Chromosomes as well as chromosomal subdomains constitute distinct units in interphase nuclei

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SUMMARY

Fluorescence in situ hybridization has demonstrated that chromosomes form individual territories in interphase nuclei. However, this technique is not suitable to determine whether territories are mutually exclusive or interwoven. This notion, however, is essential for understanding functional organizations in the cell nucleus. Here, we analyze boundary areas of individual chromosomes during interphase using a sensitive method based on replication labeling and immunocytochemistry. Thymidine analogues IdUrd and CldUrd were incorporated during S-phase into DNA of Chinese Hamster fibroblasts. Cells labeled with IdUrd were fused with cells labeled with CldUrd. Fused nuclei contained both IdUrd or CldUrd labeled chromosomes. Alternatively, the two labels were incorporated sequentially during successive S-phases and segregated to separate chromosomes by culturing the cells
one more cell cycle. Metaphase spreads showed IdUrd-, CldUrd- and unlabeled chromosomes. Some chromatids were divided sharply in differently labeled subdomains by sister chromatid exchanges. With both methods, confocal imaging of interphase nuclei revealed labeled chromosomal domains containing fiber-like structures and unlabeled areas. At various sites, fiber-like structures were embedded in other territories. Even so, essentially no overlap between chromosome territories or between subdomains within a chromosome was observed. These observations indicate that chromosome territories and chromosomal subdomains in G_1-phase are mutually exclusive at the resolution of the light microscope.

Key words: Chromosome, Interphase nucleus, Nuclear organization, DNA replication

INTRODUCTION

Chromosomes have been shown to form territories in interphase nuclei (for review see Cremer et al., 1993). Within these territories separate domains are formed by chromosome arms (Dietzel et al., 1998; Schertan et al., 1998) and by early and late replicating chromatin (Visser et al., 1998) representing R and G bands, respectively (Zink et al., 1999).

In addition to chromosome territories, the cell nucleus contains many other compartments (for reviews see Lamond and Earnshaw, 1998; Schul et al., 1998; Spector, 1993; Van Driel et al., 1995). Transcription, for example, occurs in several hundreds of foci (Jackson et al., 1993; Wansink et al., 1993), and DNA replication foci show well-orchestrated spatial and temporal distribution patterns throughout S-phase (Manders et al., 1992; Nakayasu and Berezney, 1989; Van Dierendonck et al., 1989). Splicing factors are concentrated in speckles distributed over the nucleus and appear to stretch out towards active genes (Misteli et al., 1997).

Spatial relationships between several of the domains mentioned above and chromosome territories have been reported recently. These studies show that replication (Visser et al., 1998) and transcription (P. J. Verschure et al., personal

communication) take place throughout chromosome territories. Moreover, nascent RNA was observed to accumulate preferentially at the surface of strongly labeled chromosomal subdomains throughout the territory (P. J. Verschure et al., unpublished). Intriguingly, speckles rich in splicing factors, coiled bodies, mRNA of an integrated virus (Bridger et al., 1998; Zirbel et al., 1993) and the few genes studied so far (Clemson et al., 1996; Kurz et al., 1996; Park and DeBoni, 1998) were observed to be localized preferentially near the periphery of chromosome territories. These spatial relationships, when specific, are likely to have a functional background, and may play a role in the regulation of the processes involved. Structure-function relationships have already been well described for nucleoli: the chromatin of nucleolus organizing regions is known to interact with proteins and RNA, forming compartments specialized to perform different tasks (Scheer and Benavente, 1990).

Before we can understand structure-function relationships within chromosomes, the spatial organization of chromosomes within their territories needs to be well documented. Thus, the application of the concept of the periphery of a territory, or for that matter of a chromosomal subdomain, makes sense only when the surface of that territory or subdomain can be defined

with sufficient accuracy. The crucial question, in this respect, is whether chromosomes are interwoven with one another at their periphery or whether they form mutually exclusive territories. Similarly, we might ask to what extent subdomains within chromosomes are interwoven.

Some data related to this topic have been published previously. Images of interphase nuclei showing juxtaposed chromosomes painted in different colors using in situ hybridization procedures do not give evidence for intermingling of territories (Cremer et al., 1996). However, the method applied is not suitable to address the problem in detail. Only few contact regions can be analyzed, while cross-hybridization to other chromosomes and incomplete staining by suppression of repetitive sequences make it difficult to accurately identify surface areas between chromosomes. Furthermore, in situ hybridization procedures may blur subtle details, despite the fact that good nuclear morphology is obtained at the light microscopic level (Robinett et al., 1996).

To address the question whether chromosomes or chromosomal subdomains are interwoven or packaged as discrete units, we designed two painting methods based on the incorporation of two different thymidine analogues into DNA during replication. Whole chromosomes or large chromosomal domains were labeled with either iododeoxyuridine (IdUrd) or chlorodeoxyuridine (CldUrd), which were visualized immunocytochemically with two different fluorochromes and imaged by confocal microscopy. Border areas between chromosome territories and chromosomal subdomains have thus been analyzed in detail in interphase nuclei.

MATERIALS AND METHODS

Cell culture and replication labeling

Chinese hamster fibroblast-like HA-1 and V79 cells were cultured in MEM with Hanks' Salts (Gibco Brl, Breda, The Netherlands) containing 10% FCS, glutamine, penicillin and streptomycin in a 37°C incubator at 2% CO_2. The undisturbed cycling time of HA-1 cells was 18 hours (with an S-phase of 6 hours) and that of V79 cells 10 hours (2 hours G_1, 5 hours S, 3 hours G_2/M; data not shown).

Replicating DNA was labeled by adding IdUrd or CldUrd (Sigma, St Louis, MO) to the medium in a final concentration of 0.5 μM according to the labeling schemes described below. HPLC studies indicated that this concentration results in a substitution of every 5th to 10th thymidine in the labeled DNA strands by the analogue. The transgression through the cell cycle was checked by FACS analysis and after each cell cycle, metaphase spreads were prepared from a subset of the cells to inspect the labeling pattern of the chromosomes. Cells collected by shake-off for this purpose were treated with 0.75 M KCl and fixed in methanol-acetic acid (3:1). For interphase analysis, cells were finally cultured on coverslips and fixed in a solution of 0.1% glutaraldehyde (Polysciences,Warrington, PA) and 0.5% Triton X-100 (Sigma) in PBS for 15 minutes. Autofluorescence was quenched by two incubations of 5 minutes with 130 mM sodium borohydrate (Sigma; Aten et al., 1992).

Cell fusion

The first method to obtain nuclei containing differently labeled chromosomes was to fuse two cell populations of which the total DNA was labeled differently. Cells were fused during mitosis to obtain G_1 nuclei containing chromosomes of both parent cells. Experiments were performed using two differently labeled cultures of HA-1 cells, or alternatively, two cultures of V79 cells.
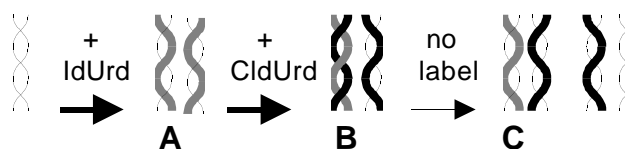

Fig. 1. Scheme for chromosome painting by thymidine analogue incorporation. (A) After one S-phase in the presence of IdUrd, metaphase chromosomes contain two chromatids labeled with IdUrd, incorporated into one strand of the DNA double helix (thick gray lines). (B) After a second S-phase in the presence of CldUrd, metaphase chromosomes contain one chromatid labeled with both IdUrd and CldUrd, and one chromatid labeled by CldUrd only (thick black lines). (C) After a third S-phase in the absence of thymidine analogues, metaphase chromosomes contain either one chromatid labeled by IdUrd and one chromatid labeled by CldUrd, or contain one chromatid labeled by CldUrd and one unlabeled chromatid (narrow lines). After mitosis these chromatids are distributed over two daughter cells and become individual chromosomes in G_1.

HA-1 cells were cultured for 32 hours and V79 cells for 10 hours in the presence of IdUrd or CldUrd to label all DNA. The cells were cultured for 90 minutes in presence of 0.1 μg/ml demecolcine (Sigma) to increase the number of cells in mitosis. The fusion protocol used is an adaptation of that used to produce hybridoma cell line preparations (Kolk et al., 1984). Briefly, mitotic cells were shaken off and both populations were mixed. They were washed in PBS supplemented with 1% glutamine, and centrifuged in a round bottom tube at 250 *g*. A prewarmed solution of 50% polyethylene glycol (PEG-6000; Merck, Darmstadt, Germany) in PBS (0.5 ml) was added and cells were incubated during 1 minute at 37°C before adding 5 ml of glutamine-containing PBS. Cells were washed in glutamine-containing PBS before plating on coverslips in culture medium. Attached HA1 cells were fixed after 18 hours of culture and V79 cells were fixed after 7 hours of culture.

Sequential labeling

Although fused cells continued to proliferate after fusion, one could argue that fusion disturbs nuclear architecture. Therefore, we used a second method as well. Cells were labeled during sequential rounds of replication: during the first S-phase, IdUrd was incorporated and during the second S-phase CldUrd was incorporated. The labels were segregated over separate chromosomes after one more round of replication in the absence of an analogue and subsequent mitosis (Fig. 1).

Specifically: HA-1 cells were grown to confluency to limit the percentage of cells in S-phase to less than 10%. Cells were reseeded and allowed to incorporate IdUrd during one full S-phase. After 14 hours, 1.5 mM hydroxy urea (Sigma) was added to the cells for another 11 hours to synchronize their cell cycles at the onset of the next S-phase. Cells were washed and cultured during one S-phase in medium containing CldUrd. After 8 hours, demecolcine was added and the cells were cultured for 1 hour before mitotic cells were collected by shake-off. After washing to remove demecolcine and CldUrd, cells were plated on coverslips in normal medium for 28 hours and then fixed. At that time cells had passed one more S-phase in the absence of label and part of the cells had arrived in G_1, now containing differentially labeled chromosomes.

V79 cells were sequentially labeled without chemical synchronization, but using consecutive mitotic cell collections by shaking off of the cells. Cells were cultured for 10 hours in presence of IdUrd. Cells were washed twice and mitotic cells were shaken off carefully without the use of demecolcine, and cultured for 13-15 hours in the presence of CldUrd. Again, cells were washed and mitotic cells were shaken off and plated on coverslips. After culturing in normal medium for 15-18 hours, cells were fixed.

Replication label detection

The thymidine analogues were detected according to Aten et al. (1992). Cells were incubated for 2 minutes in 0.07 M NaOH to denature DNA, and washed in PBS containing 0.05% Tween-20 (PBT). Nonspecific antibody binding was blocked by 10% BSA (Sigma) in PBS. IdUrd and CldUrd were specifically detected by specially selected antibodies originally raised against BrdUrd: a mouse-derived antibody from Becton and Dickinson (San Jose, CA; diluted 1:4 in PBT), and a rat-derived antibody from Seralab (Crawley Down, Sussex, England; diluted 1:100 in PBT). The antibodies were applied in combination during a 30 minutes incubation. Cross reaction of the antibodies with either CldUrd or IdUrd, respectively, was minimized by washing the cells during 6 minutes with a Tris-buffered 0.5 M NaCl solution containing 0.5% Tween (pH 8), followed by washes in PBT. Nonspecific staining was blocked by 50% normal goat serum (Dako, Glostrup, Denmark) in PBS. Then, the cells were incubated for 30 minutes in a solution of PBT containing goat anti-mouse antibodies labeled with Texas Red and goat anti-rat antibodies labeled with FITC (Jackson ImmunoResearch, West Groove, PA). Cells were washed in PBT, counterstained with 4′,6-diamino-2-phenylindole (DAPI; Sigma) and embedded in Vectashield (Vector Laboratories, Burlingame, CA). During the whole procedure, drying of the cells was avoided to retain nuclear morphology of interphase nuclei as optimally as possible. As morphological control, cells labeled with CldUrd and immuno-stained, were compared with unlabeled cells fixed and stained with the DNA stain TOPRO-3 (Molecular Probes, Leiden, The Netherlands).

Imaging and analysis

Metaphase preparations were visually inspected with respect to labeling using a Ortholux fluorescence microscope (Leica, Wetzlar, Germany) and images were recorded with a cooled CCD camera (Lambert Instruments, Leutingewolde, The Netherlands).

For interphase analysis, only cells were selected that were not in mitosis as determined microscopically after staining by DAPI and, in the case of the sequentially labeled cells, had arrived in the fourth G_1-phase as indicated by the presence of some unlabeled regions (Fig. 1). From 39 fused and 47 sequentially labeled interphase nuclei, optical sections were obtained with the use of a confocal laser scanning microscope (CLSM; Leica Fluovert, ×63, NA 1.4) with a voxel size of $50 \times 50 \times 167$ nm. Images of FITC and Texas Red labeling were recorded simultaneously by two photomultipliers. Chromatic shift between FITC and Texas Red was measured with multi-color beads (Molecular Probes) embedded under the same conditions as the samples, and the images were corrected for this shift before further processing. Cross-talk between the fluorochromes was determined with samples containing the two labels without spatial overlap. The images were corrected for cross-talk by subtracting the measured cross-talk percentage of the red images from the green images and vice versa, using ScilImage software (Van Balen et al., 1994). They were then restored, applying a measured point spread function, by the Maximal Likelihood Estimate procedure of Huygens software (Scientific Volume Imaging BV, Hilversum, The Netherlands) on a Silicon Graphics workstation. The two sets of optical sections of each cell were overlaid in artificial colors. Optical sections and 3-D representations were inspected visually using Imaris software (Bitplane AG, Zurich, Switzerland). Linescans of the intensity values of voxels on a pre-set line in the images were made using ScilImage software.

RESULTS

Nuclei containing chromosomes that were labeled either by IdUrd or by CldUrd were stained immunocytochemically using antibodies that discriminated specifically between the two
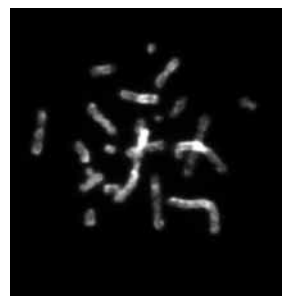

Fig. 2. IdUrd labeled metaphase chromosomes before fusion. Thymidine analogues label all chromatin when incorporated into the DNA during a complete S-phase.

thymidine analogues. These fluorescence immuno-labeled nuclei showed the same nuclear morphology and chromatin structure as non-immunolabeled nuclei that were stained with the DNA stain TOPRO-3 (data not shown). Previous observations of Manders et al. (1999, and E. M. M. Manders, personal communication) indicate that replication foci visualized by immuno-fluorescence labeling of halogenated thymidine analogues were similarly distributed in nuclei and had the same morphology as those observed in vivo, using directly fluorescence-conjugated thymidine analogues. We, therefore, conclude that the nuclear structures we observe are not artefacts introduced by the fixation and detection procedures.

Chromosomes in fused cell nuclei do not intermingle

Our first strategy to investigate chromosome contact regions was by fusing cells whose DNA that was tagged with either IdUrd or CldUrd. Immuno-fluorescence labeling of metaphase spreads before fusion showed that all chromosomes were uniformly labeled (Fig. 2). Mitotic cells containing IdUrd-DNA were fused with mitotic cells containing CldUrd-DNA. The cells were fixed 7 hours later when they had proceeded into the next interphase. Fused nuclei displayed a mosaic pattern of domains containing exclusively IdUrd-DNA or exclusively CldUrd-DNA. Very little overlap was observed between adjacent domains (Fig. 3). This indicates that in fused nuclei no intermingling occurred between chromosomes derived from differently labeled parent nuclei.

The borders between the IdUrd-containing chromosomes and CldUrd-containing chromosomes were remarkably well-defined. Linescans of the two fluorochrome signal intensities crossing the border area between two chromosomes confirmed that there is limited overlap between bordering chromosomes. It is known that two adjacent fluorescent objects have some optical overlap, due to the limited resolution of the light microscope. To evaluate this imaging effect we made linescans of fused cells whose nuclei were not fused but positioned side by side (Fig. 4). Here, too, IdUrd-containing chromatin was located adjacent to CldUrd-containing chromatin, but physically separated by nuclear envelopes. The apparent overlap of the different nuclei in this linescan was similar to that observed for adjacent chromosomes in fused nuclei (compare Figs 3 and 4). Thus, in heterokaryons, the chromatin of one chromosome does not intermingle in any detectable way with that of other chromosomes.

Fused cells continued to proliferate. After a round of replication and cell division in absence of thymidine analogues, unlabeled chromosomes re-appeared next to labeled

Fig. 3. Chromosome territories after fusing IdUrd labeled cells with CldUrd labeled cells. The contact areas between IdUrd (red) and CldUrd (green) labeled regions represent the interface between chromosome territories. (A-C) V79 fibroblast at 7 hours after fusion; (D-F) HA-1 fibroblast which has proceeded through another round of replication and mitosis at 18 hours after fusion. (A and D) Single optical sections, showing clear separations between chromosome territories. The overlapping regions (orange) correspond to sites where IdUrd and CldUrd labeled chromosomes were juxtaposed axially. This can be observed in the cross views B and E (at the site indicated by the small arrow), indicating that most of the overlap is due to the lower spatial resolution in axial direction. (C and F) Linescans of the label intensities through IdUrd (red continuous line) and CldUrd (green dotted line) labeled chromosome territories at the sites indicated by the large arrows. The asterix indicates a transition perpendicular to the border of laterally juxtaposed chromosomes, whereas the dot indicates a site where the chromosomes are juxtaposed axially. Bar, 1 µm.
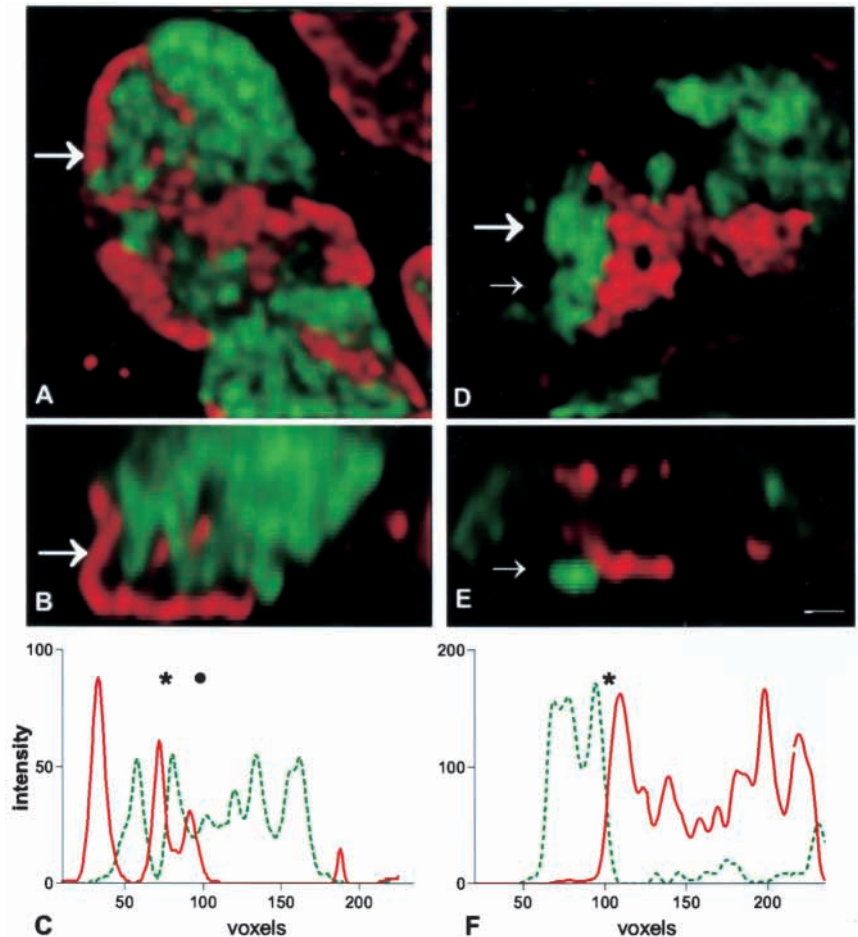
chromosomes (see step B-C in Fig. 1) and were observed as several unlabeled regions in interphase nuclei. Borders between IdUrd- and CldUrd-containing chromosomes in cells that had passed a full cell cycle were similar to those in cells fixed in the first interphase after fusion (Fig. 3D-F). This indicates that the heterokaryons were fully viable and that the observed confinement of chromosomes to individual territories was not due to the fusion process.

Chromosome subdomains do not intermingle either

In our second approach to obtain differently labeled chromosomes we took advantage of the semi-conservative character of DNA replication and labeled the cells during successive cell cycles (see Fig. 1). Metaphase spreads of the labeled cells showed that after one full S-phase of IdUrd incorporation, both chromatids contained IdUrd (Fig. 5). In the second metaphase, after one full S-phase of CldUrd incorporation, both chromatids contained CldUrd, but only one of them contained IdUrd as well. In the third metaphase, after

one S-phase in absence of thymidine analogues, IdUrd and CldUrd became segregated to opposite chromatids. A considerable number of sisterchromatid exchanges (SCE) occurred during the labeling process, resulting in a harlequin
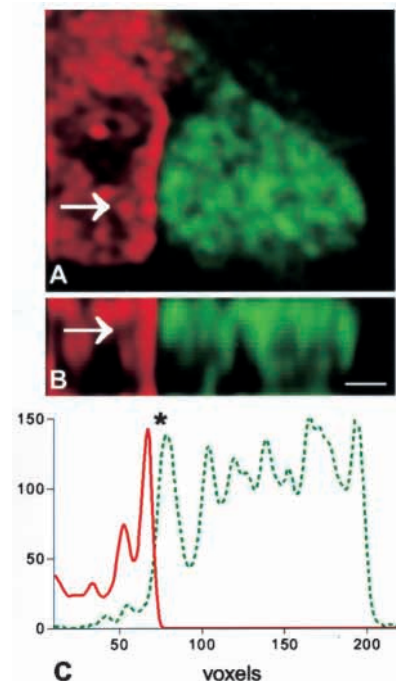

Fig. 4. Reference for visual overlap. Two juxtaposed nuclei in a fused cell, one labeled with IdUrd and the other one with CldUrd, illustrating intensity transitions for two labeled domains that are physically separated by nuclear envelopes. (A) Single optical section; (B) axial cross view at the site of the arrow; (C) linescan at the site of the arrow. This intensity transition is comparable to those observed at the border area between two chromosomes within one nucleus, as shown in Fig. 3. Bar, 1 µm.

Fig. 5. Metaphase preparations after successive rounds of replication in the presence of label. (A) After the first S-phase in presence of IdUrd: both chromatids are labeled with IdUrd (red), (B) after the second S-phase in presence of CldUrd: both chromatids are labeled with CldUrd (green), and one is labeled with IdUrd as well (red overlaid on green, thus appearing as orange), (C) after the third S-phase in the absence of label: IdUrd and CldUrd are segregated to individual chromatids (see also Fig. 1). In B and C, SCEs are visualized by color-jumps within a chromatid. In the G_1-phase following C, the chromatids are segregated becoming individual chromosomes that are labeled completely by IdUrd, CldUrd or are unlabeled, or may be subdivided in chromosomal domains created by SCE.
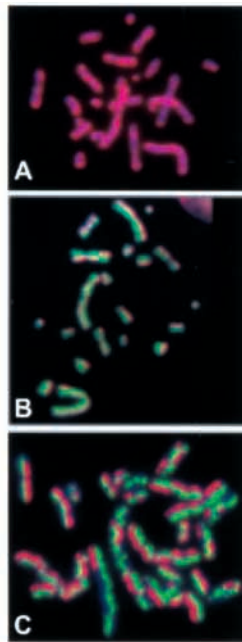
entering the fourth G_1-phase, the chromosomes each consist of a single chromatid. These G_1-phase chromosomes were, thus, labeled entirely with a single thymidine analogue or they were without any label, or they were divided by SCEs into several subdomains that contained exclusively IdUrd, CldUrd or no label.

The interphase nuclei displayed a mosaic pattern of distinct domains that contained either IdUrd or CldUrd or that were without label (Fig. 6). Such domains corresponded either to complete chromosome territories or to chromosomal subdomains formed by SCEs. In rare cases, a small region of overlap was observed (Fig. 6D-F), suggesting that, locally, a tighter contact may exist between chromosomes and/or chromosomal subdomains. Linescans through juxtaposed domains gave results similar to linescans made through chromosome territories in fused nuclei, confirming that there was little overlap between adjacent domains. These findings demonstrate that, essentially, neither chromosome territories nor chromosomal subdomains formed by SCEs intermingle.

Chromatin structures within domains

Chromatin that contained IdUrd or CldUrd showed intensely and less intensely labeled regions, frequently resembling fiber-like structures with diameters ranging from the limit of light microscopic resolution (0.2 µm) to 0.6 µm (Fig. 7, and also apparent in Figs 3, 4 and 6). We never observed well-resolved fiber-like structures containing both labels, indicating that these structures were not created by mixing chromatin fibers

staining of metaphase chromosomes with sharp transitions between chromosomal subdomains that were labeled with different thymidine analogues (Fig. 5). After mitosis, when


Fig. 6. Chromosomal domains in sequentially labeled interphase nuclei. Contact areas between IdUrd (red) and CldUrd (green) labeled regions represent border regions either between chromosome territories or between chromosomal subdomains created by SCEs. (A-C) V79 fibroblast, (D-F) HA-1 fibroblast. (A and D) Single optical sections showing mostly distinct domains and a rare overlapping region (in the line of the arrow in D); (B and E) crossview at the site of the arrow, (C and F) linescans at the site of the arrow. The red continuous line represents the IdUrd (Texas Red) fluorescence and the green dotted line the CldUrd (FITC) fluorescence. The asterix indicates a transition perpendicular to the border of laterally juxtaposed chromosomes, whereas the dot indicates a site where the chromosomes were juxtaposed axially. The overlapping region is indicated by the cross. The linescans support the notion that most chromosomal domains are not interwoven with one another. Bar, 1 µm.
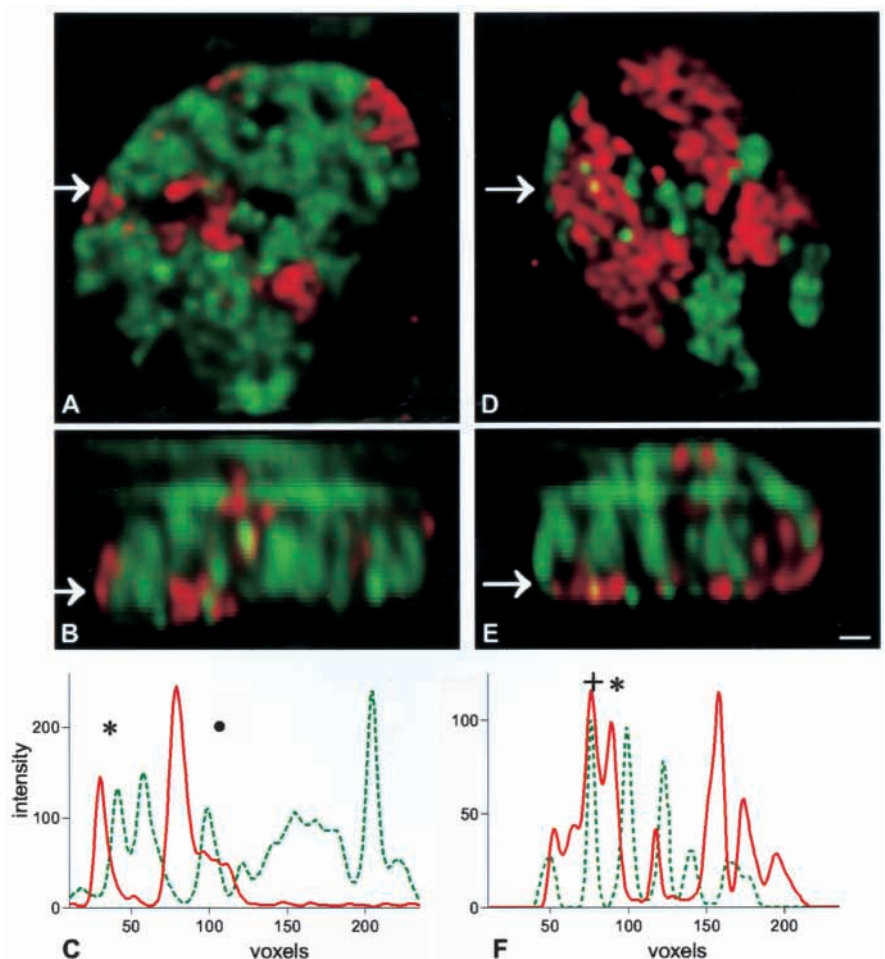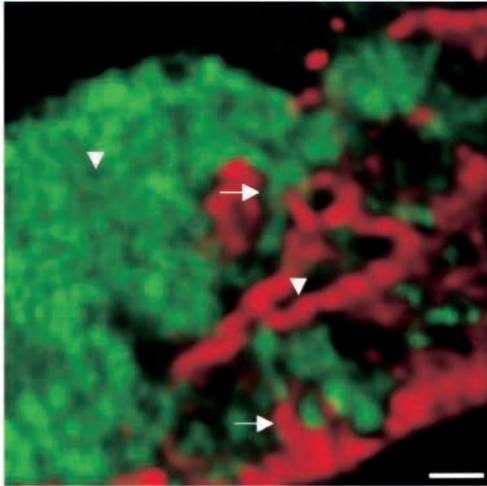
Fig. 7. Labeled chromatin frequently resemble fiber-like structures. These fiber-like chromatin structures are either red or green (arrows and arrowheads), indicating that they are not formed by mixing chromatin originating from different chromosomes. Fiber-like structures are sometimes embedded in other chromosomes (arrows). This indicates that chromosomes form irregular territories with extensions that may intrude into other chromosomes without intermingling. Single optical section of a nucleus containing IdUrd and CldUrd labeled chromosomes, 7 hours after fusion. Bar, 1 μm.

from different chromosomes or different chromosomal subdomains. Fiber-like structures sometimes were embedded in differently labeled regions and in some cases were seen to encircle one another. This indicates that chromosome territories are irregularly shaped with extensions that may in part penetrate into other chromosomes without intermingling (Fig. 7).

DISCUSSION

The aim of the present study was to establish whether chromosomes and chromosomal subdomains in interphase nuclei are separate and distinct entities or that chromatin of different chromosomes is interwoven. This structural data is essential for elucidating functional organizations in the cell nucleus. The two approaches used to label chromosomes, namely (a) by fusing nuclei containing IdUrd-DNA with nuclei containing CldUrd-DNA and (b) by labeling cells sequentially with IdUrd and CldUrd, showed that chromosome territories are distinct structures and contain separate subdomains. Furthermore, we noticed singularly labeled fiber-like structures with diameters ranging from the limit of light microscopic resolution (0.2 μm) up to 0.6 μm. Since these fiber-like structures were sometimes embedded in other chromosome territories, our results show that chromosomes may in part penetrate into one another without mixing their chromatin. Chromosome territories are thus irregularly shaped distinct units containing subdomains that do not intermingle.

For a more detailed analysis, we have made linescans over lateral boundaries between chromatin domains labeled with the two fluorochromes. The crossing-over point in these intensity curves was close to half the intensity of the maximum. A

crossing-over point at 50% of maximal intensity corresponds to two non-overlapping objects. We confirmed this by analyzing intensity transitions between chromatin of two juxtaposed nuclei in a fused cell separated by two nuclear envelopes. Obviously, low concentrations of labeled DNA below the detection level may have been present but not visualized. It can thus not be excluded that some chromatin from one chromosome or domain intrudes into another chromosome or domain. However, if so, only small amounts of DNA are involved. While taking this into account, we conclude that, at light microscopic resolution, chromosomes form mutually exclusive territories with distinct surfaces in the interphase nucleus, and that also within territories the chromatin is not interwoven but forms discrete domains and fiber-like structures.

At some boundaries, a small degree of overlap between chromosomes or chromosomal subdomains was observed. It is likely that this overlap was mostly optically, due to the limited resolution of the light microscope in axial direction. However, it is impossible to determine whether this was the only cause. In fact, we observed some rare small regions where chromatin of different domains seemed to be closely associated. These sites may correspond to SCEs that occurred in highly compacted chromatin regions or to certain nuclear processes that involve a temporally and locally limited intermingling of chromatin. We, therefore, conclude that in some cases the chromatin of different chromosomes or subdomains may be interwoven locally beyond light microscopic resolution, while the remainder of the chromosomes form separate and distinctly organized territories.

Discreteness of chromosome territories

Discreteness may well be a common feature of chromosome territories in eukaryotic interphase nuclei, and not only in the two cell lines investigated here. Studies on specialized polytene chromosomes in *Drosophila* salivary gland interphase nuclei, which can be observed directly by light microscopy, revealed that these chromosomes were maintained in separate spatial domains without looping around one another (Hochstrasser et al., 1986). In other polytene tissues, chromosomes arms did sometimes loop around one another, but remained recognizable as individual polytene fibers (Hochstrasser and Sedat, 1987). In human cells, less detailed studies showed that when FISH painted chromosomes were adjacent to each other, the bulk of the territories were apart (Cremer et al., 1996). Bridger et al. (1998) introduced *Xenopus* vimentin, an intermediate filament, into human nuclei. Interestingly, these filaments formed an interconnecting channel-like system after polymerization, almost exclusively outside painted chromosome territories. Since growing cytoplasmic intermediate filaments are known to avoid intracellular barriers (Franke et al., 1978), the authors concluded that the vimentin filaments indicate areas of diminished chromatin density at the borders of chromosome territories. These data support our conclusion that chromosome territories are mutually exclusive. A recent observation of two intermingled chromosomes during leptotene, a well-defined meiotic cell cycle stage in spermatogenesis (Schertan et al., 1998), indicates that in special situations chromosomes may be interwoven. This emphasizes the remarkable discreteness of chromosome territories in general.

Discreteness of chromosomal domains within chromosome territories

Sequential labeling of cells yielded chromosomes divided into several differently labeled domains linearly along the metaphase chromosome, due to SCE. SCE is a naturally occurring process where sister chromatids are exchanged during or immediately after replication without loss of genetic information (Cortés et al., 1993). The boundaries between these domains were thus distinct, corresponding to a sudden change of label. Previously investigated subdomains containing early or late replicating chromatin (Ferreira et al., 1997; Visser et al., 1998; Zink et al., 1998, 1999), were likely to display some non-labeled chromatin in between domains, due to a chase period in mid S-phase separating early from late replication labeling. Thus, the discreteness that we observed for SCE-induced domains in interphase nuclei unambiguously demonstrates a high degree of compartmentalization within chromosome territories.

While early and late replicating chromatin generally corresponds to euchromatin and heterochromatin domains (Craig and Bickmore, 1993), SCEs create differently labeled chromosomal segments that cut in part through these domains. We therefore conclude that even within domains of functionally related chromatin, such as early and late replicating chromatin domains, the chromatin is not interwoven.

In addition to the discreteness of domains and territories, we observed a substructure of intensely and less intensely labeled regions, which in some places formed fiber-like structures with a diameter ranging from the resolution limit of the microscope (0.2 μm) to 0.6 μm. Similar substructures were also observed in vivo (Belmont et al., 1989; Li et al., 1998; Robinett et al., 1996; Zink et al., 1998) and in painted chromosome territories (P. J. Verschure et al., unpublished). These fiber-like structures may correspond to a higher level of chromatin folding, resulting in chromonema fibers as proposed by Belmont and Bruce (Belmont, 1997; Belmont and Bruce, 1994). Since interweaving of chromatin fibers with a diameter below the light microscopic resolution would result in a double-labeled structure, we conclude that chromomena fibers are not closely interwoven with another.

Internal organization of chromosome territories

The question remains how chromomena fibers are organized within a territory. Computer simulations by Münkel and Langowski (1998) are interesting in this respect. The authors concluded that chromatin which is folded according to the Giant Loop/Random Walk model (Sachs et al., 1995) is highly intermingled. A slight adaptation of the model, the Multiloop Subcompartment model (Münkel and Langowski, 1998), however, predicts non-overlapping chromosomal subdomains. Our data fit in this latter model that attributes simple polymere characteristics to chromatin. Also, several models have been described that are based on a functional organization of chromosome territories. Some of these models postulate a space between chromosome territories, such as the Interchromatin Domain model (Cremer et al., 1993, 1995; Zirbel et al., 1993) or a network of channels, such as models by Razin and Gromova (1995) and Zachar et al. (1993). These models are also compatible with our findings that chromosomes and chromosomal subdomains are mutually exclusive discrete units.

In summary, we demonstrate that chromatin forms distinct structures at several levels of organization and that these structures are not interwoven with one another as observed with light microscopy: the chromosome territory itself, chromosomal subdomains within territories and fiber-like chromatin structures. This suggests a strict organization of the chromatin in several subunits. These findings, also, provide a solid basis for further studies elucidating structure-function relationships concerning the higher order chromatin organization in the interphase nucleus.

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