Introduction

One of the marvels of living mammalian cells is that the 2-meter-long genomic DNA can be replicated inside a nucleus that is only 10 μm in diameter. At present, it is only possible to speculate how this amazing macroscopic process is performed, but it presumably involves the highly sophisticated hierarchy in which the DNA is packaged inside the nucleus. DNA forms nucleosomes in conjunction with histone proteins, and is then further coiled to form a 30-nm fiber. The 30-nm fiber appears to be looped and anchored to the nuclear matrix, where the replication machinery may assemble (for reviews, see Cook, 1998; Razin, 1999; Munkel et al., 1999). We previously showed that a plasmid bearing a mammalian replication origin efficiently generated a chromosomal homogeneously staining region (HSR). Here, we analyze the replication of this artificial HSR and show that it was replicated at the last stage of S phase. The HSR was composed of plasmid repeats only; nonetheless, we found that replication sites pulse-labeled during late S phase appeared as bands in the mitotic HSR and their number was dependent on the length of the HSR. Therefore, replication bands might not arise from sequence information per se. To understand the chronological order of appearance of replication sites, we performed a double pulse-chase experiment using IdU and CldU. Replication of the entire HSR required 100-120 minutes. During this period, the replicated sites appeared as bands at the first and last stages, but in between were apparently scattered along the entire HSR. An analysis of S-phase nuclei revealed that the replication started at the periphery of the globular HSR domain, followed by initiation in the internal domain. The replicated HSR appeared as a ring or a pair of extended spirals in late G2-phase nuclei. To account for these findings, we present a model in which the HSR is folded as a coiled-coil structure that is replicated from the outside to the inside in S phase nuclei.

Key words: Homogeneously staining region, Replication, Chromosome band, Heterochromatin

Summary

The chromosomal G/R bands are alternating domains differing in their nucleotide sequence biases. The bands are also related to the time of replication: pulse-labeling during S phase makes the replication sites as visible as replication bands that are close to the G/R bands in mitotic chromosomes. We previously showed that a plasmid bearing a mammalian replication origin efficiently generated a chromosomal homogeneously staining region (HSR). Here, we analyze the replication of this artificial HSR and show that it was replicated at the last stage of S phase. The HSR was composed of plasmid repeats only; nonetheless, we found that replication sites pulse-labeled during late S phase appeared as bands in the mitotic HSR and their number was dependent on the length of the HSR. Therefore, replication bands might not arise from sequence information per se. To understand the chronological order of appearance of replication sites, we performed a double pulse-chase experiment using IdU and CldU. Replication of the entire HSR required 100-120 minutes. During this period, the replicated sites appeared as bands at the first and last stages, but in between were apparently scattered along the entire HSR. An analysis of S-phase nuclei revealed that the replication started at the periphery of the globular HSR domain, followed by initiation in the internal domain. The replicated HSR appeared as a ring or a pair of extended spirals in late G2-phase nuclei. To account for these findings, we present a model in which the HSR is folded as a coiled-coil structure that is replicated from the outside to the inside in S phase nuclei.

Key words: Homogeneously staining region, Replication, Chromosome band, Heterochromatin

Macroscopic folding and replication of the homogeneously staining region in late S phase leads to the appearance of replication bands in mitotic chromosomes

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Research Article

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R band is replicated earlier and the G band later in S phase (Watanabe et al., 2002). This, for example, results in the replication sites in early S phase appearing in bands in the mitotic chromosome (replication bands) that are similar to the R bands. Interestingly, the chromosomal replication bands can also be detected in the cells of cold-blooded vertebrates (Schmid and Guttenbach, 1988; Boron, 2003) and plants (Sparvoli et al., 1994), in whose chromosomes G/R bands are not evident. This suggests that the replication bands might not have arisen evolutionarily from sequence-based chromatin structure but might have arisen for other epigenetic reasons, such as the folding of chromatin inside the nucleus when it is replicated. If so, this evolutionary adaptation might produce G/R bands with the above-mentioned sequence bias in mammalian cells.

We previously showed that, in a human tumor cell line (COLO 320HSR), the chromosomal homogeneously staining region (HSR) shows replication bands within, despite its lack of G/R bands (Shimizu et al., 2001b). The HSR and the double minutes (DMs) are two major cytogenetic manifestations of gene amplification associated with the malignant transformation of many human tumor cells (for reviews, see Knuttila et al., 1998; Schwaab, 1999). The DMs are autonomously replicating, extrachromosomal chromatid composed of several million base pairs of circular DNA. Importantly, the HSR is a tandem repeat of a sequence derived from the DMs (Shimizu et al., 2001b). Therefore, the presence of replication bands in the HSR suggests that the bands might not have arisen from the sequence information per se, but might have arisen from the folding pattern of the HSR chromatid inside the nucleus during its replication. Unfortunately, we could not further investigate this argument using this experimental system, because the sequence complexity of the HSR generated during malignant transformation is very high.

However, we have recently found that a plasmid with both a mammalian replication origin and a nuclear matrix attachment region is efficiently amplified and generates DMs and an HSR in cells of a human colorectal carcinoma cell line (Shimizu et al., 2001a). Our analysis of the amplification mechanism revealed that the transfected plasmid was multimerized into tandem repeats, and that the multimer recombined with the pre-existing DMs or the chromosomal arm. At the chromosomal arm, the plasmid repeat efficiently induced the breakage-fusion-bridge (BFB) cycle, which led to the generation of an artificial HSR composed of the plasmid repeat only (Shimizu et al., 2003). In the BFB cycle model, a chromosomal breakage followed by replication and end-to-end fusion of sister chromatids generates a mitotically unstable dicentric chromosome, which leads to another breakage close to the first one. Multiple cycles of BFB lead to the multiplication of the genes near the breakage.

Our aim in this study was to gain insight into the chromosomal replication bands by examining this artificial HSR. If sequence information is not required for the generation of replication bands, then even an artificial HSR containing only plasmid repeats should have the replication bands. We therefore reasoned that the artificial HSR would be ideal for studying how the replication bands might be generated, because it is composed of simple repeats of a known plasmid sequence and because we know how it was generated.

### Materials and Methods

**Cell culture and pulse-labeling of halogenated thymidine analogs**

Clones 22 and 24 used in this study were obtained and characterized as described previously (Shimizu et al., 2003). In brief, these clones were obtained by transfecting pSFVdhfr plasmids into cells of the human COLO 320DM (CCL 220) colorectal carcinoma cell line of neuroendocrine origin, followed by selection with blasticidin. pSFVdhfr (11 kbp) has a replication origin sequence (Ori\(\beta\), 4.6 kb) from the DHFR genomic region, and the blasticidin resistance gene. The 4.6-kb sequence also contains a region showing nuclear matrix attachment region activity (Shimizu et al., 2001a). Fluorescence in situ hybridization (FISH) using the plasmid as a probe showed that clones 22 and 24 have plasmid repeats on the long HSRs. Genomic Southern blot hybridization showed that the plasmid sequences are arranged as somewhat disrupted direct repeats in the HSR. Chromatin fiber FISH showed that a long plasmid array with no interruption in the HSR is present in clones 22 and 24 (Shimizu et al., 2003).

The cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum. 5-bromo-2′-deoxyuridine (BrdU), 5-iodo-2′-deoxyuridine (I\(\mu\)U) (both Sigma, St Louis, MO) or 5-chloro-2′-deoxyuridine (CldU) (ICN Pharmaceuticals Inc., Irvine CA) was added to logarithmically growing cultures to a final concentration of 20 \(\mu\)M. After pulse-labeling and chasing for the indicated times, cells were harvested and fixed with paraformaldehyde (PFA) or processed for the metaphase spread preparation according to our previously published protocol (Shimizu et al., 2001b).

**FISH and the detection of incorporated thymidine analogs**

FISH and the detection of incorporated thymidine analogs by indirect immunofluorescence were performed according to a modified version of our previously published protocol (Shimizu et al., 2001b). Digoxigenin (DIG)- or biotin-labeled plasmid probes were prepared by by PCR amplification of a 500-bp fragment encompassing the Col \(E1\) sequence, using the linearized pSFVdhfr plasmid DNA as a template and DIG-dUTP or biotin-dUTP. We found that a probe of 500-bp length was enough to detect the HSR and that longer probes interfered with the detection of the incorporated halogenated thymidine analog by hybridizing with a longer region. A DIG-labeled \(Alu\) probe was generated by PCR using the same protocol as previously described (Shimizu et al., 2001b). A DIG-labeled micronuclei probe was prepared using DNA in the purified micronucleus as a template. DMs were selectively incorporated into the micronuclei formed at the cytoplasm. The selective incorporation into the micronuclei were related to the behavior of acentric DMs during the cell cycle (Tanaka and Shimizu, 2000). We have developed a method to purify the micronuclei and showed that their DNA stained the amplified sequences on the DMs (Shimizu et al., 1996). In this study, the purification of micronuclei from parental untransfected COLO 320DM cells (Shimizu et al., 1996), the preparation of DIG-labeled probes (Shimizu et al., 2001b) and the hybridization (Shimizu et al., 2001b) were performed according to the previously published protocols.

After the hybridization, the slides of metaphase-chromosome-spread or PFA-fixed cells were blocked with 3% Block Ace (Dainippon Pharmaceuticales, Osaka, Japan) in 0.2% Tween 20 and 1×SSC for 30 minutes at 37°C. The hybridized DIG-probe was detected with an FITC-conjugated goat anti-DIG polyclonal antibody Fab fragment (Roche Diagnostics, Basel, Switzerland), and the hybridized biotin-probe was detected with Alexa Fluor 594- or Alexa Fluor 647-conjugated streptavidin (Molecular Probes, Eugene, OR). The slides were incubated with the antibody or streptavidin (at 10 \(\mu\)g/ml) and 1% Block Ace in phosphate buffered saline (PBS) for 1 hour at 37°C, and then extensively washed with PBS and 0.05% Tween 20 in PBS.
The incorporated BrdU was detected with a mouse monoclonal anti-BrdU antibody (5 μg/ml) (Roche Diagnostics) and Alexa Fluor 568-conjugated goat antimouse IgG (10 μg/ml) ( Molecular Probes) in Block Ace (1% in PBS). For the double pulse-chase experiment, using IdU and CldU, the incorporated CldU was detected with a monoclonal rat anti-BrdU antibody (6 μg/ml) (OBT0030; Oxford Biotechnology); IdU was detected with a monoclonal mouse anti-BrdU antibody (1.5 μg/ml) (Becton Dickinson, catalog number 347580), both antibodies were diluted in Block Ace (1% in PBS). The rat antibody binds to both BrdU and CldU but not to IdU, whereas the mouse antibody strongly binds to BrdU and IdU but only weakly to CldU. Therefore, we first incubated the samples with rat antibody for 1 hour at 37°C to detect CldU, and then with mouse antibody for 1 hour at 37°C to detect IdU. After the primary antibody reaction, we washed the slides with a high-salt buffer (0.5 M NaCl, 0.5% Tween 20, 36 mM Tris-HCl, pH 8.0) to remove non-specifically bound mouse anti-BrdU antibody (Tanaka and Watanabe, 2003). The bound rat or mouse primary antibody was detected with Alexa Fluor 488-conjugated donkey anti-rat IgG (H+L) (Molecular Probes) or Alexa Fluor 594-conjugated F(ab´2) fragment of goat anti-mouse IgG (H+L) (Molecular Probes). The slides were incubated with 10 μg/ml of these antibodies in 1% Block Ace in PBS for 1 hour at 37°C, and then washed extensively with PBS and 0.05% Tween 20 in PBS.

Images from the metaphase chromosomal spreads were obtained using a Leica DC300F cooled CCD camera equipped with a Zeiss Axiosvert 135 microscope and a 100× objective (Plan-Neofluor, 1.30, oil) (Zeiss). The images from the PFA-fixed cells were obtained with a BioRad MRC600 confocal system using the same microscope and objective. The acquired digital images were merged using Adobe Photoshop version 4.0J.

Results

The plasmid repeat sequence in the HSR is replicated at the last stage of S phase

We examined the time point of replication of a plasmid repeat by using cells that had the repeat at the HSR (clone 22) (Shimizu et al., 2003). The logarithmically growing cultures were pulse-labeled for 30 minutes with BrdU, to specifically label the replicated DNA, and were chased in fresh medium for several lengths of time (Fig. 1A). After harvesting the cells, we prepared metaphase chromosomal spreads, and simultaneously detected the incorporated BrdU and the plasmid sequences. When the cells were chased for 4 hours, the metaphase cells represented those cells that were in late S phase at the time of BrdU pulse-labeling. At that time, the incorporated BrdU was detected at the HSR (Fig. 1B). However, when cells were chased for 12 hours, metaphase cells represented those cells that were in early S phase at the time of BrdU labeling, and the BrdU signal was never detected at the HSR (Fig. 1C). We counted the number of metaphase cells containing BrdU-labeled HSRs and the number of metaphase cells containing BrdU-labeled chromosomes by examining more than 20 metaphase cells at each time point (Fig. 1D). The chromosomal BrdU labeling increased between 2 and 6 hours after the release, which showed that the length of G2 phase was about 4 hours in this culture (see open circles in the figures). By contrast, the
fraction of BrdU labeling decreased to 50% after 14 hours, corresponding to the time from the start of S phase to metaphase (G2 plus S phase). The BrdU labeling at the HSRs peaked at about 4-6 hours (Fig. 1D) suggesting that the plasmid repeats in HSR were replicated during late S phase.

To determine exactly when HSR replication takes place, we compared the time point of replication of HSR relative to that of centromeres and telomeres, which are known to replicate late. We did the same pulse-chase experiment as shown in Fig. 1B-D, except for fixing the chase time at 4 hours. The HSRs in a total of 86 metaphases were sorted into seven groups, according to the BrdU labeling-pattern at the HSR, at the centromere, and at the telomere on the long arm and short arm (Fig. 1F). The incidence of each pattern is noted in Fig. 1E. In pattern 1, the HSR had not been replicated whereas telomeres and other late-replicating chromatin had been. Replication of the HSR and the centromere then began and continued sequentially (patterns 2 to 5). After the other heterochromatin had finished replicating (pattern 6), replication of the HSR continued and the S phase finished (pattern 7). Therefore, all HSRs were replicated at the final stage of S phase.

The transfected plasmid had a replication origin from the DHFR locus normally replicated at early S phase. Therefore, our data suggest that the time point of replication was not primarily determined by the sequence information, but rather by the chromatin context. Consistent with the late replication, HSRs in these cells appeared highly condensed (resembling heterochromatin) as reported for artificial HSR (Li et al., 1998). Gene expression from the plasmid-encoded gene was almost entirely suppressed (N.S. and K.S., unpublished data obtained by using

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**Fig. 2.** The HSR is composed of a homogeneous array of transfected plasmid sequences. (A-A') Metaphase spreads prepared from clone-22 cells were simultaneously hybridized with the DIG-labeled plasmid probe and the biotinylated Alu probe; the hybridized probes were detected with different fluorescent colors. DNA was counterstained with DAPI (A'). The plasmid probe brightly and uniformly stained the HSR (two-headed arrow). (A'') At the HSR, the Alu probe never produced a signal, whereas it brightly labeled highly repetitive Alu sequences on the chromosomes. The signal appeared in bands at the chromosomes, because the Alu sequences were concentrated in the R-band (Matera and Ward, 1992). (B-B'') The metaphase was simultaneously hybridized with the DIG-labeled DM-painting micronuclei probe and the biotin-labeled plasmid probe. (B') The first probe hybridized with the amplicon already present in the parental COLO 320DM cells, and it painted the DMs in clone-22 cells (compare arrowheads in B and B''), but it never hybridized to the HSR (compare two-headed arrow in B and B''). The DM-derived sequence flanking the HSR is indicated by an arrow (B'').

**Fig. 3.** The HSR shows reproducible replication bands that depend on the HSR length. Clone-22 (A,B,E,F) and clone-24 cells (C,D,G,H) were analyzed in the same experiment as shown in Fig. 1H. The replication sites pulse-labeled in late S phase appeared as bands in the mitotic HSR. The arrows in A to D indicate the positions of the bands. The number of bands appeared to be proportional to the length of the HSR. A portion of the clone-22 and clone-24 cells had pairs of homologous HSRs. Such pairs within a single cell had similar replication bands (E-H). In E, the orientation of the HSR is shown as two arrows. For panels G and H, two microscope fields of the identical metaphase cell were digitally assembled to form one panel.
competitive RT-PCR), which might be owing to the 'repeat-induced gene silencing' (McBurney et al., 2002).

The HSR is composed of a homogeneous array of plasmid repeats
Using genomic Southern blot hybridization and fiber FISH, we previously found that the HSR in clones 22 and 24 is composed of a continuous array of plasmid sequences (Shimizu et al., 2003). To further confirm the homogeneity of the HSR, we next examined the HSR using dual-color FISH. We simultaneously hybridized an Alu probe and the plasmid probe to metaphases prepared from clone-22 cells, and detected the hybridized probes using secondary antibodies with different fluorescent colors. The transfected plasmid did not contain the Alu sequences, but the plasmid probe brightly and uniformly stained the HSR (Fig. 2A). The Alu probe did detect the highly repetitive sequences in the normal chromosomal arm but did not produce any signal at the HSR, indicating that there are no detectable genomic sequences in the HSR. We then simultaneously hybridized the DM-specific probe and the plasmid probe to the metaphase spreads (Fig. 2B). The former probe was prepared from DNA in the purified micronuclei from the parental COLO 320DM cells, and it specifically hybridized with the amplified sequences on DMs that were present in the untransfected cells (see also Materials and Methods). The probe did not produce any hybridization signal inside the HSR, whereas it did stain the DMs appearing in the same metaphase. The probe produced a signal in the region flanking the HSR, which suggests that the HSR was generated by a mechanism involving the recombination between the plasmid sequences and the DMs (Shimizu et al., 2001a). Essentially the same result was obtained for clone 24 (data not shown). Therefore, considering also our previously published results, we concluded that the HSR was composed only of plasmid repeats.

The HSR has reproducible replication bands that depend on its length
As we had predicted, we were able to detect replication bands even in an HSR composed of a homogeneous array of plasmid sequences (Fig. 1F, patterns 2, 3, and 6). However, the length of the HSR was variable among these monoclonal cells, because the BFB cycle was still ongoing (Shimizu et al., 2003). Examination of the variously sized HSRs suggested that the number of replication bands was almost proportional to the length of the HSR (Fig. 3A-D). Furthermore, these images suggest that the spacing of each band is constant.

Whereas most of the clone-22 and clone-24 cells had one chromosome with one HSR, a portion of the cells contained two homologous chromosomes with two homologous HSRs,
probably products of chromosomal non-disjunction. Within such pairs of homologous HSRs, the pattern of the replicated sites appeared to be quite similar (Fig. 3E-H), suggesting that the replication of the HSR proceeded in a pre-defined manner and not in a stochastic manner.

Sequential alteration of the replication bands, analyzed with a double pulse-chase experiment

The progression of the replication sites in the S-phase nucleus should reflect alterations of the replication bands during mitosis. Therefore, we performed a double pulse-chase experiment using IdU and CldU to analyze the progression of the replication sites. The procedure is shown in a diagram in Fig. 4A. Logarithmically growing clone-22 cells were pulse-labeled with IdU, chased for varying lengths of time, pulse-labeled with CldU, and further chased in fresh medium. The cells were harvested 4 hours after the IdU-labeling, and the incorporated IdU and CldU, as well as the HSR, were simultaneously detected in metaphase spreads (Fig. 4C-I). To determine the time required for the replication of the entire HSR, we examined several interval times between IdU and CldU labeling, and obtained the frequencies of HSRs that were labeled with both IdU and CldU in each case. As expected, the frequencies decreased as the interval time increased (Fig. 4B).

At an interval of 100 minutes, the HSR still had both IdU and CldU labeling (Fig. 4C). In this interval time, both labels appeared as a limited number of bands. At the 120-minute interval, only one out of 20 HSRs had both labels, and the signal from each label was very weak (Fig. 4B, parenthesized number). Therefore, we conclude that replication of the entire HSR requires between 100 minutes and 120 minutes.

To understand the chronological order of HSR replication, we next examined the images obtained when intervals between IdU and CldU labeling had been shorter. After initiation of HSR replication, the CldU signal appeared as bands (Fig. 4D and E) 20 and 40 minutes after IdU labeling, which produced little or no signal. However, bands detected at the last stage of the HSR replication disappeared within 40 minutes after IdU labeling (Fig. 4I). The CldU bands were sustained or only slightly shifted during short interval times (Fig. 4H). Between early and late HSR replication, the replicated sites appeared as small granules scattered throughout the HSR (Fig. 4F). From this smeared pattern, the replication bands re-appeared after a 40-minute chase (Fig. 4G). Therefore, during the 120 minutes of HSR replication, the replicated sites initially appeared as bands, became scattered and then reappeared as bands.

The globular HSR domain is replicated from the periphery to the interior within the late S-phase nucleus

We next examined how the HSR is replicated by directly analyzing the S-phase nucleus. Initially, clone-22 cells were pulse-labeled with BrdU and immediately fixed with PFA. We detected the HSR and the incorporated BrdU simultaneously by using confocal microscopy. When the HSR was associated with a BrdU signal, it was within a globular subchromosomal domain inside the nucleus (Fig. 5A-C). These cells were apparently in late S phase, because the BrdU signal was detected at only a few locations in the internal large heterochromatin islands. Serial confocal images of 0.68-μm intervals in the z-axis were obtained for each HSR domain (right panels). There were three types of BrdU-labeling: (A) at the peripheral region, (B) at the whole domain and (C) at the internal region. Bars, 5 μm.
Cells were then immediately fixed with PFA and examined using confocal microscopy. Representative images are shown in Fig. 6. If the HSR incorporated both IdU and CldU, the IdU-labeled sites (red) were always outside of the CldU-labeled sites (green). Therefore, the replication site moved from the surface to the internal region of the HSR-domain during the double pulse-labeling. From these images alone, it might be conceivable that the replication sites are fixed at the interior and that the chromatin move inside the domain. However, the images of BrdU labeling in Fig. 5A and B exclude this possibility because the HSR domain was replicated either at the periphery or the whole domain at the time of fixation. Therefore, we concluded that the chromatin did not move substantially, but the replication was initiated sequentially from the periphery to the internal sites within the HSR domain. The shape of the HSR domain in the confocal optical section was circular (Fig. 6B; in eight out of 20 nuclei examined), oval (Fig. 6A; in six out of 20 nuclei examined), or rod-shaped (Fig. 6C; in six out of 20 nuclei examined). Because the shape appeared to be independent of the replication stage that was revealed by the IdU and CldU labeling, the shape in the optical section appeared to reflect the orientation of nuclei. The three-dimensional shape might therefore be closer to a disk than to a sphere.

Shape of the HSR domain in the late G2 phase nucleus
We have examined how the HSR domain is replicated in late S phase and how the replicated sites appear in the mitotic chromosome, and have further shown how the HSR appeared between these stages. While examining the cells in the double pulse-chase experiment (Fig. 4), we detected either IdU or CldU at the HSR in some interphase cells. These cells should have been in late G2 at the time of harvest because we chased the cells for 4 hours after the IdU labeling, and the HSR is replicated at the end of S phase. In such nuclei, the HSR typically appears as a ring (Fig. 7A) or a bar (Fig. 7B), that look like a coiled spiral and a pair of extended spirals, respectively. Among 12 HSRs labeled with either IdU or CldU, three HSRs were the former shape, and nine were the latter shape. It is possible that the coiled spiral structure might unfold to the extended pair of spirals, because the former shape was frequently labeled only with CldU (two out of three cells) whereas the latter shape was labeled with only IdU (seven out of nine cells). However, the precise chronological sequence of the HSR-shape at G2 phase should be clarified in the future study using, for example, the fluorescently labeled HSR in living cells.

Discussion
In this study, we examined whether replication bands are derived from sequence-specific information, or whether they are secondary to the three-dimensional folding of the chromosome. Sites that are replicated during S phase appeared as replication bands in the mitotic chromosome when pulse-labeled, and are usually related to the G/R bands in the normal chromosome arm. However, the fact that these bands can be detected in the homogeneously repeated plasmid array strongly argues that the replication bands are generated independently of sequence information. As described above, the replication bands might originate as a simple reflection of how the chromatin is folded and replicated inside the nucleus, and any association of G/R-band sequence bias with replication bands might be a consequence of evolutionary adaptation. Therefore,
replication bands in the HSR might manifest the fundamental nuclear architecture of mammalian cells.

The HSR appears as a globular subchromosomal domain while being replicated (Figs 5, 6). This is consistent with a previous report on a similar artificial HSR (Li et al., 1998). Within the HSR domain, our double pulse-chase experiment using IdU and CldU showed that replication was initiated sequentially from the periphery to the internal sites (Figs 5, 6). This finding suggests that the HSR-chromatin is most probably folded to go in and out of the domain periodically. As a result, replicated sites would appear at defined times as bands in the subsequent mitotic chromosome, where the chromatin is linearly extended and tightly packed. Such periodicity of folding seems to be constant within homogeneous chromatin, because the width and spacing of the bands in the mitotic chromosome appear to be constant and the number of bands was almost proportional to the length of the HSR (Fig. 3A-D).

Folding with a constant periodicity is most probably explained by symmetrical folding, such as a coiled-coil structure (Fig. 8). The model hypothesizes a giant HSR coil, which is further coiled to form a disk-shaped or a ball-shaped structure, consistent with the confocal analysis of the HSR-domain (Figs 5, 6). The coiled-coil structure might appear as the structure looking like a coiled spiral and a pair of extended spirals at late G2 phase when the chromatin partially condenses (Fig. 7). Yokota et al. proposed the 'random walk/giant loop model' for the general chromosome arm in G0-G1 nuclei, and predict the giant loop to be several Mb in size (Yokota et al., 1995). Assuming that one turn of the coil in our model corresponds to the giant loops of their model, the two models might be topologically compatible.

In our model, replication proceeds from the outside to the inside of the coiled-coil domain, leading to the appearance of mitotic replication bands. As noted above, this model is
consistent with our observation that the number of replication bands is almost proportional to the length of the HSR. That a pair of homologous HSRs within a single cell shows similar replication bands (Fig. 3E–H) also supports the model, because it implies that HSRs of similar length are folded and replicated similarly at a defined stage in S phase. Furthermore, our data show that the replication bands are most evident at the initial and the final stage of HSR-replication, whereas replicated sites appear to be scattered in the HSR during the middle stage (Fig. 4). This result matches well the prediction of our model (Fig. 8 and see below). However, the initial appearance of the bands was not a synchronous event, because a number of bands appeared earlier than others (Figs 3F–H, 4D). It is currently unclear what mechanism initiates the replication of a specific loop, but it might be related to the orientation of the HSR domain inside the nucleus.

Topologically, the coiled-coil structure suggests that the chromatin is loosened at the periphery of the domain, which might be a reason why replication preferentially initiates at the periphery. This idea seems to be consistent with the report that active and inactive chromatin were differentially stained by an anti-acetylated histone antibody, and alternated along the oncogenesis-associated HSR in a mouse melanoma cell line (Nicol and Jeppesen, 1996). The acetylated active chromatin might correspond to the peripheral loosened region, because active chromatin is usually loosened. At the middle stage of HSR replication, the replication sites do not appear as bands but are scattered (Fig. 4), which is consistent with our model (Fig. 8B). However, considering the topology, the internal region of the coiled-coil structure is probably tightly packed, and might need to be unfolded during its replication (Fig. 8C). Using similar artificial chromatin, Li et al. showed that the HSR was initially replicated in a loose ball-shaped structure and then in a more extended fibrous structure (Li et al., 1998). The first structure corresponds well to our observations, whereas we were not able to find the second one. The above suggests that to completely unfold the coil at the end of HSR-replication would produce a fibrous structure, whereas our HSR might be only partially unfolded (Fig. 8C). This discrepancy might be explained by differences in the HSR-structure at the molecular level.

Our previous Southern blot analysis showed that clones 22 and 24 had about 10,000 copies of the 11-kb PSFVdhfr plasmid sequence per cell (Shimizu et al., 2003). Considering that the cells at G2 phase had the duplicated HSR and that a portion of cells had pairs of homologous HSRs, we estimated the length of the HSR in these clones to be about 80 Mb. An HSR of average size in these clones showed seven to ten replication bands (Figs 1, 3, and 4); therefore, one band approximately corresponds to 10 Mb. If the 30-nm fiber was directly folded as a coiled-coil structure, a length of 10 Mb is too long for one turn because the HSR-domain is 2.3 μm in diameter (Figs 6, 7). Therefore, there should be an intermediate folding structure between the 30-nm fiber and the coiled-coil structure. We have drawn the hypothetical replicon clusters that might exist in such an intermediate structure in the diagram of our model shown in Fig. 8. One replicon cluster might approximately correspond to 1 Mb (Berezney et al., 2000; Berezney, 2002). Furthermore, previous works have characterized 1Mb domains as units of higher-order chromosome organization and coordinated replication (Zink et al., 1998; Zink et al., 1999).

Therefore, our coiled-coil model might correspond to the spatial arrangement of such domains.

We have developed a model explaining how an artificial HSR composed of plasmid repeat sequences is folded and replicated in the nucleus. Many of the large pulse-labeled late-S-phase replication-foci have a ring- or horseshoe-like appearance (Nakayasu and Berezney, 1989; O’Keefe et al., 1992), which is very close to our observation that the replication sites initially surround the HSR domain (Figs 5, 6). Therefore, the model might be applicable to late-replicating heterochromatin in general, and the analysis of a simple repeat structure might contribute to our understanding of fundamental nuclear structure.

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References

Berezney, R. (2002). Regulating the mammalian genome: the role of nuclear architecture. Adv. Enzyme Regul. 42, 39-52.
Berezney, R., Dubey, D. D. and Huberman, J. A. (2000). Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. Chromosoma 108, 471-484.
Boron, A. (2003). Replication banding patterns in the spined loach, Cobitis taenia L. (Pisces, Cobitidae). Genetica 119, 51-55.
Cook, P. (1998). Duplicating a tangled genome. Science 281, 1466-1467.
Furey, T. S. and Haussler, D. (2003). Integration of the cytogenetic map with the draft human genome sequence. Hum. Mol. Genet. 12, 1037-1044.
Gardiner, K. (1995). Human genome organization. Curr. Opin. Genet. Dev. 5, 315-322.
Knuttila, S., Bjorkqvist, A. M., Autio, K., Tarkkanen, M., Wolf, M., Monni, O., Szymanska, J., Larramendi, M. L., Tapper, J., Pere, H. et al. (1998). DNA copy number amplifications in human neoplasms – review of comparative genomic hybridization studies. Am. J. Pathol. 152, 1107-1123.
Lercher, M. J., Urrutia, A. O., Pavlicek, A. and Hurst, L. D. (2003). A unification of mosaic structures in the human genome. Hum. Mol. Genet. 12, 2411-2415.
Li, G., Sudlow, G. and Belmont, A. S. (1998). Interphase cell cycle dynamics of a late-replicating, heterochromatic homogeneous staining region: precise choreography of condensation/decon densation and nuclear positioning. J. Cell Biol. 140, 975-989.
Matera, A. G. and Ward, D. C. (1992). Oligonucleotide probes for the analysis of specific repetitive DNA sequences by fluorescence in situ hybridization. Hum. Mol. Genet. 1, 535-539.
McBurney, M. W., Mai, T., Yang, X. and Jardine, K. (2002). Evidence for repeat-induced gene silencing in cultured Mammalian cells: inactivation of tandem repeats of transfected genes. Exp. Cell Res. 274, 1-8.
Munkel, C., Elhs, R., Dietzel, S., Zink, D., Mehring, C., Wedemann, G., Cremer, T. and Langowski, J. (1999). Compartmentalization of interphase chromosomes observed in situ and experiment. J. Mol. Biol. 285, 1053-1065.
Nakayasu, H. and Berezney, R. (1989). Mapping replicational sites in the euchromatic cell nucleus. J. Cell Biol. 108, 1-11.
Nicol, L. and Jeppesen, P. (1996). Chromatin organization in the homogeneously staining regions of a methotrexate-resistant mouse cell line: interspersion of inactive and active chromatin domains distinguished by acetylation of histone H4. J. Cell Sci. 109, 2221-2228.
Niimura, Y. and Gojobori, T. (2002). In silico chromosome staining: reconstruction of Giemsa bands from the whole human genome sequence. Proc. Natl. Acad. Sci. USA 99, 797-802.
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 alpha-satellite DNA sequences. J. Cell Biol. 116, 1095-1110.
Parada, L. and Misteli, T. (2002). Chromosome positioning in the interphase nucleus. Trends Cell Biol. 12, 425-432.
Razin, S. V. (1999). Chromosomal DNA loops may constitute basic units of
the eukaryotic genome organization and evolution. Crit. Rev. Eukaryot. Gene Expr. 9, 279-283.
Saccom, S., Federico, C. and Bernardi, G. (2002). Localization of the gene-richest and the gene-poorest isochores in the interphase nuclei of mammals and birds. Gene 300, 169-178.
Schmid, M. and Guttenbach, M. (1988). Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 97, 101-114.
Scah, M. (1999). Oncogene amplification in solid tumors. Semin. Cancer Biol. 9, 319-325.
Shimizu, N., Kanda, T. and Wahl, G. M. (1996). Selective capture of acentric fragments by micronuclei provides a rapid method for purifying extrachromosomally amplified DNA. Nat. Genet. 12, 65-71.
Shimizu, N., Miura, Y., Sakamoto, Y. and Tsutsui, K. (2001a). Plasmids with a mammalian replication origin and a matrix attachment region initiate the event similar to gene amplification. Cancer Res. 61, 6987-6990.
Shimizu, N., Ochi, T. and Itonaga, K. (2001b). Replication timing of amplified genetic regions relates to intranuclear localization but not to genetic activity or G/R band. Exp. Cell Res. 268, 201-210.
Shimizu, N., Hashizume, T., Shingaki, K. and Kawamoto, J. K. (2003). Amplification of plasmids containing a mammalian replication initiation region is mediated by controllable conflict between replication and transcription. Cancer Res. 63, 5281-5290.

Sparvoli, E., Levi, M. and Rossi, E. (1994). Replicon clusters may form structurally stable complexes of chromatin and chromosomes. J. Cell Sci. 107, 3097-3103.
Tanaka, T. and Shimizu, N. (2000). Induced detachment of acentric chromatin from mitotic chromosomes leads to their cytoplasmic localization at G1 and the micronucleation by lamin reorganization at S phase. J. Cell Sci. 113, 697-707.
Tanaka, T. and Watanabe, T. (2003). Spatiotemporal sites of DNA replication in macro- and micronuclei of the ciliate Paramecium caudatum. Chromosome Res. 11, 153-164.
Watanab, Y., Fujiyama, A., Ichiba, Y., Hattori, M., Yada, T., Sakaki, Y. and Ikemura, T. (2002). Chromosome-wide assessment of replication timing for human chromosomes 11q and 21q: disease-related genes in timing-switch regions. Hum. Mol. Genet. 11, 13-21.
Yokota, H., van den Engh, G., Hearst, J. E., Sachs, R. K. and Trask, B. J. (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.
Zink, D., Cremer, T., Saffrich, R., Fischer, R., Trendelenburg, M. F., Ansgor, W. and Stelzer, E. (1998). Structure and dynamics of human interphase chromosome territories in vivo. Hum. Genet. 102, 241-251.
Zink, D., Boerfleth, H., Vissler, A., Cremer, C. and Cremer, T. (1999). Organization of early and late replicating DNA in human chromosome territories. Exp. Cell Res. 247, 176-188.