Spatial Distributions of Early and Late Replicating Chromatin in Interphase Chromosome Territories

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The surface area of chromosome territories has been suggested as a preferred site for genes, specific RNAs, and accumulations of splicing factors. Here, we investigated the localization of sites of replication within individual chromosome territories. In vivo replication labeling with thymidine analogues IdUrd and CldUrd was combined with chromosome painting by fluorescent in situ hybridization on three-dimensionally preserved human fibroblast nuclei. Spatial distributions of replication labels over the chromosome territory, as well as the territory volume and shape, were determined by 3D image analysis. During late S-phase a previously observed shape difference between the active and inactive X-chromosome in female cells was maintained, while the volumes of the two territories did not differ significantly. Domains containing early or mid to late replicating chromatin were distributed throughout territories of chromosome 8 and the active X. In the inactive X-chromosome early replicating chromatin was observed preferentially near the territory surface. Most important, we established that the process of replication takes place in foci throughout the entire chromosome territory volume, in early as well as in late S-phase. This demonstrates that activity of macromolecular enzyme complexes takes place throughout chromosome territories and is not confined to the territory surface as suggested previously.

Key Words: chromosome territory; replication; X-chromosome; FISH; nuclear organization.

INTRODUCTION

The territorial organization of chromosomes in the interphase nucleus has been well documented [1–3]. During the past few years the three-dimensional architecture of individual chromosome territories has become an area of active investigation [4–8].

Such studies have been started with the goal to explore whether the folding and packaging of chromatin fibers that constitute an individual chromosome territory result in a functionally relevant higher order organization. In electronmicroscopic studies of interphase chromatin a hierarchy of chromatin fibers of increasing thickness has been described. However, the evidence for large scale chromatin arrangements beyond the level of the 10- and 25- to 30-nm fibers, respectively, is still controversial (e.g. [9–12]; for a review of the older, but still relevant literature see [13]).

Studies employing fluorescence in situ hybridization with whole chromosome paint and subregional probes in combination with three-dimensional microscopy and quantitative image analysis are still in an early and mostly descriptive stage. Several observations have suggested that the periphery of the chromosome territory plays a special topological and functional role. Kurz et al. [6] have demonstrated that two active genes and one inactive gene were preferentially localized at the surface of the respective chromosome territories in contrast to two noncoding sequences. Zirbel et al. [4] noted newly synthesized viral RNA near the surface of chromosome territories. These authors have also started to investigate the topological relationships of chromosome territories with other macromolecular domains demonstrating that prominent local concentrations of Sm antigen, a constituent of snRNP splicing factors, were preferentially localized at chromosome territory surfaces. Most likely, these focal accumulations are storage sites from which splicing factors can be recruited to transcription sites [14, 15]. Further studies are clearly needed to decide whether the preferential localization of genes, specific RNAs, and accumulations of splicing factors at the chromosome territory outer surface is a generally notable phenomenon indicating a specific functional role of the chromosome territory surface.
The two X-chromosome territories in somatic female cell nuclei provide a particularly useful model for exploring potential differences in the higher order organization of individual chromosome territories. One X-chromosome in female cell nuclei (Xa) is transcriptionally active in a way that corresponds to the single X-chromosome in male cells (Xmale). The other X-chromosome (Xi) becomes inactivated at an early stage of development except for genes located in the pseudoautosomal regions and a few other sites [16, 17]. Notably, the Xi-territory also replicates later in S-phase than its active homologue [18, 19]. Recently, Eils et al. [5] studied human amniotic fluid cell nuclei and demonstrated that the Xi-territory generally has a rounder shape and/or a smoother surface than the corresponding Xa-territory. The cause of this shape difference is unknown, but might be related to the different functional states of the two X-territories. Notably, a comparison with the shape of chromosome 7 territories showed that the latter corresponded closely to the shape of the Xa-territory. The study of Eils et al. [5] was performed with nonsynchronized cell cultures where most cells were apparently in the G1-phase of the cell cycle. One aim of the present study was to analyze whether the shape difference between Xa- and Xi-territories was maintained also during late S-phase, i.e., at a time where the Xi-territory was still heavily replicating in contrast to the Xa-territory.

The second and major aim of this study concerned the three-dimensional localization of the DNA replication process in individual X- and autosome chromosome territories. In eukaryotic cell nuclei DNA replication is concentrated in discrete foci. These can be visualized after incorporation of [3H]thymidine with autoradiography [20] or by immunohistochemistry after incorporation of biotin-labeled thymidine [21] or halogenated thymidine analogues BrdUrd [22], iododeoxyuridine (IodUrd), and chlorodeoxyuridine (CldUrd) [23, 24]. Each focus contains several replication forks [25] and is active for approximately 1 h [26, 27]. During S-phase progression the spatial distribution of replication foci changes from a few hundred small foci throughout the nucleus in early S-phase to several larger foci at the end of S-phase [26, 28]. Three [21] to five [29] distinct patterns can be recognized sequentially. Metaphase chromosomes derived from cells labeled with thymidine analogues in the first half of S-phase show a banding pattern similar to R-bands, whereas cells labeled in the second part of S-phase give rise to metaphases with a G-band pattern [30]. Furthermore, most active genes are replicated early in S-phase, in contrast to heterochromatin, which is replicated later in S-phase [31–33].

In the present study we combined two cytochemical techniques, replication double labeling, allowing detection of DNA synthesized at two separate stages of S-phase in the same nucleus, and chromosome painting. Using this approach we tried to answer the following questions. First, does the process of DNA replication occur throughout the chromosome territory volume or preferentially at the chromosome territory surface? The latter possibility would support a major functional significance of the chromosome territory surface but also necessitate major chromatin movements during replication of a given territory. Second, do Xa- and Xi-territories differ in the three-dimensional distribution of early and late replicating chromatin domains? Chromosome 8 territories were studied for comparison with a similarly sized autosome.

MATERIALS AND METHODS

Cell culture and replication labeling. Human primary foreskin fibroblasts (kindly provided by R. van de Wijngaard, AMC, Amsterdam, the Netherlands) and female oral cavity derived fibroblasts (GF 032, kindly provided by P. Tomakidi, Kopfklinik, Heidelberg, Germany) were cultured on coverslips in DMEM supplemented with 10% FCS. The S-phase of both cell types lasted 8–10 h (data not shown). Replicating DNA of nonsynchronously growing cells was pulse labeled by adding thymidine analogue IodUrd (end concentration 10 μM; Sigma, St. Louis, MO) to the medium. Cells were washed twice and cultured in normal medium during the chase period until a second pulse was given by adding CldUrd (Sigma) [23]. The labeling pulses and chase period were dependent on the experiment and are described under Results. Directly after the second pulse the cells were fixed with 4% formaldehyde and permeabilized by two saponin and Triton X-100 washes and after a glycerol incubation times dipped shortly in INa [5]. This fixation has been shown to maintain the 3D structure of the nuclei and the positions of intranuclear structures under these conditions and is suitable for FISH [34].

DNA probes and chromosome in situ suppression hybridization. A DOP-PCR library of the human X-chromosome and a plasmid DNA library for chromosome 8 (pBS8) were kindly provided by Dr. J. Gray (University of California, San Francisco, CA) (for pBS8 see [35]). The probes were labeled with biotin-16–dUTP (Boehringer Mannheim, Mannheim, Germany) using standard nick-translation [36], denatured for 5 min at 75°C, and allowed to preanneal for 30 min at 37°C before hybridization. Slides were pretreated by an overnight incubation in sodium thiocyanate, a mild pepsin digestion, and an overnight incubation in 50% formamide in SSC. Slides were denatured for 2 min at 72°C, excess formamide was shaken off, and the probe was applied without allowing the cells to fall dry. Hybridization was performed at 37°C for 48 h [5].

Detection of DNA probe and replication label. During the detection procedure the temperature was not raised above 37°C and SSC concentrations were lowered to obtain optimal signals of both the hybridized probe and the replication label. Slides were washed three times in 50% formamide/1× SSC and three times in 0.05× SSC, both at 37°C. The biotin-labeled probes were detected in CY5.
using an avidin–biotin amplification (biotin conjugated goat-anti-avidin, Vector, 1:200) and washing in 2× SSC–Tween at 37°C. The thymidine analogues were detected by antibodies specially selected to discriminate between IdUrd and CldUrd (respectively, mouse anti-BrdU, 7580, 1:3 in PBS–TWEEN; Becton Dickinson, San Jose, CA, and rat anti-BrdU, Mas 250c; Seralab, Crawley Down, Sussex, England, 1:100) [23]. To minimize cross-talk between IdUrd and CldUrd cells were washed for 6 min in a Tris-buffered 0.5 M NaCl solution with 0.5% Tween (pH 8). IdUrd was visualized using Texas red conjugated to goat anti-mouse and CldUrd using FITC conjugated to goat anti-rat (Jackson ImmunoResearch, 1:100). Barr body staining was performed with 4',6-diamino-2-phenylindole (DAPI). Slides were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA). During the complete procedure air drying was carefully avoided to preserve the 3D structure as well as possible.

Image acquisition. For imaging, nuclei were selected which showed two non-touching chromosome territories and were labeled and fixed in specific stages of the cell cycle. The cell cycle stage of each individual cell was deduced from the incorporation of the labeled nucleotides and the chase period applied. The selection criteria are summarized in Table 1.

Series of optical sections were recorded using a LEICA (TCD 4) confocal laser scanning microscope equipped with a Plan Apo 63× 1.4 air immersion lens and an argon-krypton laser (excitation lines of 488, 568, and 647 nm). Simultaneous acquisition of all three colors was not possible, as only two photon multipliers were available. However, we were able to make consecutive frame scans of each color before moving in the axial direction, circumventing the problem of random Z-shift between the colors caused by the step motor. A chromatic shift of 225 nm was detected between FITC and both TRITC and CY5, which was corrected during the image processing [37]. An advantage of scanning nonsimultaneously is that cross-talk is minimal as each color is excited only by its own optimal wavelength. Images were sampled with a voxel size of 0.1 μm lateral and 0.25 μm axial. DAPI images for Barr body identification were obtained with a CCD camera. Images were stored on CD.

Image analyses. The voxel-based image processing and analysis were performed with the software package Scilimage [38]. The Voronoi-based software has been described in detail in [5, 39]. The software was run on Unix workstations (Silicon Graphics Indy and Indigo, SGI, U.S.A.).

Image segmentation. Chromosome territories were defined using two image segmentation procedures. For voxel-based analysis a small uniform filter was applied and a threshold was selected in the gray value images such that the resulting mask visually represented the chromosome territory best, without many holes, missing areas, or spreading into the background. In the Voronoi-based analyses the stack of optical serial sections from a given nucleus was iteratively tessellated into polyhedra (for a detailed description see Eils et al. [39, 40]). Each polyhedron represents a subvolume with voxels of similar gray value intensities. For segmenting the image to define the chromosome territories the following threshold constraint was used: two neighboring polyhedra were regarded to belong to the same object if the associated mean gray value of all voxels belonging to each polyhedron exceeded a common preset gray value threshold.

A range of thresholds was used around the threshold representing the chromosome territory best. The threshold level influenced the absolute values of the measurements, but not the relative results obtained by our analysis, comparing Xi and Xa pairwise within the same cell.

In the analysis presented here, the replication label images were not segmented; the gray values of all voxels belonging to the chromosome territory mask were used.

Replication ratio measurements of the X-chromosomes. The replication timing difference between Xa and Xi was analyzed by comparing the amount of label incorporated in early and in mid-late S-phase in the two territories. The total intensity (the sum of the gray values of all pixels belonging to one territory) of the early label was divided by the total intensity of the mid/late label, resulting in the replication ratio (RR) of that territory:

$$RR = \frac{\text{Σ intensities first label}}{\text{Σ intensities mid-late label}}$$

The RR of Xa was compared with the RR of Xi in the same nucleus. Shape differences between active and inactive X-chromosome territories. Using the Voronoi tessellation approach morphological parameters as volume (V), surface area (S), and the roundness factor (RF) were calculated for each territory as described in detail in [5, 39]. The RF is defined as

$$RF = 36\pi^2(V^2/S^3) \quad 0 < RF \leq 1.$$  

This dimensionless parameter expresses to what extent the shape of an object diverges from a perfectly round sphere with a perfectly smooth surface (RF = 1).

Distribution of replication signals within chromosome territories. First, the smallest distance of each voxel in a chromosome territory to the territory surface was determined using an Euclidean distance transform routine [41]. Then the territory was divided in 100-nm-thick concentric shells, starting from the territory surface and going inward (10–15 shells per territory). The concentration of replication signal was determined in each shell (summed intensities of replication label/voxels in shell) and normalized by dividing the concentration in each shell by the average shell concentration of that territory to allow comparison between labels and territories. The replication concentrations were plotted in graphs as function of the distance to the territory surface. To compare distributions, linear replication concentration curves were fitted to these data points with a least squares method. The slope of the curve is indicative of the trend of the distribution.

RESULTS

Replication Double Labeling and Chromosome Painting

The combination of replication double labeling and chromosome painting on 3D interphase nuclei became possible after adjusting the interphase hybridization detection protocol by lowering the temperature and the salt concentration in the washing steps, as described under Material and Method section. This resulted in chromosome paint signals similar to those obtained with the original protocol [5]. No difference was observed between replication foci outside and inside the hybridized chromosome territories (Figs. 1 and 2). The replication patterns observed in these experiments were similar to those in nonhybridized cells (data not shown) and to those described in the literature [21, 23, 28, 29]. Manders et al. (personal communication, manuscript in preparation) have, at the light microscopic level, observed no difference in appearance between replication foci detected in glutaraldehyde fixed cells and those labeled by incorpo-
rating fluorescently tagged nucleotides into the DNA and
detected in living cells. However, as a result of the addi-
tional hybridization procedure in our experiments, some
of the larger replication foci appeared more fragmented
than similar foci in nonhybridized, glutaraldehyde-fixed
nuclei. Robinett et al. [42] also showed that hybridization
maintained overall size and shape of the domains but
may blur fine detail. We are aware that small artifacts
may have been introduced by our procedure, but since
our analyses were independent of small changes in do-
main appearance, we believe our results to closely reflect
the in vivo situation.

Discriminating between Xa and Xi Territories
in Female Cells

For the purposes of this study it was essential to
discriminate in female cells hybridized with an X-Chro-
mosome paint between the Xa and the Xi. The stan-
dard method identifies Xi with the Barr body [43],
visualized by DAPI. However, not in all cells a Barr
body was distinguishable. Therefore, we used the dif-
fERENCE in replication timing between Xa and Xi as an
independent means for identification. Xa replicates
more in early S-phase than Xi and should incorporate
more replication label in early S-phase than in mid to
late S-phase, compared to Xi. The replication ratio (RR,
amount of label incorporated in early S-phase/amount
of label incorporated in mid-late S-phase) is expected to
be higher for Xa than for Xi.

Nonsynchronized growing cells were labeled for 1.5 h
with IdUrd, chased for 4 h, labeled for 1.5 h with
CldUrd, and fixed. Triple-labeled cells (X-Chromosome
paint and the two replication labels) had obviously
incorporated the IdUrd at the earliest beginning of S-phase and CldUrd
in mid S-phase. At the other extreme, a cell may have
incorporated IdUrd 4 h after the onset of the 9-h
S-phase and CldUrd at the very end of S-phase (see
Table 1). Twenty-nine triple-labeled cells, having two
nontouching X-chromosome territories, were selected
for further analysis. In 23 of these cells a Barr body
was recognized in the DAPI-counterstained images,
colocalizing in all cases with one of the two X-Chromo-
somes. In each nucleus the RR of the two territories
were calculated and compared.

In 22 of 23 cells, the RR measured for the Xi territory
as defined by the Barr body was lower than the RR
of the Xa territory; in one cell RRXi was slightly higher
than RRXa. The differences between RR (Xa) and RR
(Xi) of the same cell were significantly different (P <
0.001) (see Table 2). This result validated the use of the
RR as an independent parameter to identify Xi in the
cells without visible Barr body (6 of 29) assuming RR
(Xi), RR (Xa).

Overall Shape Difference between Xi and Xa
Territories Is Maintained during Mid–Late
S- and G2-Phase

Xa and Xi territories have different shapes in inter-
phase, but similar volumes as has been demonstrated
previously by Eils et al. [5]. Our question was whether
this shape difference may temporarily disappear dur-
ing replication and whether a volume difference may
become notable. We determined the volume and the
roundness factor of the X-chromosomes in 29 cells fixed
in the second half of S-phase and in four cells fixed in
G2-phase (see Table 1 for the cell cycle information).
The volumes of Xa and Xi territories were not signifi-
cantly different in cells fixed in the second half of
S-phase (P = 0.1) or in G2 (see Table 2). In 18 of 29 cells
Xa had a larger territory than Xi, while on average the
volume of Xa territory was 1.1 times larger (SD = 0.4)

<table>
<thead>
<tr>
<th>Observation</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>IdUrd applied</td>
<td>Conclusion</td>
</tr>
<tr>
<td>IdUrd added in</td>
<td>CldUrd added in</td>
</tr>
<tr>
<td>No 1-3 h</td>
<td>Yes</td>
</tr>
<tr>
<td>Yes 4-7 h</td>
<td>Yes</td>
</tr>
<tr>
<td>Yes 1-4 h</td>
<td>No</td>
</tr>
</tbody>
</table>

Note. Nonsynchronized fibroblasts with an S-phase of 8–10 h and a total cell cycle time of more than 20 h were labeled with IdUrd and
CldUrd and a variable chase period without label between the pulses. Labels were only incorporated by cells which were replicating during
the pulse period (S-phase). Cells were fixed immediately after the second (CldUrd) pulse. Based on the presence or absence of IdUrd and
CldUrd signals (observation) in combination with the duration of the chase period, the phase of the cell cycle at the times of labeling and
fixation can be determined (conclusion).
than the Xi territory in the same nucleus. The roundness factor (RF) is indicative for the shape of a territory. A larger RF corresponds to a smoother surface and/or rounder territory. In mid–late S-phase, when Xi is replicating heavily, Xi still had a higher RF than Xa in 24 of 29 cells. The RF (Xa) and RF (Xi) differed significantly ($P < 0.001$). Also in G2 cells the RF (Xi) was higher than RF (Xa) (see Table 2).

**Early and Late Replicating Chromatin Is Found in Separate Domains**

Cells labeled for 1.5 h in the first half of S-phase with IdUrd and with CldUrd in the second half of S-phase showed areas of IdUrd incorporation and areas of CldUrd incorporation and unlabeled areas. However, in all cases very little overlap between the two replication markers was observed, as can be seen in Fig. 2a. This indicates that in interphase stretches of several Mbp DNA are organized in separate domains (i.e., sub-volume of a chromosome territory) consisting of either early replicating chromatin or of late replicating chromatin.

**Sites of Replication Are Distributed Throughout Territories**

A major goal of this work was to determine whether replication takes place preferentially near the surface of chromosome territories or throughout the territo-
Table 2

Summary of Differences between Xa and Xi in Replication Ratio, Volume, and Roundness Factor

<table>
<thead>
<tr>
<th>Cell Cycle</th>
<th>Xa mean (SD)</th>
<th>Xi mean (SD)</th>
<th>Xa = Xi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication ratio (RR)</td>
<td>Second half of S (n = 23)</td>
<td>1.1 (0.4)</td>
<td>0.7 (0.3)</td>
</tr>
<tr>
<td></td>
<td>Second half of S (n = 29)</td>
<td>38 (16)</td>
<td>34 (13)</td>
</tr>
<tr>
<td></td>
<td>G2 (n = 4)</td>
<td>36 (18)</td>
<td>41 (15)</td>
</tr>
<tr>
<td>Volume (V), in μm³</td>
<td>Second half of S (n = 29)</td>
<td>0.06 (0.03)</td>
<td>0.11 (0.05)</td>
</tr>
<tr>
<td></td>
<td>G2 (n = 4)</td>
<td>0.09 (0.5)</td>
<td>0.12 (0.04)</td>
</tr>
</tbody>
</table>

Note. Replication ratios (RR; only in cells fixed in the second half of S-phase) of Xa and Xi were compared in cells where Xi was identified by Barr body, to justify the use of RR as Xi-identifier in cells without recognizable Barr body. The morphological parameters volume and roundness factor were compared between Xa and Xi in cells fixed in the second half of S- or in G2-phase.

a Identified by Barr Body, when present (n = 23), otherwise by RR.
b Wilcoxon signed rank test.

Replication DNA in nonsynchronous male fibroblasts was labeled with a short 10-min pulse of thymidine analogue IdUrd and after a chase period of varying length (1–7 h) a short pulse of CldUrd was applied. Individual cells had incorporated either both labels, the first label only, the second label only, or no label. Table 1 summarizes the labeling schemes and consequently the cell cycle phase in which the cells were labeled and fixed. The second label, CldUrd, labeled the sites of replication at the moment of fixation. The first label, IdUrd, labeled DNA which was replicated several hours earlier and could have moved from the sites where the replication process took place. Chromosomes 8 or the X-chromosome were painted.

Sites of replication were observed in foci throughout the territories of chromosome 8 and Xmale. This distribution (but not the size or the shape of the foci) was independent whether the label was incorporated in the first half of S-phase or in the second half of S-phase, since it was observed both in cells fixed in early S-phase (showing only CldUrd) and in cells fixed in mid-late S-phase (which contained both replication labels; data not shown). Figure 1 shows sites of replication in early S-phase together with chromosome 8 territories.

In the female cells longer stretches of chromatin were labeled during the 1.5-h pulse. Part of the labeled chromatin may have moved from the site of replication during the labeling period. However, CldUrd incorporated just before fixation remained connected to the site of replication. Figure 2d indicates replication in mid-late S-phase in the X-chromosomes in female cells.

Distribution of Early and Late Replicating Chromatin within X-Chromosome Territories

To analyze the distribution of early and late replicating chromatin in chromosome territories the X-chromosomes in female cells are a good model system. Xa and Xi are functionally distinct chromosomes that replicate differently, while comparisons between these genetically identical chromosomes can be made within one nucleus.

Xi and Xa were identified either by Barr body or replication ratio in female fibroblasts which had incorporated IdUrd for 1.5 h in early S-phase and CldUrd in mid-late S-phase (see Table 1). In contrast to the data obtained with shorter pulse labels, described above, the labeled chromatin in these experiments may have moved during the labeling and chase periods. Manders et al. [27] showed that, within 1 h, replicated DNA and sites of replication moved apart at a rate of 0.5 μm/h. Most of the chromatin in the domains labeled here thus represents not the sites of replication themselves, but rather a region at which early or mid-late replicating chromatin is located at a specific time after replication. However, the domains labeled by the second label would still be connected to the site or replication. To analyze the distribution of the domains over the territories, the chromosome territories were subdivided into 10–15 shells of 100 nm wide, starting at the territory surface and going inward. The concentration of replication label in each shell was calculated and, after normalization, plotted as a function of the distance from the territory surface toward the interior.

Within Xa, both early and mid–late replicating domains were found throughout the chromosome territory, in the periphery as well as in the interior. This can be seen in Fig. 2 for the Xa-territory located on the right-hand side of the image and is quantitatively represented in Fig. 3a. Here, both replication labels were found in equal concentrations at the periphery and in the interior, resulting in a horizontal replication concentration curve. In contrast, in the majority of Xi-territories we observed a difference in distribution between early and mid–late replicating chromatin. Here, the first label was found preferentially near the periphery, resulting in a replication concentration curve slop-
ing down, whereas the second label was more concentrated in the interior of the territory, resulting in a rising curve as in Fig. 3b (see also the Xi territory on the left-hand side in the image in Fig. 2).

For each territory the distribution of early replicating chromatin was compared with the distribution of mid–late replicating chromatin by subtracting the value of the slope of the curve fitted to the data points of the mid–late replicating chromatin from the value obtained for the early replicating chromatin. A value around zero indicates that early and mid–late replicating chromatin are distributed in a similar manner through the territory. A negative value is obtained when more early than mid–late replicating chromatin is located near the periphery. In Xa, there was no difference between the distributions of early and mid–late replicating chromatin (the average difference in slope of $0.02 \pm 0.08$ (SEM) did not deviate significantly from 0 ($P = 0.8$, one-sample $t$ test, $n = 29$)), whereas in Xi the early and mid–late replicating chromatin showed a significantly different distribution ($P < 0.001$), with an average difference in slope of $-0.5 \pm 0.1$ (SEM).

We were concerned that early replication foci near the territory surface could belong to surrounding chromosomes and not to the Xi-territory to which they were assigned. However, the peripheral distribution of early replication that was still observed in Xi-territories outlined by high threshold levels that would unlikely contain much chromatin belonging to adjacent territories. In conclusion, although some of the replication signals near the territory surface may belong to surrounding territories, the early replicating chromatin of Xi was preferentially located near the territory surface in the majority of the cells.

To compare the X-chromosomes with an autosome we analyzed chromosome 8, which has a DNA content similar to that of X-chromosomes. Early and late replicating chromatin were found throughout both of the chromosome 8 territories, with distributions similar to those in Xa (Fig. 4). Here too, the early and late replicating chromatin were not distributed significantly differently (average difference in slope of $-0.06 \pm 0.35$ (SEM)).

**DISCUSSION**

Our aim was to establish how nuclear processes are organized in chromosome territories in interphase nu-
clei. Three-dimensionally preserved nuclei of male human fibroblasts were used to investigate the spatial distribution of DNA replication foci in individual chromosome X- and chromosome 8 territories. For this purpose chromosome painting was combined with in vivo double replication labeling employing incorporation of the halogenated nucleotides IdUrd and CldUrd. Furthermore, in female cells, we compared the distribution of domains containing early or mid–late replicating chromatin directly within the active and inactive X-chromosome territory in the same nucleus. In addition, we obtained data about the volume and shape/surface structure of these chromosome territories.

Earlier, a significant difference in the roundness factor (RF) was noted between the two X-chromosome territories in nuclei of nonsynchronized amniotic fluid cells, which are mostly in the G1 phase of the cell cycle, suggesting that Xi was rounder and/or smoother than Xa [5]. Here the same shape difference was observed in female fibroblasts fixed in mid–late S-phase, a period characterized by heavy replication in XI (in contrast to Xa that replicates mostly earlier in S-phase). Our data furthermore confirm the previous observation that Xa- and XI-territories have similar volumes [5]. Xa was on average slightly, but not significantly, larger than Xi. In fact, in the second half of S-phase the Xa-territory would have been duplicated somewhat more than XI-territory, due to the difference in replication timing. The similarities in roundness factor and volume ratio between cells in interphase in general [5], and in mid–late S-phase specifically, as studied here, suggest that replication of chromosomes does not require a drastic overall decondensation, although more subtle chromatin decondensation phenomena cannot be excluded.

Chromatin labeled during the first half of S-phase and chromatin labeled during the second half of S-phase were organized in domains which were well separated at the light microscopy level. Similar observations were made previously in Chinese hamster cells by Manders et al. [27]. These replication labeled domains, with diameters ranging between approximately 400 and 800 nm [44, 45], are likely to correspond to metaphase chromosome bands [30] and may thus contain a few hundred kilobase pairs up to a few megabase pairs of DNA. Recent studies [46] have provided further evidence for the structural persistence through the cell cycle of domains consisting of DNA replicated during the same period [44, 45]. These, and our, findings imply that interphase chromosome territories are compartmentalized in subchromosomal domains that consist of chromatin with a similar replication timing. These domains might possibly serve as higher order units for other functions during other stages of interphase and in terminally differentiated cells as well.

When we analyzed the distribution of these domains with respect to the chromosome territory, the early and the mid–late replicating chromatin domains were found to be distributed in a similar way throughout the entire territories of chromosome 8 and of X in male cells and in Xa in female cells. However, the Xi in female cells showed a distribution of the early and late replicating chromatin domains that deviated from that of its genetically identical, but functionally different counterpart, Xa. A decrease in the concentration or early replicating chromatin from the periphery toward the interior in Xi, in contrast to the equally distributed domains in Xa, indicates a preferential localization of early replicating chromatin near the territory surface in Xi.

The analysis of the sites of replication (visualized by a 10-min replication label pulse immediately before fixation) showed that replication takes place throughout the entire territories of chromosome 8 and X in male fibroblasts. Chromatin labeled by longer 1.5-h pulses may in part have moved from the site of synthesis, but would still be connected with the replication site via the DNA replicated immediately before fixation. The observation that mid–late replicating chromatin domains are located in the interior as well as near the surface area of the X-chromosome territories of female cells indicates that the process of replication in mid and late S-phase takes place throughout these territories as well. These results clearly demonstrate that replication takes place in the interior as well as near the periphery of the chromosome territory.

To date, studies which concerned the localization of genes, viral mRNA, and speckles rich in splicing factors in relation to 3D chromosomal architecture showed all these signals near the surface of chromosome territories [4, 6]. These results suggest a preferential localization of nuclear processes at the periphery of the chromosome territories. Our results, in contrast, clearly show that DNA replication activity is not limited to the territory surface area. Interestingly, active genes generally replicate early in S-phase [31], and transcription and early replication take place in similar compartments in the nucleus, although Wansink et al. [47] did not observe replication and transcription simultaneously in the same foci in human bladder carcinoma cells. Moreover, electron microscopic studies using Drosophila embryo DNA extracts showed RNA transcripts within replicons [48]. It is, in this respect, highly relevant that, with the exception of XI, early replicating chromatin is found throughout chromosome territories, and not preferentially near the periphery of the territory, where the few genes studied so far were observed. Recently it was hypothesized that essential nuclear functions might take place within an interchromosomal domain (ICD) space at the surfaces of chromosomal domains [4, 34]. Our results are com-
patible with the adaptations of this model, where it is assumed that ICD channels extend from the territory surface into the territory interior and between subchromosomal domains [46, 49, 50]. Older, autoradiography electron microscopic studies (reviewed by [51]) showed that DNA synthesis takes place in dispersed chromatin close to the periphery of condensed chromatin. After a chase period of 7 h the radioactivity was observed mostly inside the condensed chromatin areas [52]. These condensed chromatin areas might correspond to the early and late replicating chromatin domains observed in our experiments that could be related to the functionally defined domains of the ICD model.

In conclusion, our data imply that activity of the macromolecular enzyme complexes for replication are not confined to the territory surface, as was previously suggested, but occur throughout chromosome territories. In the light of these findings it will be interesting to investigate how other nuclear processes that depend on macromolecular enzyme complexes are distributed throughout the territory.

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