

DNA replication-dependent intranuclear relocation of double minute chromatin

Nobuo Itoh and Noriaki Shimizu*

Faculty of Integrated Arts and Sciences, Hiroshima University, 1-7-1 Kagamiyama, Higashi-hiroshima, 739-8521, Japan

*Author for correspondence (e-mail: shimizu@ipc.hiroshima-u.ac.jp)

Accepted 10 September; published on WWW 28 October 1998

SUMMARY

Double minutes (DMs) seen in a substantial fraction of human tumors are the cytogenetic manifestation of gene amplification which renders the tumor cells advantageous for growth and survival. DMs are acentric and atelomeric chromatin composed of circular DNA. In this study, we found they showed a remarkable relocation inside the nucleus which was spatially and temporally coupled to DNA replication. Using the human COLO 320DM tumor line, we detected DMs by fluorescence in situ hybridization followed by confocal examination. The location of multi-copy DMs was evaluated statistically by an easy method developed in this study. By examination of a synchronized culture, we found that DMs preferentially located at the nuclear periphery during G₁-phase of the cell cycle, which is consistent with the location at M-phase. The peripheral DMs were in contact with the nuclear lamina which was shown by the simultaneous detection of DMs and lamin protein. The peripheral location persisted until the cells

reached the G₁/S-boundary, then the DMs relocated promptly to inward once the DNA replication started. The relocation was obvious using two different probes that detect DMs, or using two different methods for the cell fixation. Furthermore, the simultaneous detection of DMs and the site of DNA replication suggested that the inward relocation of peripheral DMs initiated just prior to the onset of DNA replication at the periphery. On the other hand, if the same amplified sequences were placed in a chromosome as an homogeneously staining region, they did not show any significant relocation during S-phase. From these and reported results, there may exist a generalized inward motion of some kind of chromatin that precedes the replication of their DNA. DMs might magnify the motion by their acentric, atelomeric or small circular nature.

Key words: Double minute chromatin, Location in nucleus, DNA replication

INTRODUCTION

Recent advances in cell biology have shown that the individual chromosomes in interphase nuclei occupy their own territories which are not intermixed each other. Most of the nuclear events such as DNA replication, transcription, splicing, DNA-damage repair or DNA recombination should be tightly related to the organization of the chromosomal territories (for recent reviews, see Strouboulis and Wolffe, 1996; Marshall et al., 1997a). Among these, DNA replication is surprising because it requires accurate replication of about 6×10^9 bp or 2 meters in length of human genome within a few hours inside a nucleus of only 10 μ m in a diameter. To achieve this, chromatin may actively be rearranged during the period of DNA replication. However, no clear evidence has been provided for such a replication-coupled movement of the entire or a portion of the chromosomal territories.

To date, studies dealing with the movement of chromatin inside the nucleus is not only limited, they are also somewhat contradictory. For example, recent study using living human and mouse cells concluded that there is no substantial movement of chromatin based on the absence of fluorescence recovery after photobleaching (Abney et al., 1997). This

conclusion is consistent with the photobleaching studies using isolated interphase nuclei (Selvin et al., 1990), or with the studies that showed centromeres were generally motionless in interphase HeLa cells (Shelby et al., 1996). Moreover, a recent report concluded that the chromatin undergoes significant but constrained motion similar to classical Brownian motion based on the observation in living yeast cells of a specific chromatin site tagged by green fluorescent protein, or based on the observation of microinjected fluorescently labeled topoisomerase II, which binds to heterochromatin in *Drosophila* embryonal cells (Marshall et al., 1997b). In contrast, other studies had shown that there is a substantial rearrangement of chromatin during interphase. These include curvilinear, three-dimensional movement of heterochromatin in interphase nuclei of living neuron cells (De and Mintz, 1986), and the occasional slow movement of a centromere in living HeLa cells (Shelby et al., 1996). The location and the rearrangement of centromeric regions was especially examined thoroughly by fluorescence in situ hybridization (FISH) or immunofluorescence. In general, centromeric sequences tend to localize to each of the perinucleolar or the nuclear peripheral regions. Such studies, employing several kinds of cell cycle-synchronized cells, showed the relocation of centromeric

regions from the nuclear periphery to the interior during the progression from G₁- to S-phase (Haaf and Schmid, 1989; Ferguson and Ward, 1992; Vourc'h et al., 1993; Tagawa et al., 1997). Quite recently, an elegant analysis on the homogeneously staining region (HSR) in living cells was reported (Li et al., 1998). The late replicating, heterochromatic HSR formed by the amplification of an introduced tandem array of lac-operator sequences showed both the decondensation and the movement from the nuclear periphery to the interior which preceded their DNA replication.

These rather disparate results on the motion of the chromatin inside the interphase nucleus might come from the analysis of the chromatin that was linked to a huge mass of chromosome that had many kinds of functional units which might constrain their motion. In this study, we focused our attention on the more simple and unique double minute (DM) chromatin body. DMs are heterogeneously sized, paired minute chromatin bodies seen in metaphases from a broad spectrum of human tumors including breast, lung, ovary or colon (for review see Benner et al., 1991). They represent a most common cytogenetic manifestation of gene amplification that is a major mechanism of oncogene activation or the acquisition of drug resistance. DMs usually do not possess a functional centromere because they lag behind the chromosomes at anaphase (Levan and Levan, 1978), are generally not stained by alpha satellite DNA, and are G- or C-band negative (Barker, 1982), with some exceptions (Hammond et al., 1994; Chen and Manuelidis, 1989). DMs of up to few mega-base pairs of DNA are considered to be circular based on ultrastructural observations (Hamkalo et al., 1985) or biochemical analyses (VanDevanter et al., 1990), and based on the absence of telomeres (Lin et al., 1990). The analysis of DMs has another advantage: the amplified sequences typically locate on DMs in biopsied specimens or in low passage culture of cell lines, whereas they generally integrate into specific chromosomal sites forming HSR as the *in vitro* passage increases (for a review see Wahl, 1989; for the COLO 320 cell line used in this study see Lin et al., 1985). Because these isogenic cells have the same amplified sequences as DMs on a chromosomal HSR, they can be used as an excellent control chromatin to DMs.

Previously, we showed that DMs tend to locate at the periphery of the nucleus in a randomly growing culture (Shimizu et al., 1998). The location inside the nucleus should be tightly related to the specific inclusion of DMs in nuclear buds or micronuclei formed at S-phase (Shimizu et al., 1996, 1998). In this study, we show that DMs are preferentially located at the nuclear periphery during G₁-phase, and are dramatically relocated to the nuclear interior which is spatially and temporally coupled to replication at the periphery.

MATERIALS AND METHODS

Cell culture and synchronization

Human COLO 320DM (CCL 220) and COLO 320HSR (CCL 220.1) neuroendocrine tumor cells (for their karyotype analysis see Lin et al., 1985) were obtained from the American Type Culture Collection and single cell subclones were obtained by limiting dilution (Von Hoff et al., 1988). The locations of amplified *c-myc* genes to DMs or HSRs were confirmed by FISH using *c-myc* cosmid DNA. The cells were grown in RPMI1640 medium supplemented with 10% fetal calf serum

(FCS). The cell cycle of the culture was synchronized by each of the following two procedures.

The synchronization from the G₁/S-boundary was performed as described (Shimizu et al., 1998) with some modifications. In brief, a logarithmically growing culture of COLO 320DM or COLO 320HSR was seeded at a cell density of 3×10⁵ cells/ml, and treated with 2 mM thymidine for 17 hours to arrest the cells at S-phase. The arrested cells were thoroughly washed and released for 12 hours in a fresh medium containing 25 μM of deoxycytidine (Sigma, St Louis, MO) to extrude all cells from S-phase. After release, aphidicolin (Sigma) was added to a final concentration of 2 μg/ml, and incubated for 17 hours to arrest the cells at the entry of next S-phase. The arrested cells were washed thoroughly and were synchronously released in fresh medium at a cell density of 5×10⁵ cells/ml.

The synchronization from prometaphase was performed by first arresting the cells at the entry of S-phase by the above protocol. The arrested cells were washed thoroughly and released for 6 hours in a fresh medium. After release, nocodazole (Sigma) was added to the culture at a final concentration of 0.4 μg/ml, and further cultured for 12 hours. The cells arrested at prometaphase were synchronously released in fresh medium. Both of the measurement of the uptake of [³H]thymidine to acid-insoluble fraction and the measurement of the mitotic index were performed as described previously (Shimizu et al., 1998).

FISH

The method for the isolation of nuclei and the fixation by paraformaldehyde (PFA) was developed based on that developed for human lymphocytes (Ferguson and Ward, 1992; Vourc'h et al., 1993), and described in our previous paper (Shimizu et al., 1998). In this study, we used essentially the same protocol as this one, except that the nuclear isolation buffer was changed to a more isotonic one. In brief, 10 ml of COLO 320DM cells were washed once in nuclear isolation buffer (NIB; 100 mM KCl, 5 mM Hepes-KOH, 10 mM MgSO₄, 3 mM dithiothreitol, pH 8.0), and suspended in 1 ml of pre-chilled NIB. To the suspension, 1 ml of NIB containing 0.5% Triton X-100 was added, mixed, and left to stand at 4°C for 10 minutes. The suspension was Dounce homogenized (loose fitting pestle, 10 strokes) at 4°C, and 3 ml of 5% PFA in phosphate buffered saline without divalent cations (PBS-) was added to the suspension. The tube was gently shaken for 15 minutes to fix the nuclei, and the reaction was terminated by the addition of 1/10 volume of 1 M Tris-HCl, pH 7.4, containing 2% BSA, followed by gentle shaking for 10 minutes. The suspension was washed twice in PBS- containing 3% FCS, and stored at 4°C for up to a few days. Cytocentrifugation on poly L-lysine (PLL)-coated glass slides, treatment with RNase A, hybridization with biotin-labeled probe, and the detection by FITC-conjugated streptavidin were done exactly as described (Shimizu et al., 1998).

In some experiments, the COLO 320DM cells were fixed directly and hybridized. In this case, the cells were cytocentrifuged on PLL-coated slides, rinsed with PBS- and fixed with 1.75% PFA in PBS- for 10 minutes at room temperature. The slides were washed once with PBS- and treated with ethanol/acetic acid (19/1) at -20°C for 3 minutes. The slides were washed once with PBS-, permeabilized by treatment with PBS- containing 0.5% Tween 20, and used for hybridization with the biotinylated probe in the same way as the isolated nuclei.

For most of the FISH shown in this report, the biotinylated probe was prepared from DNA in the purified micronuclei from COLO 320DM cells and used as a DM-painting probe. This probe exclusively stains DMs among metaphase spreads prepared from COLO 320DM cells with negligible signals on normal chromosomes, even though the signal was amplified by a biotin-avidin sandwich (Shimizu et al., 1996, 1998, see also Fig. 3A). If we used this probe, we did not employ the signal amplification strategy because a strong signal was obtained. In some experiments, *c-myc* cosmid DNA (Shimizu et al., 1996) was biotinylated and used as a probe that detects sequences

amplified on DMs. In this case we employed the signal amplification strategy.

Simultaneous detection of DMs and nuclear lamin protein was done based on the fact that the antigenicity of lamin resists the denaturation conditions used in the FISH procedure. Namely, the cells fixed directly by the above protocol were subjected to FISH to detect the sequences on DMs, which was followed by the indirect immunofluorescence detection of lamin using anti-lamin primary rabbit antibody and Texas Red-conjugated anti-rabbit IgG. The anti-lamin antibody that detects all three subtypes of human lamin was a kind gift from Dr Larry Gerace at Scripps Research Institute (CA, USA). Simultaneous detection of DMs and the site of DNA replication by using FISH and immunofluorescence that detects pulse-labeled BrdU, respectively, was done in the same way as already described (Shimizu et al., 1998). In brief, cells were pulse labeled with 10 μ M BrdU (Sigma) for 30 minutes just before the harvest of the cells. Nuclei were isolated and hybridized with the DM-painting probe as described above, and the site of DNA replication inside the nucleus was visualized by indirect immunofluorescence using anti-BrdU monoclonal antibody (Pharmingen, San Diego, CA) and rhodamine-conjugated anti-mouse Ig (Boehringer-Mannheim).

Images were obtained using a Bio-Rad MRC600 confocal system on a Zeiss Axiovert 135 microscope. Most images were obtained using a $\times 63$ objective (Zeiss, Apochromat, 1.40, oil), and zoom factor 2. The acquired digital images were expressed as pseudocolors, and merged using Adobe Photoshop version 2.0.1 or 3.0.5 (Adobe Systems Inc., Mountain View, CA).

Measurement of the location of DMs inside the nucleus and the statistical expression of the results

We intended to measure the location of DMs inside each nucleus and treat the data from many nuclei statistically. For this objective, we chose 2-dimensional focal planes for the analysis instead of the 3-dimensional reconstructed images which were usually employed in previous reports. Whereas the method employing 3-dimensional reconstruction is required for the localization of the specific chromatin

region, the procedure needed specialized computer software and a huge memory to treat the data from many nuclei statistically. Instead, the number of DMs present in the cells used in this study is so huge (43.6 \pm 27.6 in COLO 320DM; Shimizu et al., 1996) that the number of DM-specific FISH signals seen in a single optical plane with narrow depth was enough to be analyzed. Therefore, we concluded that the analysis in a 2-dimensional plane was sufficient for the evaluation of the location of such multiple chromatin bodies.

FISH using isolated nuclei and a DM-painting probe was performed as described above and coplanar PI (DNA) and FITC (hybridized signal) images intercepting the center of the nucleus were obtained from randomly chosen nuclei. The outline of the procedure is shown in Fig. 1. The digital images were merged using Adobe Photoshop 2.0.1, saved in TIFF format, opened in Canvas 3.0.5 (Deneva Software Inc., Miami, FL), and further merged with the pre-drawn lattice as shown in Fig. 1A. The lattice was drawn as follows: each nucleus was divided into 10 subregions according to the distance from the center. A series of concentric ovals each passing through the middle of each subregion were drawn. The lattice was composed of these ovals and 36 radial lines each of which is 10 degree rotating. The shape and the size of lattice was manually modified so that the outer edge of the lattice may fit the outer edge of the PI-image of the nucleus. For the measurement, we noted the lattice points that coincide with the FISH signal (Fig. 1A; bolded dots). From these data, we approximate the amount of DMs in each nuclear subregions as the hatched area shown in Fig. 1B. In actual fact, the number of bold dots in each of the subregions was multiplied by the ratio of the area of each subregion, expressed as $n^2-(n-1)^2 = 2n-1$, where n is the subregion number counted from the center. The amounts of DMs in each of the subregions that were calculated thus far were finally converted to a percentage by calling the total amount of signal that appeared in the measured nucleus as 100%. A graph obtained for a model nucleus (Fig. 1A) is shown in Fig. 1C. The theoretical random distribution line based on the area of each nuclear position is also shown in the same graph. Using this procedure, the actual results from some representative nuclei are given in Fig. 2 and show that the obtained

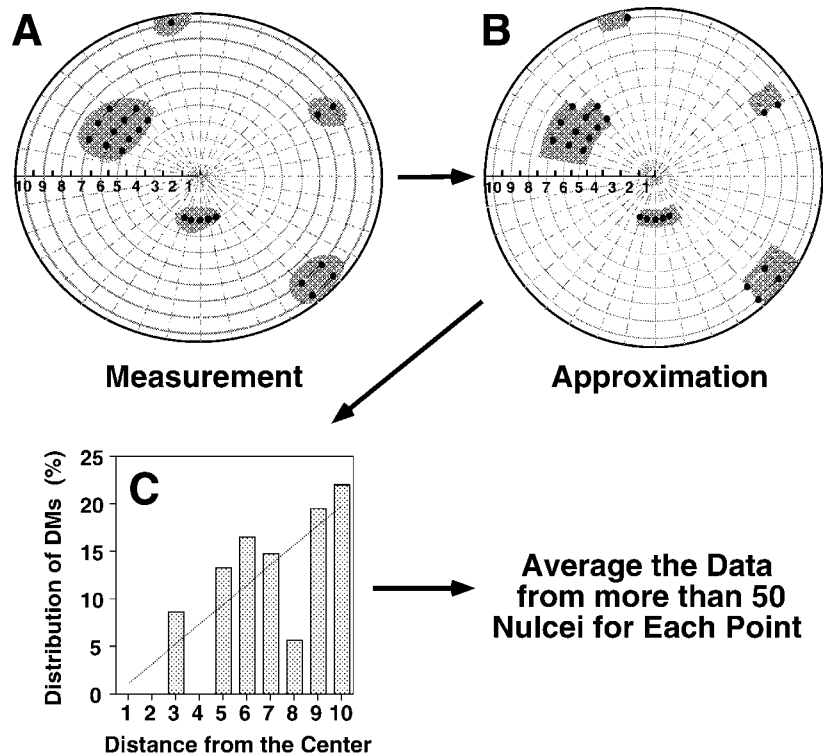
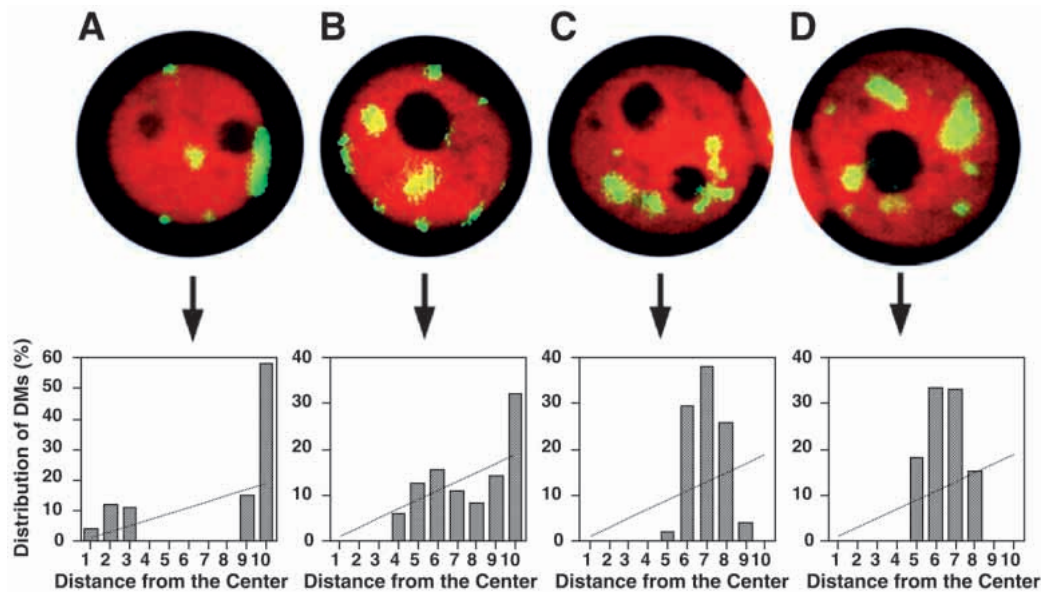


Fig. 1. How to estimate the location and amount of DMs inside the nucleus. The outlined procedure for the measurement and expression of the location of DMs inside the nucleus is illustrated. The theoretical random distribution line based on the area of each nuclear position is also shown in C. For a detailed explanation, see Materials and Methods.

Fig. 2. Measurement of the location of DMs in 4 representative nuclei. Nuclei from logarithmically growing COLO 320DM cells were hybridized with a DM-painting probe and the confocal sections that intercept the center of each nucleus were taken. In this figure, four nuclei (A to D) showing different localization of DMs are shown together with the results from the measurement described in Fig. 1 and Materials and Methods. The theoretical random distribution line based on the area of each nuclear position is also shown in the graph. The obtained graphs showed good representation of the original images.



graphs well represented the actual images. To express the location of DMs statistically, we obtained these results from 50 randomly chosen nuclei for each point, and simply averaged them.

RESULTS

Localization of DM sequences in the interphase nucleus

DMs in the human COLO 320DM tumor cell line were visualized by FISH using a probe prepared from DNA in purified micronuclei from the same cells. This probe exclusively paints DMs seen in metaphases of these cells, with no apparent background on normal chromosomes (Shimizu et al., 1996, 1998; see also Fig. 3A). By using this probe, the hybridized signal was so intense and specific that the entire chromatin territories of DMs were sharply illuminated in interphase nuclei. Fig. 3C shows an example of the image obtained from logarithmically growing COLO 320DM cells. The DM distribution displayed multiple patterns; i.e. DMs in some nuclei located preferentially at the periphery as reported previously (Shimizu et al., 1998), whereas they located to the interior or both the periphery and the interior in other nuclei. From this observation, we postulated that the multiple patterns seen in the localization of DMs may reflect the cell cycle-dependent relocation of DMs. To address this possibility, we examined the location of DMs in cell cycle synchronized COLO 320DM cells.

DMs preferentially locate at the nuclear periphery during G₁-phase

The COLO 320DM culture was synchronized by two successive rounds of treatment with aphidicolin which assembled almost all the cells at the G₁/S-boundary, followed by treatment with nocodazole which arrested the cells at prometaphase (see Fig. 4A and Materials and Methods). The cells were then synchronously released by replacing the medium with fresh medium. As shown in Fig. 4B, the mitotic index at time 0 (just before release) was 47%, and the viability

of the cells at 6 hours after release was 68% as determined by the Trypan Blue extrusion method. This indicated the level of synchronization achieved by this method was not so high, presumably because the nocodazole was somewhat toxic to the cells. Anyway, the mitotic index decreased drastically until 3 hours after release, then gradually decreased to nearly 0 at 12 hours after release. On the other hand, the uptake of [³H]thymidine to acid insoluble fraction was very low during the first 9 hours, then it increased and peaked at 21 hours after release. Therefore the cells from 3, 9, and 18 hours after release were considered to be enriched with the cells of early G₁-, late G₁- and S-phase, respectively, and were analyzed by FISH to examine the location of DMs. From these images, it was suggested that DMs were preferentially at the peripheral location during G₁-phase (a representative image is shown in Fig. 3E), whereas they located more inside at S-phase. To express this observation more quantitatively and statistically, we measured the location of DMs by the method described above. The results from 50 randomly chosen nuclei for each time point were averaged and expressed as the distribution of DMs in each of the nuclear subregion. Fig. 4C to E shows the results from such analysis. At both early G₁ (3 hours) and late G₁ (9 hours), DMs obviously located preferentially at the nuclear periphery. However, if the cells entered S-phase (18 hours), the localization drastically changed and the majority of DMs were detected more inside the nuclei. These results showed that DMs localized peripherally during G₁-phase and relocated to the interior during S-phase. We next examined the latter issue more precisely.

DMs move to the nuclear interior during progression through S-phase

The COLO 320DM cells were assembled at the G₁/S-boundary by two successive rounds of arrest at S-phase by aphidicolin (see Fig. 5A and Materials and Methods). The arrested cells were released into fresh medium and DNA synthesis was monitored by the uptake of [³H]thymidine. As shown in Fig. 5B, the DNA synthesis at time 0 (just before the release) was nearly zero, whereas it increased

Fig. 3. Detection of amplified sequences by FISH. Sequences amplified in COLO 320 cells were stained by hybridization with a biotinylated probe followed by detection of the hybridized probe with FITC-streptavidin. The probe exclusively stained many DMs seen in the metaphase from COLO 320DM cells (A; some DMs were arrowed). Arrowheads indicate a marker chromosome seen in COLO 320 cells. The probe hybridized to opposite arms of this metacentric chromosome, suggesting the presence of a large chromosomal inversion. The probe also exclusively stained HSRs in metaphases from COLO 320HSR cells (B; arrowed). The marker chromosome common to DM cells is indicated by an arrowhead. FISH using this probe detected the amplified sequences in PFA-fixed interphase nuclei. (C and D) Representative confocal images of nuclei from logarithmically growing cultures of COLO 320DM and COLO 320HSR cells, respectively. The representative confocal images of nuclei from a synchronized culture of COLO 320DM cells are shown in E to G. E is from the culture 9 hours after release from the nocodazole block and represents G₁-phase nuclei. F are from the culture arrested by aphidicolin and represent G₁/S-boundary nuclei. G are from the culture 3 hours after release from the aphidicolin block and represent mid-S-phase nuclei. Bars, 10 μ m.

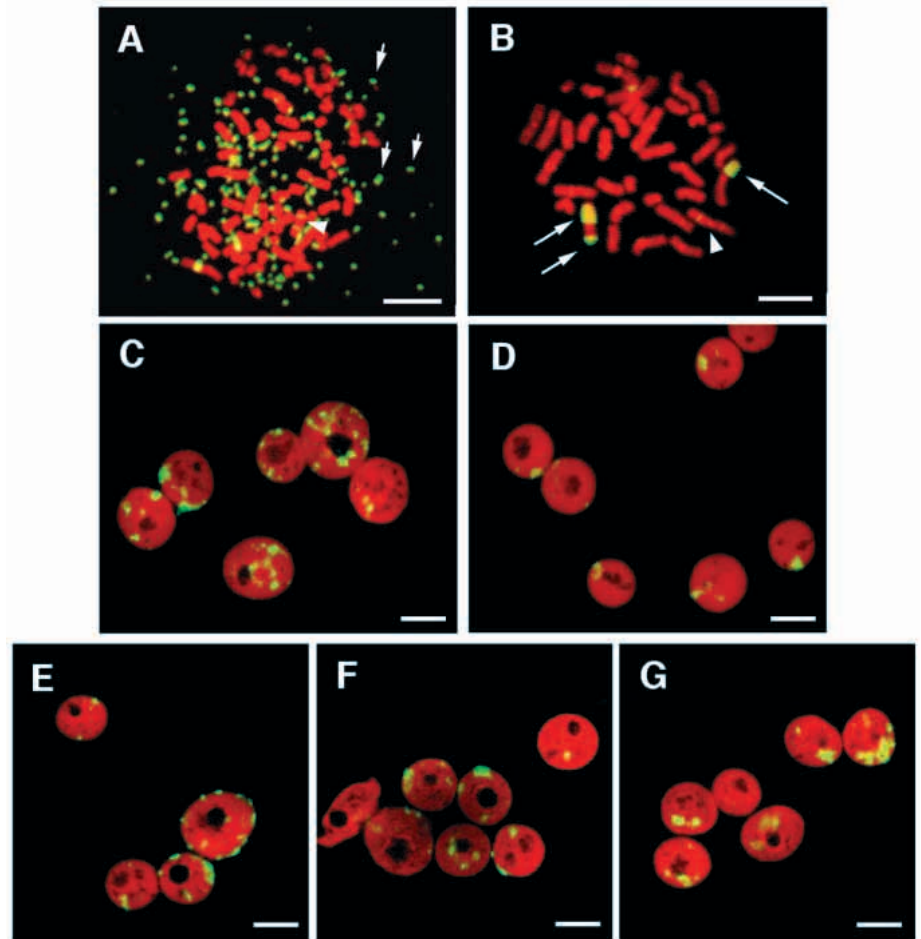
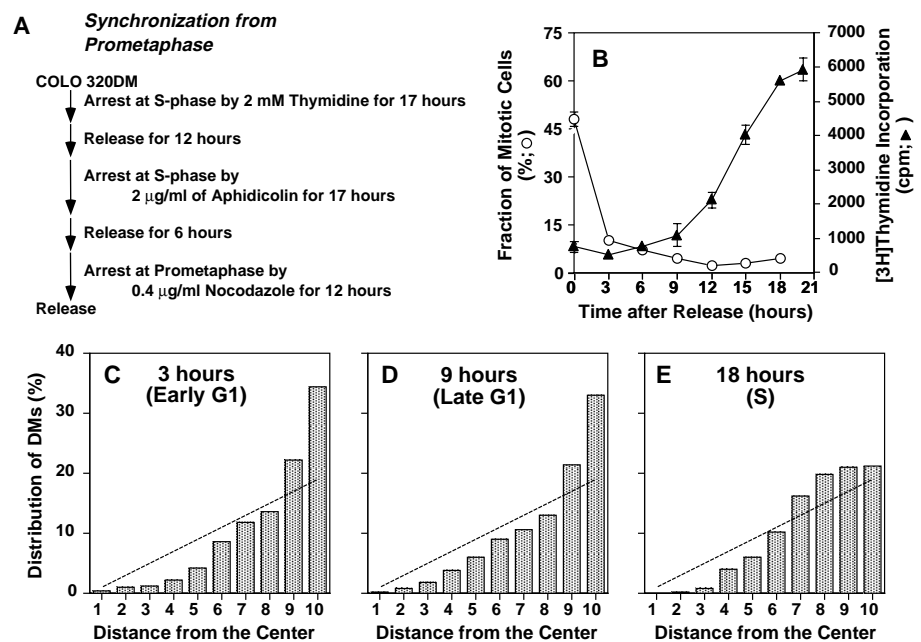


Fig. 4. DMs locate to the nuclear periphery at the G₁-phase of the cell cycle. COLO 320DM cells were synchronized from M-phase by the procedure outlined in A (for more detail, see Materials and Methods). The synchronous progression through the cell cycle was monitored by measuring the mitotic index and the incorporation of [³H]thymidine, and is shown in B. The nuclei from the culture of 3 (C), 9 (D), and 18 (E) hours after release from the nocodazole block, which represent early G₁-, late G₁-, and S-phase, respectively, were hybridized with the DM-painting probe as shown in Fig. 3C to G, and the location of DMs was measured as shown in Figs 1 and 2. The results from 50 randomly chosen nuclei per each time point were averaged and expressed in each graph. The theoretical random distribution line based on the area of each nuclear position is also shown.



dramatically after the release and peaked at 4 hours after release. The cell viability was more than 95%, and the level of synchrony determined by in situ detection of pulse-labeled

BrdU was at least 80% (see Fig. 8), indicating that a sufficient level of synchrony was achieved. The cells at 0, 1, 3 and 6 hours after release, which represented the G₁/S-boundary,

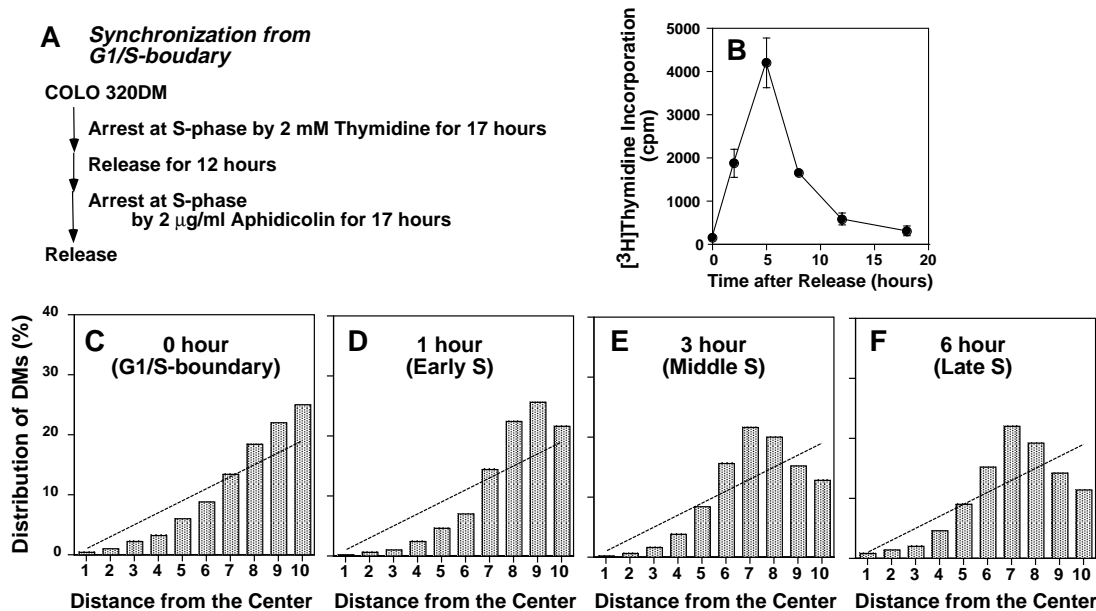


Fig. 5. DMs relocate to the nuclear interior after the start of DNA replication. COLO 320DM cells were synchronized from the G₁/S-boundary by the procedure outlined in A (for more detail see Materials and Methods). The synchronous progression through S-phase was monitored by the incorporation of [³H]thymidine and shown in B. A portion of the culture was removed at 0 (C), 1 (D), 3 (E), and 6 (F) hours after release from the aphidicolin block, which represents the G₁/S-boundary, early, middle and late S-phase, respectively. The nuclei from each time point were hybridized with the DM-painting probe and the location of DMs was measured and expressed as in Fig. 4. The theoretical random distribution line based on the area of each nuclear position is also shown.

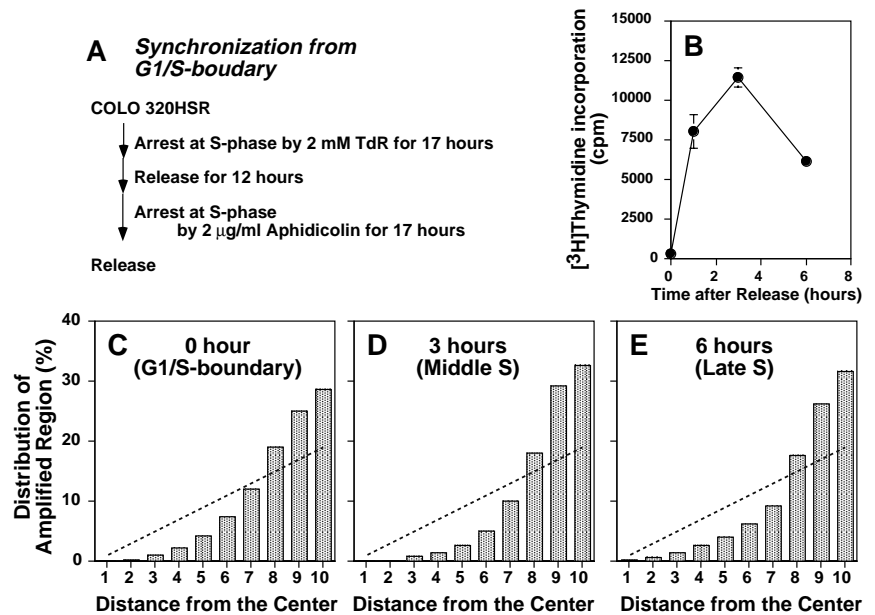
early, middle and late S-phase, respectively, were analyzed by FISH. The localization of DMs in 50 randomly chosen nuclei was measured and averaged as in Fig. 4. The results obtained (Fig. 5C to F) showed a drastic movement of DMs after the initiation of DNA-replication. At the G₁/S-boundary, DMs were located in a similar position as in G₁-phase (Fig. 4C,D), whereas they started to relocate at 1 hour after release, and moved more inward at 3 hours after release. The result from 6 hours was almost the same as for 3 hours. These results suggested that the relocation started at very early S-phase and finished in mid-S-phase.

The relocation of DMs thus observed may be due to either the small acentric nature of DMs or to the nature of amplified sequences on them. To address this issue, we next examined the position of HSR in nuclei from the COLO 320HSR cell line. The DM-painting probe prepared from purified micronuclei also brightly stained the HSR seen in the metaphases from COLO 320HSR cells (Fig. 3B). This indicated that the same amplified sequences as in DMs were placed at a chromosomal site, as anticipated. The HSR located on both arms of a large metacentric chromosome in most of the metaphases, which corroborates a previous report (Lin et al., 1985), as well as on one arm of a metacentric chromosome in a portion of the cell population. In the interphase nuclei from randomly growing COLO 320HSR cells, mostly a single large territory of HSR was detected by FISH (Fig. 3D). We synchronized these cells from the G₁/S-boundary in the same way as the DM cells and examined the localization of HSR-territory in the interphase nuclei. The incorporation of [³H]thymidine showed that a good synchronization was achieved as in the case of COLO 320DM (Fig. 6B). FISH and the statistical analysis of the location of

the amplified sequences was performed as previously performed for DMs. Fig. 6C to E shows the results. The distribution of the amplified sequences on HSR was somewhat higher at the nuclear periphery. However the distribution did not show any change during the progression through S-phase, which was previously seen if the same sequences were placed on DMs (Fig. 5).

We did these analyses using the PFA-fixed isolated nuclei that had been used in the previous studies to examine the location of centromeres in T-lymphocytes (Ferguson and Ward, 1992; Vourc'h et al., 1993). The method provided semi-spherically fixed nuclei that were easy to measure and evaluate the location of amplified sequences in 2-dimensions. Furthermore we used a DM-painting probe prepared from micronuclei purified from COLO 320DM cells. The probe produced a strong signal that paints the entire chromatin of DMs and was easy to analyze. However, to avoid the miss-conclusion which might come from the use of isolated nuclei or the DM-painting probe, we next examined the location of DMs by using intact cells fixed directly with PFA, and by using a *c-myc* cosmid probe which detects amplified sequences on DMs in these cells. Furthermore, the location of the nuclear membrane was detected by simultaneous staining of the nuclear lamin protein, a major constituent of lamina, by immunofluorescence. Some of the representative images were shown in Fig. 7. The results obtained from the cells arrested at the G₁/S-boundary showed that most of the cells have DMs at the most peripheral part of the nuclei, and that these DMs were just in contact with the nuclear lamina (Fig. 7A). As the cells progress through S-phase, the cells having DMs at such a remarkable position disappeared, and most of the cells had DMs more internally or apart from the

Fig. 6. Sequences amplified on chromosomal sites do not show a substantial relocation during DNA replication. COLO 320HSR cells were synchronized from the G_1/S -boundary using the same procedure as for COLO 320DM cells (A). The synchronous progression through S-phase was monitored by the incorporation of [3 H]thymidine and shown in B. A portion of the culture was removed at 0 (C), 3 (D), and 6 (E) hours after release from the aphidicolin block which represent G_1/S -boundary, middle and late S-phase, respectively. The nuclei from each time point were hybridized with the DM-painting probe which stains chromosomal HSR in these cells (see Fig. 3B), and the location of the signal from HSR were measured and expressed as in Figs 4 and 5. The theoretical random distribution line based on the area of each nuclear position is also shown.

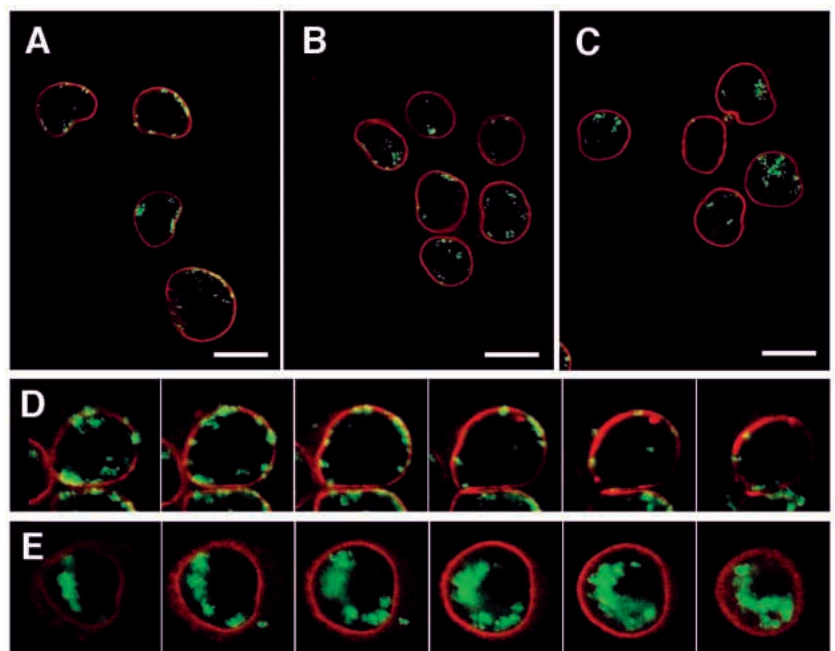


lamina (Fig. 7B,C). As explained in Materials and Methods, we concluded that the 2-dimensional analysis of a nucleus with a conserved shape was sufficient for the localization of multi-copy DMs. To confirm this conclusion, we obtained a set of confocal sections and examined the 3-dimensional distribution of DMs. In Fig. 7D and E, a representative image from the cells at the G_1/S -boundary and mid-S-phase, respectively, is shown. From these images, the relocation of DMs from the periphery to internally was evident in each of the optical sections. Taken together, these results showed that the relocation of DMs during S-phase was also seen in intact cells fixed in situ, by using a *c-myc* probe, and in 3-dimensions.

The expulsion of DMs from the nuclear periphery precedes peripheral DNA replication

We next examined more precisely the relationship between the relocation of DMs and DNA replication, by simultaneous detection of DMs and the site of DNA replication (Fig. 8). COLO 320DM cells were synchronized from the G_1/S -boundary using the same procedure as for Fig. 5. The site of DNA replication was detected in situ by pulse-labeling the cells with BrdU and the detection of incorporated BrdU by anti-BrdU antibody followed by confocal examination. We confirmed that the labeling pattern progressed essentially the same way as reported (O'Keefe et al., 1992), and classified these into 5 patterns (Fig. 8A, see red signal). These patterns

Fig. 7. Simultaneous detection of the amplified *c-myc* sequences and the lamin protein in intact COLO 320DM cells. COLO 320DM cells synchronized as in Fig. 5 were directly fixed by PFA and ethanol/acetic acid, and hybridized with biotinylated *c-myc* cosmid DNA. After the detection of the hybridized probe by FITC-conjugated streptavidin, the lamin protein was detected by an indirect immunofluorescence using rabbit anti-lamin antibody which recognizes all three subtypes of human lamin. The bound antibody was detected by a Texas Red-conjugated anti-rabbit Ig. The cells arrested at the G_1/S -boundary frequently contained amplified *c-myc* (green pseudocolor) at the nuclear periphery (A). Note these peripheral DMs are just in contact with lamin (red pseudocolor). If the cells were released from the G_1/S -boundary, DMs relocated to more interior at 2 (B), and 6 (C) hours after the release. Bars, 10 µm. The cells treated as above were also analyzed the three-dimensional distribution of DMs by obtaining a set of confocal optical sections. A representative image from the cells arrested at the G_1/S -boundary (D) or 4 hours after release (E) shows the peripheral and the internal location of DMs, respectively, in each of the optical sections. The sequential images were in increments of 1.44 µm from bottom (left) to top (right).



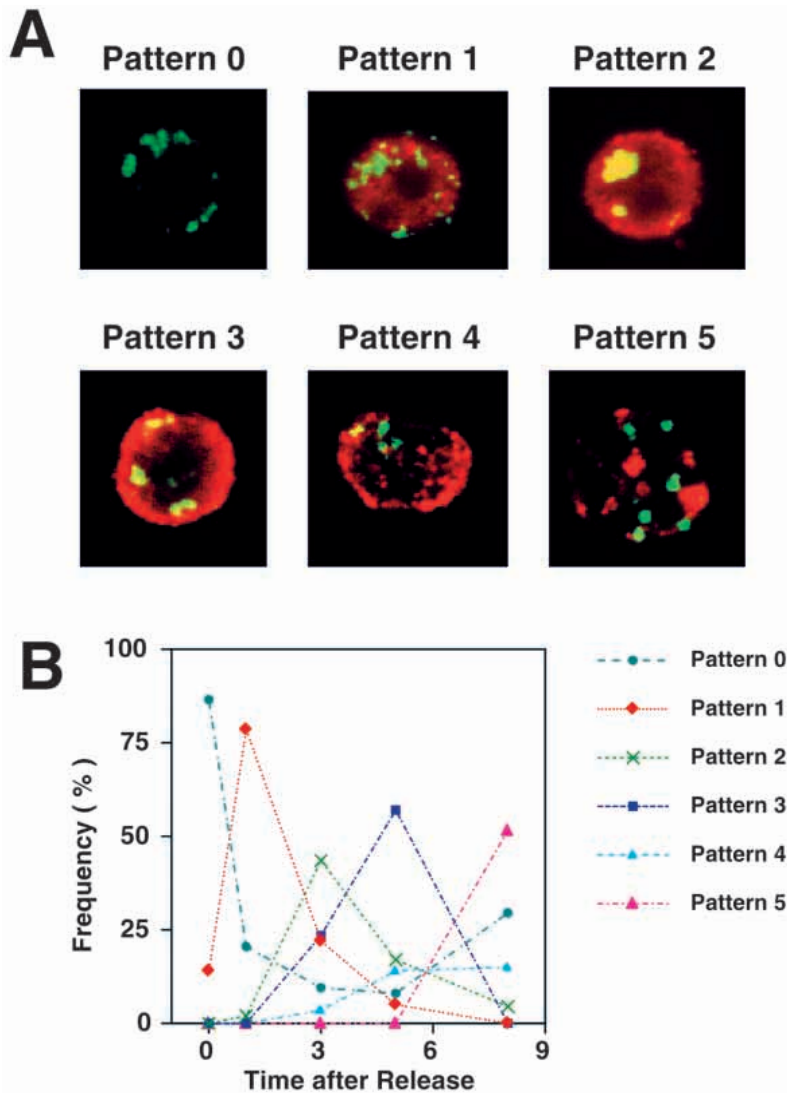


Fig. 8. DMs start to relocate to the nuclear interior before the start of the replication of the peripheral heterochromatin. COLO 320DM cells were synchronized as the same way as in Fig. 5, and the cells were pulse-labeled with BrdU just prior to the harvest. DMs in the nuclei were detected by FISH using DM-painting probe as before, and the site of DNA replication was detected by the indirect immunofluorescence using anti-BrdU mouse antibody and rhodamine conjugated anti-mouse antibody. The pattern of BrdU-labeling was classified into 6 patterns; i.e. pattern 0 (no labeling), 1 (only at the internal euchromatin), 2 (spread of the labeling to peripheral heterochromatin), 3 (almost exclusive labeling at the peripheral heterochromatin), 4 (both the peripheral and internal heterochromatin) and 5 (only at the internal heterochromatin). The representative images are shown in A. In these images, the site of DMs is pseudocolored in green and the site of DNA replication is in red. The site where green and red overlapped turned to be yellow. At each time point after release from the aphidicolin block, the frequencies of each pattern scored from 100 nuclei were plotted (B). Compare this graph to Fig. 5.

progressed sequentially after the release from the aphidicolin block (Fig. 8B). The location of DMs was detected by FISH (green signal) which was simultaneously done with the detection of BrdU (red signal). The representative merged images are shown in Fig. 8A. Most DMs locate at the periphery of the nuclei in pattern 0 (no labeling with BrdU). On the other hand, most of the nuclei showing pattern 1 or later had DMs at a deeper position. Furthermore, as shown in Fig. 5, the relocation of DMs begins 1 hour after release and most of the DMs moved to the inside before 3 hours after release. By comparing this result with Fig. 8B, it was shown that DMs began to move when pattern 1 (labeling at inner euchromatin) predominated the population, and finished the relocation before pattern 2 (initiation of the labeling at the peripheral heterochromatin) predominated. Therefore, it was strongly suggested that the relocation of peripheral DMs to the interior precedes the replication at the peripheral heterochromatin. Consistent with this result, if we released the cells from the G₁/S-boundary in the presence of actinomycin D, which inhibits DNA and RNA synthesis, we observed the initiation of DNA replication and the block at pattern 1 of replication. In that case, the relocation of DMs was not seen (data not shown).

This observation also supports the theory that the relocation is coupled to peripheral DNA replication.

DISCUSSION

In this report we showed that acentric DMs harboring amplified genetic sequences localized to the nuclear periphery during G₁-phase. Furthermore and more importantly, we found the peripheral DMs showed a remarkable relocation during S-phase that was spatially and temporally coupled to the replication at the periphery. Previously, we reported that peripheral DMs were selectively incorporated into nuclear buds which led to the formation of a micronucleus during S-phase (Shimizu et al., 1998). The specific inclusion of DMs into micronuclei was involved in the elimination of DMs from tumor cells which led to the differentiation or the reversion of the tumor cell phenotype, and the process had therapeutic importance (Von Hoff et al., 1992; Eckhardt et al., 1994; Shimizu et al., 1994). The peripheral location of DMs at G₁ should have important implications for selective incorporation into micronucleation at S-phase. On the other hand, one may

argue that the change in the distribution of DMs shown in this report might be an apparent result coming from the removal of the peripheral DMs by budding. However, the frequency of budding at S-phase was only 5% at most, whereas the relocation was obvious in the majority of the nuclei (Figs 4, 5, 7). Therefore, the change in the distribution of DMs should reflect the actual relocation of DMs; i.e. most of the peripheral DMs move inward before the initiation of DNA replication at the periphery while, and almost at the same time, a portion of DMs happened to be excluded from the nucleus by specific inclusion into nuclear buds and micronuclei. The precise mechanism of these events awaits further investigation.

Peripheral localization of DMs at G₁-phase

DMs located preferentially at the nuclear periphery during G₁-phase. This may be consistent with the report that the territories of DMs are independent from other chromosomal territories because DMs can be isolated by pulsed-field gel electrophoresis without prior strand-cleaving treatments (Chen and Manuelidis, 1989). The location of DMs at G₁-phase might reflect the peripheral localization of DMs at metaphase (Levan and Levan, 1978; Shimizu et al., 1998). Despite their acentric nature, DMs are maintained with surprising stability in tumor cells. This stability was explained by their attachment to the ends of normal chromosomes during M-phase followed by segregation to daughter nuclei by a 'hitch-hike mechanism' according to initial observations in fixed cells (Levan and Levan, 1978) and to recent observations in living cells (Kanda et al., 1998). Therefore, attachment of DMs to the ends of the chromosomes might lead to their peripheral localization when the nuclear membrane reforms. This is consistent with a notion that the localization of chromatin inside the interphase nucleus at least in part reflects the localization at M-phase (Comings, 1968, 1980). For example, early replicating, gene-dense chromatin locates internally, whereas late replicating genetically less active chromatin localizes at the periphery of the mitotic figure, and this tendency is common to the localization in the interphase nucleus (Hens et al., 1982). Interestingly, a recent report showed that an extrachromosomal nuclear plasmid of the papilloma virus was segregated to daughter nuclei by just the same hitch-hike mechanism as DMs (Lehman and Botchan, 1998). This suggests that the viral plasmid may also behave like DMs in interphase nuclei.

The localization of chromatin inside the nucleus has been frequently related to the replication timing, and the site of replication showed a typical transition during the progression through S-phase (O'Keefe et al., 1992; or Fig. 8 of this report). Usually the nuclear periphery is enriched with late replicating heterochromatin that may protect the inner euchromatin from genotoxic attack from outside the nucleus (the 'body-guard hypothesis'; Hsu, 1975). On the other hand, DMs are usually C- or G-band negative (Barker, 1982) and composed of early replicating chromatin (Carroll et al., 1991, 1993). Therefore, the euchromatic DMs might be put at an unusual position at G₁-phase which was probably caused by their localization at mitosis. It might be noteworthy that several lines of evidence suggested that extrachromosomal genetic elements such as DMs might be suffering extensive recombination events. Recombination of sequences in DMs has been frequently reported. For example, about half of the *c-myc* genes amplified on DMs in COLO 320DM cells are in a truncated form,

whereas most of the amplified *c-myc* genes in COLO 320HSR cells were intact (Schwab et al., 1986; Shtivelman and Bishop, 1989). Based on the 'bodyguard hypothesis', the active recombination suggested for DMs may be related to their peripheral localization in the nucleus.

Motion of DMs at S-phase

In this report, we showed that DMs located to the nuclear periphery at G₁-phase and relocated to the interior at S-phase. Our results suggested that DMs move inwards just prior to the onset of DNA replication at the peripheral heterochromatin. The same kind of inward relocation during S-phase has been reported in other instances. Centromeric regions located peripherally at G₁ or G₀, then relocated to a more interior position when the cells entered S-phase (Ferguson and Ward, 1992; Vourc'h et al., 1993; Tagawa et al., 1997); however, there were some contradictory reports (Bartholdi, 1991; Shelby et al., 1996). The minichromosomes in *Trypanosoma* have both centromeres and telomeres and are also located at the nuclear periphery from G₁ to early S-phase then relocated to the interior at late S-phase (Ersfeld, 1997). Furthermore, a recent elegant experiment using living cells showed that late replicating heterochromatic HSR, which was generated by the amplification of an introduced lac-operator, located to the nuclear periphery at G₁ to early S-phase and relocated to a more interior position just before their replication (Li et al., 1998). Therefore, these results together with ours on DMs suggest there is a generalized inward motion of some kind of chromatin during S-phase. This inward motion most probably precedes the replication of the corresponding chromatin, based on our result on DMs and the reported result on artificial HSR (Li et al., 1998).

When we actually examined the location of centromeres by hybridizing a pan-centromeric alpha-satellite probe to cell cycle-synchronized COLO 320DM cells, the relocation of centromeric sequences during the transition from G₁ to S-phase was not so obvious compared to the relocation of DMs (N. Itoh et al., unpublished observation). Furthermore, the same amplified sequences as in DMs did not show any significant relocation in our analysis if they were placed in a normal chromosome as HSR (Fig. 6). This result seems to be contradictory to the previous report that described the inward motion of HSR (Li et al., 1998). One possible explanation comes from the difference in the structure of the HSRs examined. Li et al. analyzed an HSR region produced by the introduction and amplification of a simple array of lactose-operator sequences, and noted that the chromatin structure of their HSR was atypical such that it is more resistant to denaturation than most chromosome regions. This may correlate with the surprisingly late replication timing of their HSR. On the other hand, the HSR in our COLO 320HSR cell line was generated during the evolution of cancer cells and the sequence complexity should be far higher. We do not know the actual replication timing of the HSR in our COLO 320HSR cell line, but it may be natural to assume that it replicates early because it is composed of the same sequence as the early replicating DMs. These apparently contradictory results might also be attributable to the method employed for the analysis. We statistically average the results from many nuclei taken from a synchronized culture. This method is suitable for the analysis of the large scale motion occurring synchronously in

the population. However, if the motion of the chromatin was not substantial or if it was occurring asynchronously, it might not be obvious in our analysis, unless it could be detected by the living cell analysis. Anyway, our analysis used in this study showed that the motion of DMs in COLO 320DM cells should be far more remarkable than among the motions of other chromosomal regions including centromere or HSR regions. One explanation is that acentric, atelomeric and small circular DMs are liberated from some kind(s) of restriction which normally restrains the motion of chromosomal regions and hinders the detection of their motion. Another interpretation of the remarkable motion of DMs may come from the early replicating euchromatic DMs localized at an unusual position at G₁-phase as a possible consequence of the mitotic configuration of this acentric chromatin. In that case, the replication of euchromatic DMs may require their movement to an internal site where euchromatin is normally replicated.

The peripheral location of DMs at G₁-phase and their inward relocation at S-phase should be related to the specific inclusion of DMs into buds and micronuclei formed at S-phase (Shimizu et al., 1998). As discussed above, the remarkable position and relocation of DMs is most possibly magnified by either their acentric, atelomeric or circular nature. Besides DMs, several kinds of genetic material that have these characteristics are known to be present in the cells. For example, the extrachromosomal closed circular (ecc) DNA, also known as small polydispersed circular (spc) DNA, is present in almost all the somatic cells examined as well as tumor cells (for review, see Gaubatz, 1990). We discussed in the above that the extrachromosomal nuclear plasmids of some kinds of virus may behave like DMs as they use the same hitch-hike mechanism as DMs at mitosis. Furthermore, plasmid vectors used in most genetic transformations of mammalian cells also share common structural characteristics with DMs. Therefore, we speculate that these genetic elements also may behave like the DMs reported here.

We are grateful to Dr Larry Gerace for kindly providing anti-lamin antibody. We also thank Tsubasa Tanaka and Takaya Ochi for their technical assistance. This work was in part supported by a Grant-in-Aid for Scientific Research (C) (#09680696) and a Grant-in-Aid for International Scientific Research (#07044271), both from the Japanese Ministry of Education to N.S.

REFERENCES

- Abney, J. R., Cutler, B., Fillbach, M. L., Axelrod, D. and Scalettar, B. A. (1997). Chromatin dynamics in interphase nuclei and its implications for nuclear structure. *J. Cell Biol.* **137**, 1459-1468.
- Barker, P. E. (1982). Double minutes in human tumor cells. *Cancer Genet. Cytogenet.* **5**, 81-94.
- Bartholdi, M. F. (1991). Nuclear distribution of centromeres during the cell cycle of human diploid fibroblasts. *J. Cell Sci.* **99**, 255-263.
- Benner, S. E., Wahl, G. M. and Von Hoff, D. D. (1991). Double minute chromosomes and homogeneously staining regions in tumors taken directly from patients versus in human tumor cell lines. *Anti-Cancer Drugs* **2**, 11-25.
- Carroll, S. M., Trotter, J. and Wahl, G. M. (1991). Replication timing control can be maintained in extrachromosomally amplified genes. *Mol. Cell. Biol.* **11**, 4779-4785.
- Carroll, S. M., DeRose, M. L., Kolman, J. L., Nonet, G. H., Kelly, R. E. and Wahl, G. M. (1993). Localization of a bidirectional DNA replication origin in the native locus and in epismally amplified murine adenosine deaminase loci. *Mol. Cell. Biol.* **13**, 2971-2981.
- Chen, T. L. and Manuelidis, L. (1989). Neuroblastoma double minutes isolated by pulsed-field gel electrophoresis without prior strand-cleaving treatments. *Genomics* **4**, 430-433.
- Comings, D. E. (1968). The rationale for an ordered arrangement of chromatin in the interphase nucleus. *Am. J. Hum. Genet.* **20**, 440-460.
- Comings, D. E. (1980). Arrangement of chromatin in the nucleus. *Hum. Genet.* **53**, 131-143.
- De, B. U. and Mintz, A. H. (1986). Curvilinear, three-dimensional motion of chromatin domains and nucleoli in neuronal interphase nuclei. *Science* **234**, 863-866.
- Eckhardt, S. G., Dai, A., Davidson, K. K., Forseth, B. J., Wahl, G. M. and Von Hoff, D. D. (1994). Induction of differentiation in HL60 cells by the reduction of extrachromosomally amplified c-myc. *Proc. Nat. Acad. Sci. USA* **91**, 6674-6678.
- Erstfeld, K. and Gull, K. (1997). Partitioning of large and minichromosomes in *Trypanosoma brucei*. *Science* **276**, 611-614.
- Ferguson, M. and Ward, D. C. (1992). Cell cycle dependent chromosomal movement in pre-mitotic human T-lymphocyte nuclei. *Chromosoma* **101**, 557-565.
- Gaubatz, J. W. (1990). Extrachromosomal circular DNAs and genomic sequence plasticity in eukaryotic cells. *Mutat. Res.* **237**, 271-292.
- Haaf, T. and Schmid, M. (1989). Centromeric association and non-random distribution of centromeres in human tumour cells. *Hum. Genet.* **81**, 137-143.
- Hankalo, B. A., Farnham, P. J., Johnston, R. and Schimke, R. T. (1985). Ultrastructural features of minute chromosomes in a methotrexate-resistant mouse 3T3 cell line. *Proc. Nat. Acad. Sci. USA* **82**, 1026-1030.
- Hammond, D. W., Hancock, B. W. and Goyns, M. H. (1994). Identification of a subclass of double minute chromosomes containing centromere-associated DNA. *Genes Chromosomes Cancer* **10**, 139-142.
- Hens, L., Kirsch, V. M., Verschaeve, L. and Susanne, C. (1982). The central localization of the small and early replicating chromosomes in human diploid metaphase figures. *Hum. Genet.* **60**, 249-256.
- Hsu, T. C. (1975). A possible function of constitutive heterochromatin: the bodyguard hypothesis. *Genetics* **79**, suppl., 137-150.
- Kanda, T., Sullivan, K. F. and Wahl, G. M. (1998). Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol.* **8**, 377-385.
- Lehman, C. W. and Botchan, M. R. (1998). Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. *Proc. Nat. Acad. Sci. USA* **95**, 4338-4343.
- Levan, A. and Levan, G. (1978). Have double minutes functioning centromeres? *Hereditas* **88**, 81-92.
- Li, G., Sudlow, G. and Belmont, A. S. (1998). Interphase cell cycle dynamics of a late-replicating, heterochromatic homogeneously staining region: Precise choreography of condensation/decondensation and nuclear positioning. *J. Cell Biol.* **140**, 975-989.
- Lin, C. C., Alitalo, K., Schwab, M., George, D., Varmus, H. E. and Bishop, J. M. (1985). Evolution of karyotypic abnormalities and C-MYC oncogene amplification in human colonic carcinoma cell lines. *Chromosoma* **92**, 11-15.
- Lin, C. C., Meyne, J., Sasi, R. and Moyzis, R. K. (1990). Apparent lack of telomere sequences on double minute chromosomes. *Cancer Genet. Cytogenet.* **48**, 271-274.
- Marshall, W. F., Fung, J. C. and Sedat, J. W. (1997a). Deconstructing the nucleus: Global architecture from local interactions. *Curr. Opin. Genet. Dev.* **7**, 259-263.
- Marshall, W. F., Straight, A., Marko, J. F., Swedlow, J., Dernburg, A., Belmont, A., Murray, A. W., Agard, D. A. and Sedat, J. W. (1997b). Interphase chromosomes undergo constrained diffusional motion in living cells. *Curr. Biol.* **7**, 930-939.
- 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.
- Schwab, M., Klempnauer, K. H., Alitalo, K., Varmus, H. and Bishop, M. (1986). Rearrangement at the 5' end of amplified c-myc in human COLO 320 cells is associated with abnormal transcription. *Mol. Cell. Biol.* **6**, 2752-2755.
- Selvin, P. R., Scalettar, B. A., Langmore, J. P., Axelrod, D., Klein, M. P. and Hearst, J. E. (1990). A polarized photobleaching study of chromatin reorientation in intact nuclei. *J. Mol. Biol.* **214**, 911-922.
- Shelby, R. D., Hahn, K. M. and Sullivan, K. F. (1996). Dynamic elastic behavior of alpha-satellite DNA domains visualized in situ in living human cells. *J. Cell Biol.* **135**, 545-557.
- Shimizu, N., Nakamura, H., Kadota, T., Kitajima, K., Oda, T., Hirano, T.

- and Utiyama, H. (1994). Loss of amplified c-myc genes in the spontaneously differentiated HL-60 cells. *Cancer Res.* **54**, 3561-3567.
- 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. *Nature Genet.* **12**, 65-71.
- Shimizu, N., Itoh, N., Utiyama, H. and Wahl, G. (1998). Selective Entrapment of Extrachromosomally Amplified DNA by Nuclear Budding and Micronucleation during S-phase. *J. Cell Biol.* **140**, 1308-1320.
- Shtivelman, E. and Bishop, J. M. (1989). The PVT gene frequently amplifies with MYC in tumor cells. *Mol. Cell. Biol.* **9**, 1148-1154.
- Strouboulis, J. and Wolffe, A. P. (1996). Functional compartmentalization of the nucleus. *J. Cell Sci.* **109**, 1991-2000.
- Tagawa, Y., Nanashima, A., Yasutake, T., Hatano, K., Nishizawa, T. J. and Ayabe, H. (1997). Differences in spatial localization and chromatin pattern during different phases of cell cycle between normal and cancer cells. *Cytometry* **27**, 327-335.
- VanDevanter, D. R., Piaskowski, V. D., Casper, J. T., Douglass, E. C. and Von, H. D. (1990). Ability of circular extrachromosomal DNA molecules to carry amplified MYCN proto-oncogenes in human neuroblastomas in vivo. *J. Nat. Cancer Inst.* **82**, 1815-1821.
- Von Hoff, D., Needham-VanDevanter, D., Yucel, J., Windle, B. and Wahl, G. (1988). Amplified human MYC oncogenes localized to replicating submicroscopic circular DNA molecules. *Proc. Nat. Acad. Sci. USA* **85**, 4804-4808.
- Von Hoff, D. D., McGill, J. R., Forseth, B. J., Davidson, K. K., Bradley, T. P., Van Devanter, D. R. and Wahl, G. M. (1992). Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. *Proc. Nat. Acad. Sci. USA* **89**, 8165-8169.
- Vourc'h, C., Taruscio, D., Boyle, A. L. and Ward, D. C. (1993). Cell cycle-dependent distribution of telomeres, centromeres, and chromosome-specific subsatellite domains in the interphase nucleus of mouse lymphocytes. *Exp. Cell Res.* **205**, 142-151.
- Wahl, G. M. (1989). The importance of circular DNA in mammalian gene amplification. *Cancer Res* **49**, 1333-1340.