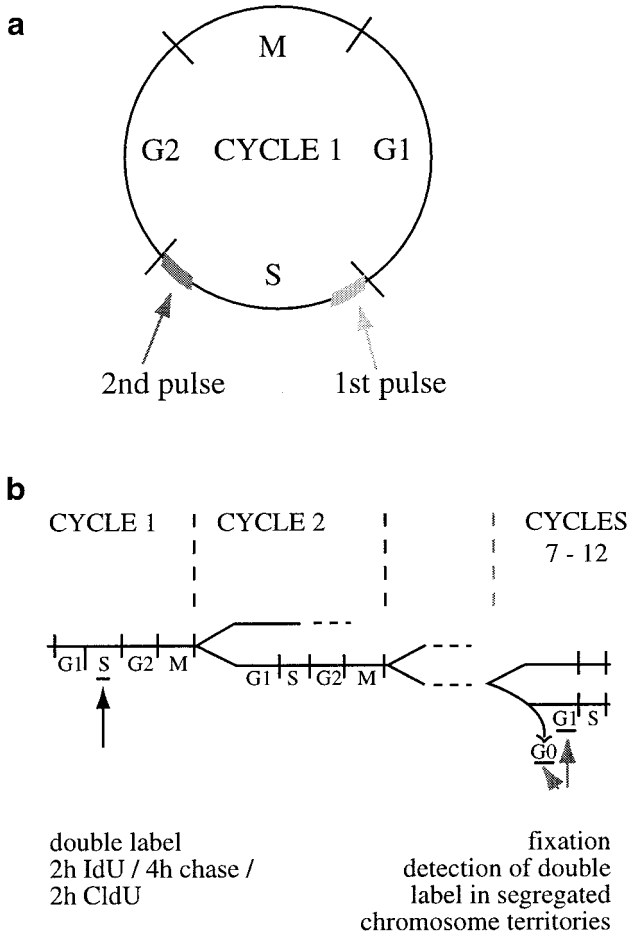

FIG. 1. Double-labeling and segregation scheme. (a) The DNA of unsynchronized human diploid fibroblasts was replication labeled with two different thymidine analogs, iododesoxyuridine (IdU) and chlorodesoxyuridine (CldU). The cells were pulsed for 2 h with IdU, chased for 4 h and pulsed a second time for 2 h with CldU. Using this time schedule it is possible to label early and late replicating DNA differently during the same S-phase. (b) Replication labeled chromosomes were segregated to daughter nuclei. The segregation occurred during growth of the labeled cells for approximately 6–11 cell cycles. After segregation, metaphase spreads were prepared (see Fig. 2) or the cells were fixed at either G_0 or G_1.

segregation of individual, replication labeled chromosomes (Fig. 2i) [3, 4, 35]. Metaphase spreads prepared from mitotic cells containing the segregated chromosomes revealed distinct banded patterns for IdU and CldU incorporation (Fig. 2). The IdU/CldU labeling patterns we observed display a high degree of similarity to the distributions of CpG island rich, early-replicating R-band (labeled by IdU) and CpG island poor, late-replicating G-band (labeled by CldU) chromatin on human metaphase chromosomes ([36], J. Craig and W. Bickmore, pers. commun.), confirming the expected incorporation patterns for IdU and CldU into early and late replicating DNA, respectively. In particular, the staining patterns display the expected differences for chromosomes with a similar DNA content but strong differences in gene density, R/G-banding patterns, and overall replication timing as examplified by chromosomes 13 and 15 or 18 and 19 [36–39] (compare Fig. 2).

In order to proof reproducible chromosome banding we pulse labeled another strain of unsynchronized human diploid fibroblasts (Hv) according to the scheme described above. Mitotic chromosomes of Hv fibroblasts confirm reproducible differential labeling of early and late replicating bands, respectively (Fig. 2). Minor differences between homolog chromosomes obtained from different mitotic spreads were noted for both fibroblast strains. This is an expected consequence of the applied labeling scheme: The timing of the two pulses is relatively flexible within S-phase (8 h pulse-chase-pulse labeling during 10 h S-phase in unsynchronized cells). Since the labeling time is substantially shorter than S-phase, the IdU/CldU labeled bands on each individual chromosome represent only a subset of all early or late replicating bands. Due to the flexibility of the labeling scheme most bands will be labeled in a larger number of chromosomes with individual chromosomes showing subsets of all labeled bands.

The question arises of whether IdU was incorporated during S-phase only into R bands before G bands and heterochromatic sequences were replicated and vice versa, whether CldU incorporation was restricted to the latter sequences. This is in particular a problem with regard to the sequences with an intermediate replication timing. Although some cross-incorporation can not be excluded, the reproducible mitotic patterns (Fig. 2), in agreement with known gene density, R/G-banding patterns, and replication timing of the corresponding chromosomes [36–39], indicate that this did not happen with regard to the majority of analyzed bands.

The Organization of Early and Late Replicating DNA within the Nuclear Territories of Chromosomes 13 and 15

Replication labeled territories were studied in interphase nuclei (Fig. 3). After segregation, the nuclei contained labeled chromosome territories surrounded by unlabeled chromatin (due to the random segregation of the individual labeled chromosomes to different daughter cells). This configuration is mandatory for the interphase analysis of individual, replication labeled chromosome territories as it prevents any confusion as to which replication label belongs to a given territory. We analyzed in detail the structural organization of early and late replicating DNA in chromosomes 13 and 15 territories. Only nuclei of G_1 or quiescent cells (G_0) were selected for the analysis (see Materials and Meth-

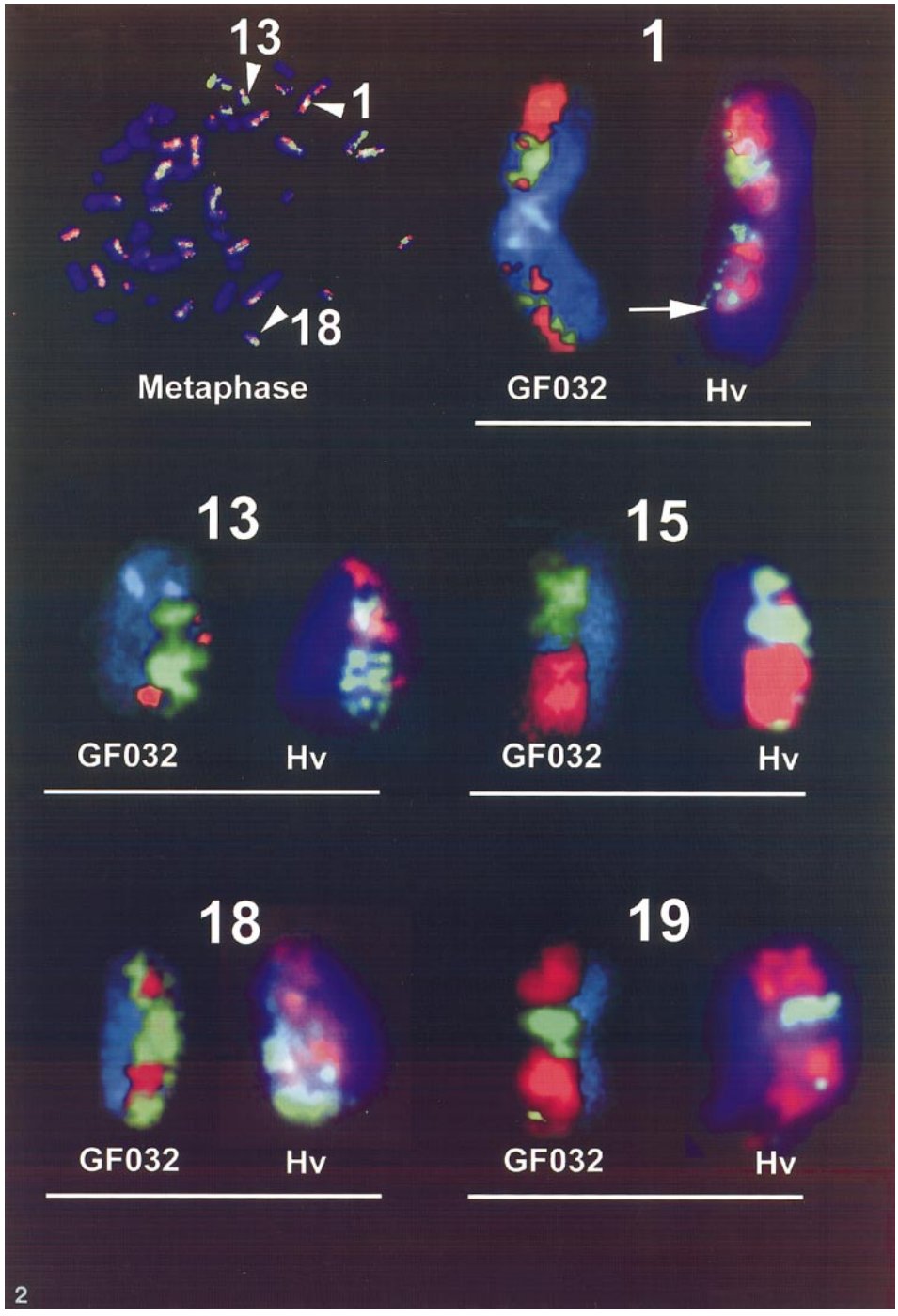

FIG. 2. Segregation of replication labeled chromosomes and differential replication labeling of individual chromosomes. The DAPI-staining is depicted in blue, the IdU-labeled DNA (early replication) in red, and the CldU-labeled DNA (late replication) in green. (Top, left) Metaphase spread prepared from a replication labeled Hv fibroblast culture at the third day after labeling (the cell cycle of Hv cells spans approx. 24 h). Note unlabeled and labeled chromosomes displaying only one labeled chromatid. Chromosomes 1, 13, and 18 of this metaphase (marked by arrowheads) are shown in detail in the other panels side by side with corresponding chromosomes of GF032 cells. In addition, chromosomes 15 and 19 from GF032 and Hv cells are shown for comparison. The chromosome examples were derived from five GF032 and two Hv metaphase spreads. Occasionally sister-chromatid exchanges (SCEs) were observed as shown for chromosome 1 (Hv, arrow marks the breakpoint).

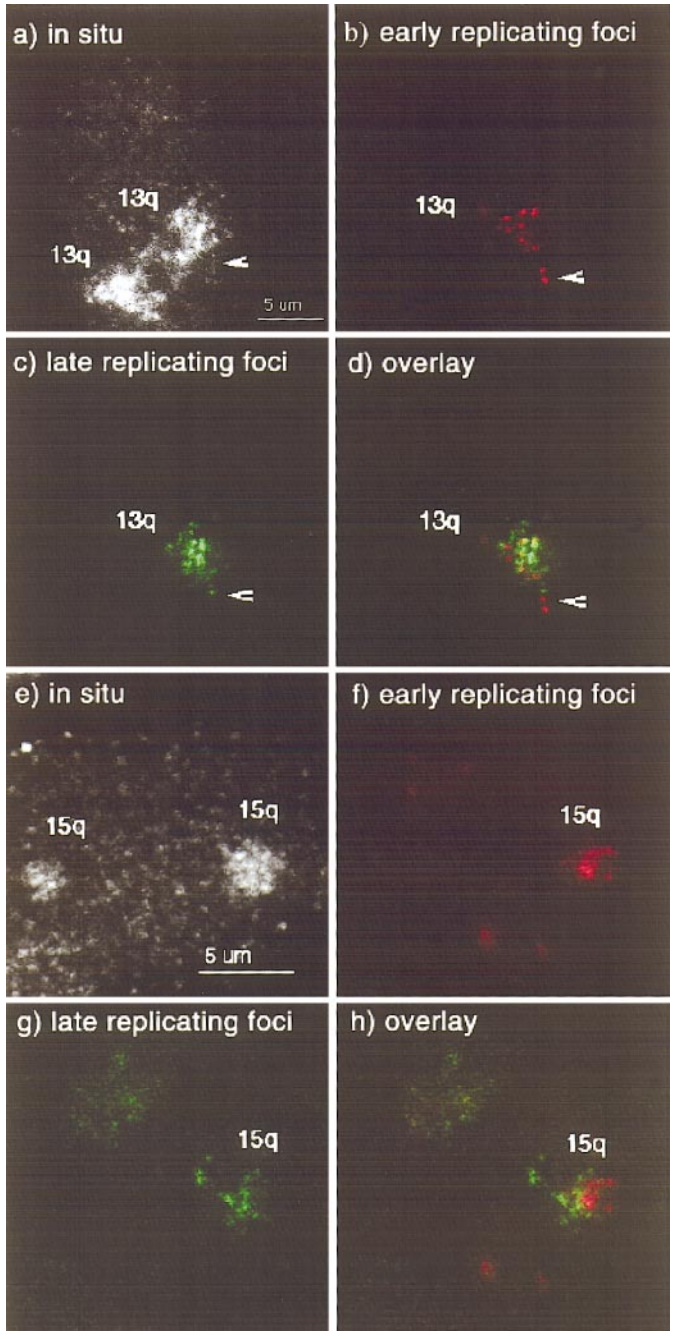

FIG. 3. Replication labeled interphase chromosome territories. (a–h) Single light optical sections selected from a whole gallery through a G_0 (a–d) and a G_1 (e–h) nucleus. For each nucleus the sections were chosen from the same nuclear plane: (a, e, white) *in situ* hybridization signal. The G_0-nucleus was hybridized with a chromosome 13*q* arm specific probe, the G_1-nucleus with a chromosome 15*q* arm specific probe. (b, f, red) IdU-labeled DNA; (c, g, green) CldU-labeled DNA; (d, h) overlay of the two replication labels. The colocalization (overlap) of red and green signals results in a yellow signal. In (d) and (h), however, very little overlap can be detected. The replication label is not distributed uniformly over the territories but shows local concentrations or foci. The colocalization of the 13*q* or 15*q* *in situ* hybridization signal with a replication labeled territory identified this territory unequivocally. The very small *p* arms of the chromosomes 13 and 15, which were not painted by the *q* arm specific microdissection probes for these acrocentric chromosomes, are not present in the sections shown here. Interestingly, the chromosome 13 territory possesses a long extension (b–d, arrowheads) with early replicating chromatin at its tip and late replicating chromatin at the basis. Due to the random segregation of replication labelled chromatids to daughter nuclei, we often note additional replication labeled, unidentified chromosome territories. For example, one such territory can be noted in the top left corner of panels (g) and (h). Note that in nuclei exhibiting one painted and replication labeled chromosome, the painted homolog typically does not show replication label (compare (a) with (d) and (e) with (h)).

ods), since at these stages a chromosome territory is entirely composed of a single chromatid. At other stages the structural arrangement of sister chromatids complicates the interpretation. No drugs were used to harvest cells at particular interphase stages in order to avoid drug induced artifacts of the nuclear structure.

Fluorescence *in situ* hybridization (FISH) with probes painting the chromosome arms 13*q* or 15*q* [30] was performed on nuclei containing replication labeled and segregated chromosome territories. In this way, it was possible to determine the chromosomal identity of replication labeled chromosome territories by their overlap with *in situ* hybridization signals (Fig. 3).

The acrocentric human chromosomes 13 and 15 have approximately the same size, shape, and DNA content [37, 38]. They differ, however, strongly in their gene densities and the content of late replicating DNA, which is particularly high for chromosome 13 (compare Fig. 2) [36, 39]. These differences in the amounts of early and late replicating DNA were also noted in the interphase territories of chromosomes 13 and 15 (compare Fig. 3) and thus confirm the correct visualization of early and late replicating DNA during interphase.

Early and Late Replicating DNA Occupies Distinct Foci within Interphase Chromosome Territories

Figures 3 and 4 show typical examples of the distribution of early and late replicating DNA within interphase chromosome territories. Confocal images that were not subject to any further processing (Fig. 3) suggested that the different chromatin types occupy distinct foci which appeared to display a different distribution within the chromosome territories.

The amount of overlap between differently replication labeled foci was determined for each chromosome 13 and 15 territory at the G_1 and G_0 stages of interphase (Fig. 5, see Materials and Methods for details on the definition of foci by the segmentation algorithm). This analysis was performed on 48 chromosome 15 territories (29 in G_1 and 19 in G_0) and 16 chromosome 13 territories (6 in G_1 and 10 in G_0). The territories selected for the analysis were chosen according objective criteria determined before selection of the territories. These criteria were as follows: territories must display both labels, territories must be identified by FISH, and selected territories are not allowed to touch a neighboring replication labeled territory in order to be able to determine a clear boundary for each territory.

Territories that fit these criteria were the product of several events that could not be influenced by the person which selected the territories. Because the chance of finding territories that fulfill all critria is low, the number of selected territories is comparably low, in particular with regard to chromosome 13. However, it has to be taken into account that the quantitative analysis was performed on the level of individual replication labeled foci. On the whole, the number of foci that entered the analysis was 1465 and 1203 for chromosome 15 at G_1 and G_0, respectively, or 212 and 598 for chromosome 13 at G_1 and G_0, respectively. These numbers are sufficient to perform a reliable statistical analysis.

The degree of overlap ranged from almost 0% to less than 15% for most of the territories (Fig. 5). For 5 of 64 territories the degree of overlap was more than 15%, with 22% being the highest value. The median degree of overlap is surprisingly low at approximately 5% for chromosome 15 territories and 10% for chromosome 13 territories. Given the limited resolution of confocal microscopy, it is not clear whether the measured 5 to 10% of overlap are due to a true intermingling of the distinct foci or whether they reflect the optical limitations.

Both Types of Foci Are More Densely Packed in G_1 Than in G_0 Territories

Visual inspection of replication labeled territories such as those shown in Fig. 4 gave the impression that packaging of foci is more compact in G_1 territories than in G_0 territories. To test this hypothesis, the median distance between every discernible focus of early replicating chromatin and all other early replicating foci, as well as the respective distances for late replicating foci, were determined for both G_1 or G_0 territories (for details, see Materials and Methods).

A statistical analysis of the distance distributions obtained for both types of foci (see Materials and Methods) revealed a significant difference between G_1 and G_0 chromosome 15 territories ($P = 0.03$ for early as well as late replicating foci, Figs. 6a and 6b). Early as well as late replicating foci are more densely packed in G_1 then in G_0, resulting in an overall compaction of the whole territory. A comparable result was obtained with regard to chromosome 13 territories ($P = 0.02$ for early replicating chromatin (Fig. 6c), for late replicating chromatin the difference was not significant ($P = 0.21$, Fig. 6d)). These data suggest that the overall chromosome structure changes at G_1 compared to that at G_0. Chromosome 15 as well as chromosome 13 territories displayed a more compact structure in G_1, where early as well as late replicating foci revealed a more dense packaging (in one of the four groups the result is only suggestive but not significant). It should be noted that the degree of overlap between the different types of foci was not affected by the change in packaging (see above).

DISCUSSION

Compartmentalization of Interphase Chromosome Territories

Early and late replicating DNA of human diploid fibroblasts was labeled differentially during the same S-phase using two thymidine analogs, IdU and CldU [5, 6]. After labeling, cells were grown for several cell cycles resulting in the formation of nuclei containing only a few replication labeled chromosome territories [3, 4, 17, 35]. Territories of chromosomes 13 and 15 were identified by FISH in nuclei of G_1 or quiescent (G_0) cells. These territories exhibited distinct foci of early and late replicating DNA. The distinct foci displayed a median overlap of only approximately 5% (chromosome 15 territories) to 10% (chromosome 13 territories) as revealed by a quantitative analysis. The average degree of overlap did not change when chromosome territories rearranged to a more compact state in G_1 compared to G_0.

Problems and Advantages of the Approach

The replication labeling/segregation approach seems to be especially useful for studying delicate structural features of chromosome territories such as their internal substructure (see below) or tiny extensions from the surface (compare Figs. 3a–3d). Such structures might have been overlooked so far because they are difficult to detect with chromosome painting [31]. The high sensitivity of the replication labeling/segregation approach argues against serious problems to visualize replication labeled chromatin in combination with *in situ* hybridization due to accessibility problems of the antibodies. Although not all incorporated thymidine analogs are expected to be recognized due to the partial renaturation of the DNA during *in situ* hybridization, the proportion of detected thymidine analogs still yields a higher sensitivity than FISH.

It should also be noted that thymidine analog pulses of 2 min mark replication foci of the same dimensions that foci display after 1 h of labeling [16, 20]. Thus, a very low density of detectable thymidine analogs within foci seems to be sufficient for visualization. There is also no hint for a severe distortion of the main outcomes of this study due to accessibility problems of the antibodies. In all four sets of experiments (chromosomes 13 and 15 at G_1 and G_0) there was a surprisingly low degree of overlap between differentially labeled foci. In the case of accessibility problems one would expect the most sensitive detection at the periphery of foci. In the case of overlap between two foci their pheripheries overlap first.

The degree of overlap was determined by quantitative image analysis. The algorithm used for the evaluation was developed for the volume-conserving segmentation of objects which are in the size range of the structures imaged in the experiments described here. Its performance was evaluated in detail with simulation data and 3D images of microspheres [32, 33]. The tests showed that the algorithm can find the mean values of object volumes and overlaps between them accurately. That means that an individual value for the volume of an object can deviate from the true value, but when many objects are analyzed, the mean values are faithfully reproduced. If the objects were so closely spaced that an extensive overlap of their mutual signals occurred due to out-of-focus blur, this was not longer true. In that case, overlaps and volumes were overestimated. However, by the segregation approach shown here, the overlap of signals from different chromosomes was effectively reduced. Care was taken to evaluate only chromosomes which were well separated from neighboring, also labeled chromosomes. Still, the overlaps found can be regarded as an upper limit to the true overlap between foci.

The distances between foci could be determined with high precision. The algorithm used a subtraction signal that stemmed from neighboring objects to calculate the intensity gravity centers with the highest accuracy possible. A detailed analysis revealed that distances in 3D can be measured with an accuracy of $\leq$60 nm [32].

Chromosome Architecture and Replicational Regulated Foci

The replication banding procedure we used labeled early and late replicating DNA. Although not all early and late replicating subchromosomal foci detected in our study necessarily correspond to metaphase chromosome R- and G-bands, a comparison of our data with published analyses of the banded human metaphase chromosome structure [36] reveals that there is indeed a good correspondence of CpG island rich R-band chromatin with IdU labeled and CpG island poor G-band chromatin with CldU labeled regions (compare Fig. 2 with [36], Figs. 1 and 2a, J. Craig and W. Bickmore, pers. commun.). The staining patterns display the expected differences for chromosomes with a similar DNA content but strong differences in gene density, R/G-banding pattern, and overall replication timing as chromosomes 13 and 15 or 18 and 19 [36–39] (compare Fig. 2). These results suggest that there is a close correspondence between the structure of mitotic chromosomes and the structure of nuclear chromosome territories.

It appears that chromosomes during mitosis as well as during other stages of the cell cycle and in quiescent cells are composed of stable foci displaying a distinct replicational regulation. A tightly regulated mainte-

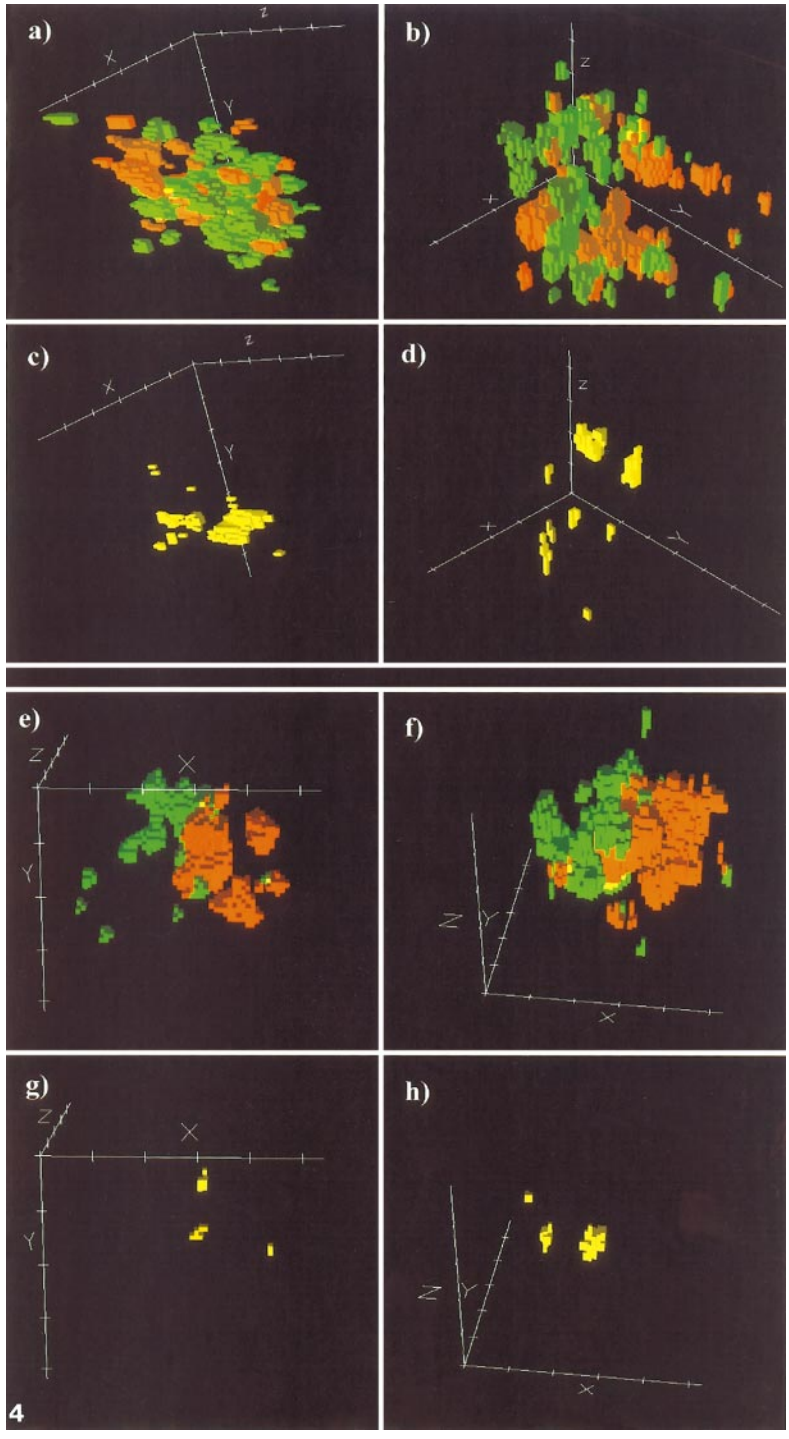

FIG. 4. 3D reconstruction of segmented confocal images. The replication label (early, red; late, green) of two G_0 (a, b) and two G_1 (e, f) chromosome 15 territories is shown in a pseudo-3D visualization. Voxels labeled simultaneously by red and green are depicted in yellow. To give a better impression the overlap is also shown exclusively below each corresponding picture (c, d, g, h). The chromosome territory shown in (e) corresponds to that depicted in Figs. 3e–3h, viewed from another angle. The axes correspond to those of the confocal microscope and the ticks denote distances of 1 μm.

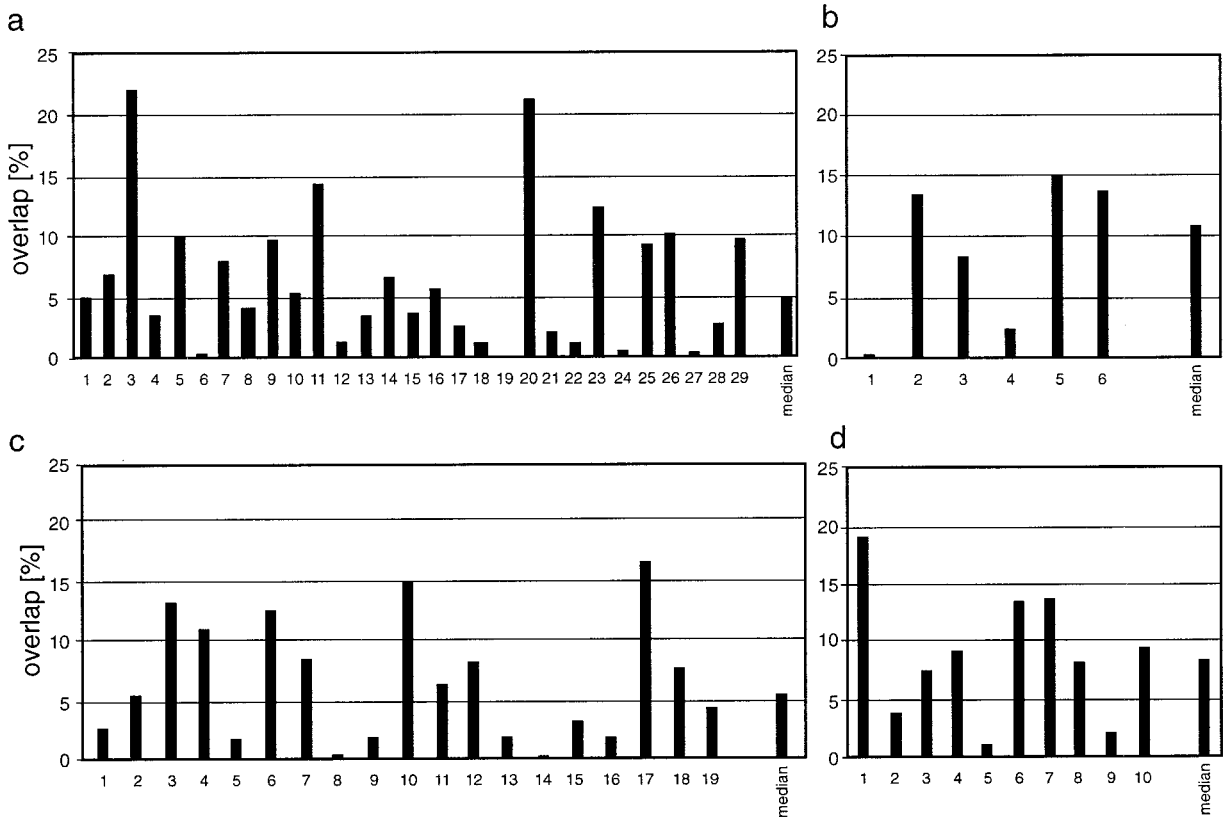

FIG. 5. The percentage of overlap (for exact definition of the overlap see Materials and Methods) was determined for 29 G_1-territories (a) and 19 G_0-territories (c) of chromosome 15, as well as 6 G_1-territories (b) and 10 G_0-territories (d) of chromosome 13. For each territory analyzed the percentage of overlap is indicated by the height of a bar. Numbers below the bars designate the individual territories. The bar at the right indicates the median percentage of overlap for all territories within one group. The analysis was performed at the level of individual replication labeled foci of each territory. The number of foci that entered the analysis was 1465 (a) and 1203 (c) for chromosome 15 territories or 212 (b) and 598 (d) for chromosome 13 territories. For nine of the chromosome 15 G_1-territories (21–29) the fluorochromes to identify the replication label were reversed (see Materials and Methods) in order to test for artifacts induced by fluorochrome usage.

nance of association between specific regions of chromatin that is not disrupted during mitosis was also noted in earlier studies [1]. Studies visualizing single foci on mitotic chromosomes of mammalian cells [3] suggest that a single chromosome band in the size range of one to a few Mbp could be composed of several foci (it is estimated that a single focus contains approximately 0.25 to 1.5 Mbp of DNA if a focus comprises on average five replicons of 50 to 300 kbp). Interestingly, similar foci were also observed on plant chromosomes [2]. Since only the mitotic chromosomes of warm blooded vertebrates display the well known banding patterns but chromosomes of all eukaryotic species including yeast display regions of distinct replicational regulation [40], the observed foci could reveal a fundamental unit of eukaryotic chromosomes in general.

The results support an important feature of the model of interphase chromosome architecture described by [8], namely the preservation of chromosomal bands or subbands as structural and functional units during interphase. The separation of early and late replicating foci is compatible with the ICD-compartment model [11–13, 41], but is difficult to explain with regard to the random walk/giant loop model [14, 42]. The random walk organization of giant chromatin loops as suggested by this model should lead to an extensive intermingling of chromosome subregions. (For a more extensive discussion of a focal chromosome organization with regard to different models of chromosome structure see [4].

Functional Aspects of a Focal Chromosome Organization

The preservation and separation of distinct foci during functionally different states of the cell as demonstrated in the present study may serve to provide a framework of chromatin modules. The foci may secure the optimal environment for the genetic information in order to meet the functional needs of the nucleus. In

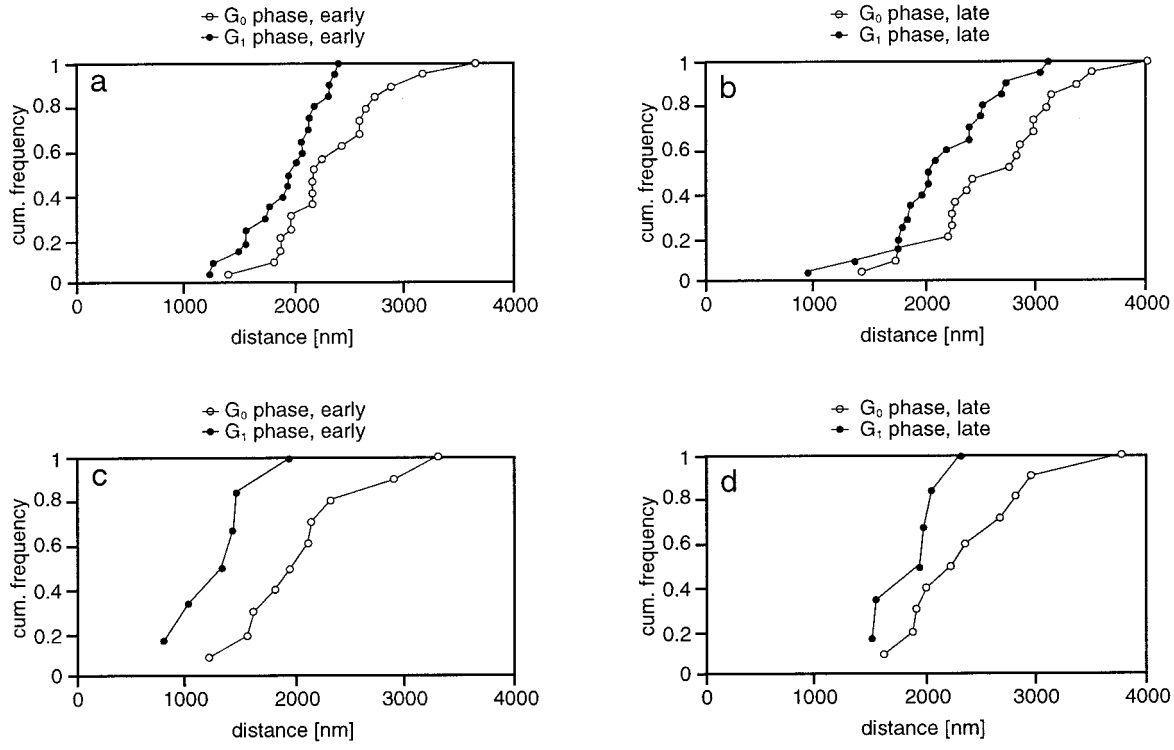

FIG. 6. Median distances between foci with the same replication timing in chromosome territories. Cumulative plots that compare the distance distribution in cells at G_1 or G_0 are shown. To ensure the statistical independence of the measurements, only one distance was chosen for each territory (of all median distances computed in a territory, the median value was taken, see Materials and Methods). Only distance distributions were compared when the same fluorochrome labeling scheme was used to avoid artifacts. (a) In chromosome 15 territories, early replicating foci are significantly closer spaced in G_1 compared to G_0. (b) The same effect was found with regard to late replicating foci. (c) Early as well as late (d) replicating foci of chromosome 13 display the same rearrangement, although the effect is not significant in (d) (see Results).

this regard it is interesting to note that the distinct foci we observed are functionally different as revealed by the distinct replication timing and the correspondence to distinct gene rich and gene poor chromosomal bands. Chromatin belonging to G-bands is expected to be transcriptionally largely inactive during interphase [44], in contrast to R-band chromatin. Thus, the foci could serve as targets not only for replicational but also for transcriptional regulation.

As the functional needs of an interphase nucleus change (for example during cell cycle or differentiation), the environment provided for the genetic information has to be organized in a different way. A modular organization of the genome might facilitate such rearrangements. Spatial rearrangements of chromosomes or chromosomal domains were frequently described in the context of changes in the functional state of cells (reviewed in [10]) and also observed in this study. Particularly interesting in this context is the formation of higher order nuclear compartments comprising early or late replicating DNA predominantly located in different chromosomal bands [35].

We already observed in the present study a rearrangement of the foci as the cells changed their functional state between G_1 and G_0. The hypothesis stated above can be tested now by direct observation of the focal chromosome organization during changes in the functional state of the cell *in vivo*. Replication labeled foci observed in chromosomes of living human cells [4, 17] were referred to as subchromosomal foci and likely correspond to replication labeled foci observed in fixed cells [1–4, 16].

In conclusion, the results of the present study support the hypothesis, that stable DNA aggregates corresponding to the well known replication foci are fundamental structural units of nuclear DNA organization and chromosomes [1–4, 16]. This applies to mammalian and plant chromosomes [1–4, 16], but may be true with regard to all eukaryotic species. Replication labeled foci (or subchromosomal foci) might not only be structural units but also functional units with

regard to both replicational and transcriptional regulation. The modular organization of the genome may facilitate the regulation of the functional architecture of the nucleus according to the functional state of the cell.

The authors are grateful to Kurt Sätzler and Roland Eils for their help with computer programming, Jeffrey Trent for providing the microdissection probes, and Pascal Tomakidi and Jan Murken for providing the fibroblast cells. We also thank Roel van Driel, Jacob Aten, Peter Lichter, and Christian Münkel for helpful discussions and Jacqueline Bornfleth and Jeff Craig for proofreading the manuscript. This work was supported by a grant from the European community (Biomed 2 BMH4-CT95-1139) and by the Deutsche Forschungsgemeinschaft (Cr 59/18-1).

REFERENCES

1. Meng, C., and Berezney, R. (1991). Replicon cluster domains persist throughout the cell cycle of mouse 3T3 cells. *J. Cell Biol.* **115,** 95a.
2. Sparvoli, E., Levi, M., and Rossi, E. (1994). Replication clusters may form structurally stable complexes of chromatin and chromosomes. *J. Cell Sci.* **107,** 3097–3103.
3. Jackson, D. A., and 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.
4. Zink, D., Cremer, T., Saffrich, R., Fischer, R., Trendelenburg, M. F., Ansorge, W., and Stelzer, E. H. K. (1998). Structure and dynamics of human interphase chromosome territories *in vivo*. *Hum. Genet.* **112,** 241–251.
5. Aten, J. A., Bakker, P. J. M., Stap, J., Boschmann, G. A., and Veenhof, C. H. N. (1992). DNA double labelling with IdUrd and CldUrd for spatial and temporal analysis of cell proliferation and DNA replication. *Histochem. J.* **24,** 251–259.
6. Visser, A. E., Eils, R., Jauch, A., Little, G., Bakker, P. J. M., Cremer, T., and Aten, J. A. (1998). Spatial distributions of early and late replicating chromatin in interphase chromosome territories. *Exp. Cell. Res.* **243,** 398–407.
7. Blobel, G. (1985). Gene gating: A hypothesis. *Proc. Natl. Acad. Sci. USA* **82,** 8527–8529.
8. Manuelidis, L., and Chen, T. L. (1990). A unified model of eukaryotic chromosomes. *Cytometry* **11,** 8–25.
9. Pienta, K. J., Getzenberg, R. H., and Coffey, D. S. (1991). Cell structure and DNA organization. **1,** 355–385.
10. De Boni, U. (1994). The interphase nucleus as a dynamic structure. *Int. Rev. Cytol.* **150,** 149–171.
11. Cremer, T., Kurz, A., Zirbel, R., Dietzel, S., Rinke, B., Schroeck, E., Speicher, M. R., Mathieu, U., Jauch, A., Emmerich, P., *et al.* (1993). The role of chromosome territories in the functional compartmentalization of the cell nucleus. *Cold Spring Harb. Symp. Quant. Biol.* **58,** 777–792.
12. Cremer, T., Dietzel, S., Eils, R., Lichter, P., and Cremer, C. (1995). Chromosome territories, nuclear matrix filaments and inter-chromatin channels: A topological view on nuclear architecture and function. *In* "Kew Chromosome Conference IV, Royal Botanic Gardens, Kew" (P. E. Brandham and M. D. Bennett, Eds.), pp. 63–81.
13. Kurz, A., Lampel, S., Nickolenko, J. E., Bradl, J., Benner, A., Zirbel, R. M., Cremer, T., and Lichter, P. (1996). Active and inactive genes localize preferentially in the periphery of chromosome territories. *J. Cell Biol.* **135,** 1195–1202.
14. Sachs, R. K., van den Engh, G., Trask, B., Yokota, H., and Hearst, J. E. (1995). A random-walk/giant-loop model for interphase chromosomes. *Proc. Natl. Acad. Sci. USA* **92,** 2710–2714.
15. Razin, S. V., and Gromova, I. I. (1995). The channels model of nuclear matrix structure. *Bioessays* **17,** 443–450.
16. Berezney, R., Mortillaro, M. J., Ma, H., Wei, X., and Samarabandu, J. (1995). The nuclear matrix: A structural milieu for genomic function. *Int. Rev. Cytol.* **162**A, 1–65.
17. Zink, D., Cremer, T., Saffrich, R., Fischer, R., Trendelenburg, M. F., Ansorge, W., and Stelzer, E. H. K. (1997). Analysis of human interphase chromosome territories *in vivo*. *Genes, Chromosomes, Genomes* **5,** 115.
18. Nakamura, H., Morita, T., and Sato, C. (1986). Structural organizations of replicon domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* **165,** 291–297.
19. Leonhardt, H., and Cardoso, C. (1995). Targeting and association of proteins with functional domains in the nucleus: The insoluble solution. *Int. Rev. Cytol.* **162**B, 303–335.
20. Nakayasu, H., and Berezney, R. (1989). Mapping replicational sites in the eucaryotic cell nucleus. *J. Cell Biol.* **108,** 1–11.
21. O'Keefe, R. T., Henderson, S. C., and Spector, D. L. (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.
22. Hassan, A. B., and Cook, P. R. (1993). Visualization of replication sites in unfixed human cells. *J. Cell Sci.* **105,** 541–550.
23. Edenberg, H. J., and Huberman, J. A. (1975). Eukaryotic chromosome replication. *Annu. Rev. Genet.* **9,** 245–284.
24. Hand, R. (1978). Eucaryotic DNA: Organization of the genome for replication. *Cell* **15,** 317–325.
25. Huberman, J. A., and Riggs, A. D. (1968). On the mechanism of DNA replication in mammalian chromosomes. *J. Mol. Biol.* **32,** 327–341.
26. Housman, D., and Huberman, J. A. (1975). Changes in rate of DNA replication fork movement during S-phase in mammalian cells. *J. Mol. Biol.* **94,** 173–181.
27. Hozak, P., Hassan, A. B., Jackson, D. A., and Cook, P. R. (1993). Visualization of replication factories attached to a nucleoskeleton. *Cell* **73,** 361–373.
28. Gerdes, J., Lemke, H., Baisch, H., Wacker, H.-H., Schwab, U., and Stein, H. (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J. Immunol.* **133,** 1710–1715.
29. Macgregor, H., and Varley, J. (1988). "Working with Animal Chromosomes," 2nd ed. Wiley, Chichester, New York, Brisbane, Toronto, Singapore.
30. Guan, X.-Y., Zhang, H., Bittner, M., Jiang, Y., Meltzer, P., and Trent, J. (1996). Chromosome arm painting probes. *Nature Genet.* **12,** 11–12.
31. Eils, R., Dietzel, S., Bertin, E., Schroeck, E., Speicher, M. R., Ried, T., Robert-Nicoud, M., Cremer, C., and Cremer, T. (1996). Three-dimensional reconstruction of painted human interphase chromosomes: Active and inactive X chromosome territories have similar volumes but differ in shape and surface structure. *J. Cell Biol.* **135,** 1427–1440.
32. Bornfleth, H., Sätzler, K., Eils, R., and Cremer, C. (1998a). High-precision distance measurements and volume-conserving segmentation of objects near and below the resolution limit in

three-dimensional confocal microscopy. *J. Microsc.* **189,** 118–136.

33. Bornfleth, H., Edelmann, P., Zink, D., Cremer, T., and Cremer, C. (in press). "Three-Dimensional Analysis of Genome Topology." Academic Press, San Diego, New York.
34. Manders, E. M. M., Verbeek, F. J., and Aten, J. A. (1993). Measurement of co-localization of objects in dual-colour confocal images. *J. Microsc.* **169,** 375–382.
35. Ferreira, J., Paolella, G., Ramos, C., and Lamond, A. I. (1997). Spatial organization of large-scale chromatin domains in the nucleus: A magnified view of single chromosome territories. *J. Cell Biol.* **139,** 1597–1610.
36. Craig, J. M., and Bickmore, W. A. (1994). The distribution of CpG islands in mammalian chromosomes. *Nat. Genet.* **7,** 376–382.
37. Morton, N. E. (1991). Parameters of the human genome. *Proc. Natl. Acad. Sci. USA* **88,** 7474–7476.
38. Mitelman, F. (Ed.) (1995). "ISCN: An International System for Human Cytogenetic Nomenclature." S. Karger, Basel.
39. Camargo, M., and Cervenka, J. (1982). Patterns of DNA replication of human chromosomes. II. Replication map and replication model. *Am. J. Hum. Genet.* **34,** 757–780.
40. Bickmore, W. A., and Sumner, A. T. (1989). Mammalian chromosome banding-an expression of genome organization. *Trends Genom.* **5,** 144–148.
41. Zirbel, R. M., Mathieu, U. R., Kurz, A., Cremer, T., and Lichter, P. (1993). Evidence for a nuclear compartment of transcription and splicing located at chromosome domain boundaries. *Chromosome Res.* **1,** 93–106.
42. Yokota, H., van den Engh, G., Hearst, J. E., Sachs, R. K., and Trask, B. (1995). Evidence for the organization of chromatin in megabase pair-sized loops arranged along a random walk path in the human G0/G1 interphase nucleus. *J. Cell Biol.* **130,** 1239–1249.
43. Craig, J. M., and Bickmore, W. A. (1993). Chromosome bands—Flavours to savour. *BioEssays* **15,** 349–354.

Received April 8, 1998
Revised version received August 2, 1998