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The nuclear position of pericentromeric DNA of chromosome 11 appears to be random in G₀ and non-random in G₁ human lymphocytes

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Abstract. The nuclear topography of pericentromeric DNA of chromosome 11 was analyzed in G₀ (nonstimulated) and G₁ [phytohemagglutinin (PHA) stimulated] human lymphocytes by confocal microscopy. In addition to the nuclear center, the centrosome was used as a second point of reference in the three-dimensional (3D) analysis. Pericentromeric DNA of chromosome 11 and the centrosome were labeled using a combination of fluorescent in situ hybridization (FISH) and immunofluorescence. To preserve the 3D morphology of the cells, these techniques were performed on whole cells in suspension. Three-dimensional images of the cells were analyzed with a recently developed 3D software program (Interactive Measurement of Axes and Positioning in 3 Dimensions). The distribution of the chromosome 11 centromeres appeared to be random during the G₀ stage but clearly non-random during the G₁ stage, when the nuclear center was used as a reference point. Further statistical analysis of the G₁ cells revealed that the centromeres were randomly distributed in a shell underlying the nuclear membrane. A topographical relationship between the centrosome and the centromeres appeared to be absent during the G₀ and G₁ stages of the cell cycle.

Introduction

The position of chromosomes has been well defined for cells in mitosis. In subsequent phases they move toward the center of the cell and align at the equatorial plate, followed by a relocation of the separated chromatids at opposite sides of the cell (Alberts et al. 1989). The highly condensed state of the chromosomes during this phase of the cell cycle, makes it comparatively easy to analyze their locations. During interphase, however, the study of

the (decondensed) chromosome position is more complicated. Using [³H]thymidine labeling and electron microscope autoradiography in synchronized human amnion cells, Comings (1968) proposed a model of the organization of the interphase nucleus in which the chromosomes are attached to the nuclear membrane at the site of initiation of DNA replication. Also, it was suggested that homologous chromosomes might be attached closer to each other than to nonhomologous chromosomes. The techniques to visualize and analyze (parts of) interphase chromosomes have evolved rapidly, revealing more accurate data on the position of chromosomes during interphase (for reviews see Manuelidis 1990; Jackson 1991; Lichter et al. 1991).

Many studies on the localization of centromeres of human chromosomes have been done by means of antibodies present in serum of patients with CREST syndrome. These antibodies are specific for kinetochore proteins enabling the detection of the centromeric regions of all chromosomes. Pair-wise arrangements of centromeres have been reported in interphase nuclei of rat-kangaroo and Indian muntjac cells, using such CREST serum antibodies (Hadlaczký et al. 1986). In murine cells, clustered and peripheral centromere distributions have been found in meiotic prophase I (Brinkley et al. 1986) and cell cycle specific distributions of centromeres have been found in human fibroblasts and lymphocytes (Bartholdi 1991; Weimer et al. 1992). The use of CREST serum antibodies, however, provides no information about the centromere distributions of specific chromosomes.

The centromere region of a chromosome contains specific, alpha satellite DNA sequences (Willard 1985; Jorgensen et al. 1986; Schulman and Bloom 1991). Today, specific DNA probes for almost every individual human chromosome are available. Using (fluorescent) in situ hybridization [(F)ISH], the analysis of the centromere distribution of individual chromosomes has been reported in several studies. Experiments performed on isolated nuclei centrifuged onto glass slides, revealed that the centromeres of chromosome 1 appeared to be

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randomly distributed in interphase nuclei of human cerebrum, whereas a pair-wise arrangement was found in human cerebellum nuclei (Arnoldus et al. 1989). A mainly peripheral location of the centromeres of chromosome 1 was seen in isolated interphase nuclei of human lymphocytes (Van Dekken et al. 1990). In this study, the nuclei were kept in suspension to preserve the three-dimensional (3D) morphology.

Random distributions of chromosome 7 centromeres have been reported in both human cerebrum and cerebellum interphase nuclei (Arnoldus et al. 1989). In isolated G_0/G_1 nuclei of human lymphocytes, however, the distributions of centromeres of chromosomes 1 and 7 were shown to be located exclusively at the nuclear periphery. Similarly, the centromeres of chromosomes 11 and 17 were found to be preferentially at the nuclear membrane. During the G_2 stage, a more interior location of these centromeres was reported, indicating a relocation of these centromeres during interphase (Ferguson and Ward 1992).

From these findings it appears that during interphase the localization of the centromeres of each individual chromosome might be cell type specific. Also, the specific interphase stage (G_0 , G_1 , S, G_2) seems to be relevant for the centromere distribution. Furthermore, the centromere distribution might be influenced by whether cells are flattened on glass slides or kept in suspension, or whether isolated nuclei are used.

An important aspect in 3D analysis, is the choice of a reference point. Usually, the position of centromeres is described in relationship to the nuclear center or relative to each other. The position of a centromere relative to a nuclear or a cellular structure might give more information about its biological role. During mitosis a clear topological relationship between centromeres and the centrosomes exists. However, during interphase this is much less clear. Recently, Funabiki et al. (1993) have found that centromeres were located near the spindle pole body throughout interphase in fission yeast.

In this study we analyzed the localization of the pericentromeric region of chromosome 11 in human lymphocytes. In an attempt to preserve the 3D morphology as much as possible, this was performed using cells in suspension (Van Dekken et al. 1990). The centromere distribution was analyzed relative to early differentiation (G_0 vs G_1 stage) of lymphocytes. The use of whole cells made it possible to choose the centrosome as an additional point of reference. To detect simultaneously the centromeres and the centrosome, a double labeling procedure was developed for cells in suspension. Using a recently developed 3D analysis software program (IMAP3D, Interactive Measurement of Axes and Positioning in 3 Dimensions; manuscript in preparation), distances were measured between the nuclear center and the centromeres, between the two homolog centromeres and between the centrosome and the centromeres. The data indicated that the centromeres of chromosome 11 are randomly distributed in G_0 lymphocytes and located near the nuclear membrane in G_1 lymphocytes. The peripheral position in G_1 cells could be described as a random distribution of the centromeres within a shell under-

lying the nuclear membrane. No significant relationship between the position of the centromeres and the centrosome was found in either the G_0 or the G_1 lymphocytes.

Materials and methods

Isolation, growth and fixation of human lymphocytes. Lymphocytes were isolated from peripheral blood with Lymphocyte Separation Medium (Organon Teknica; Durham, N.C.). They were either directly fixed in phosphate buffered saline (PBS) with 2% (w/v) paraformaldehyde, pH 7.4 (20 min, room temperature) and stored in 70% ethanol at -20°C , or cultured in Alpha Modification of Eagle's Medium (Flow Laboratories, Irvine, Scotland) with 10% (v/v) fetal calf serum. Cells were stimulated to enter the cell cycle by addition of 1% (v/v) phytohemagglutinin (PHA, Wellcome Diagnostics; Dartford, UK). After 65 h of stimulation, 10 $\mu\text{g}/\text{ml}$ bisbenzimidazole no. 33342 (Hoechst 42, Riedel-de Haen; Hannover, Germany) was added and the culture was incubated for 1 h at 37°C . Next, the cells were washed twice in PBS containing 10 $\mu\text{g}/\text{ml}$ Hoechst 42. Finally, 1.2 $\mu\text{g}/\text{ml}$ propidium iodide (PI) was added for flow cytometric analysis.

Flow cytometric analysis and sorting. Cells were analyzed on the RELACS-2 flow cytometer (Van den Engh and Stokdijk 1989), equipped with two lasers for dual beam flow cytometry. The first laser (Coherent Innova 90; Palo Alto, Calif.) was set at 488 nm (300 mW) to excite PI. PI fluorescence was detected through a BP630P10 emission filter (Corion; Holliston, Mass.). The second laser (Spectra Physics, Series 2000; Mountain View, Calif.) was set at ultraviolet wavelengths (351 nm and 364 nm; 150 mW) to excite Hoechst 42 that was detected through a 408 long pass plus 450 short pass filter (Schott Glaswerke; Mainz, Germany). The same setup was used to sort the PI negative lymphocytes in the G_1 stage of the cell cycle. The sorted cells were fixed and stored as described above.

Preparation of the biotinylated DNA probe. A nick translation was performed at 16°C for 3 h with biotin-16-dUTP (Boehringer; Mannheim, Germany) on 1 μg pLC11.A (Waye et al. 1987). Next, 1 μg of sonicated herring sperm DNA was added and the DNA was precipitated in ethanol (30 min, -20°C). After washing in 70% ethanol, the probe was dissolved in 100 μl H_2O and stored at -20°C . For in situ hybridization on whole cells in suspension the length of the probe fragments must range between 150 and 350 bp (Lawrence and Singer 1985; Hulspar and Bauman 1992). The length of the biotinylated probe fragments was determined by gel electrophoresis followed by Southern blotting. The blot was developed using streptavidin-alkaline phosphatase, according to standard procedures.

Fluorescent in situ hybridization and tubulin labeling. Fixed cells were pelleted and resuspended in PBS to allow rehydration for 20 min at room temperature (RT). After treatment with RNase A (Boehringer; 20 $\mu\text{g}/\text{ml}$, 25 min, 37°C) the cells were permeabilized in 0.1 N HCl, 0.01% (v/v) Triton X-100 (Pierce; Rockford, Ill.) (20 min, RT) and resuspended in 2 times standard sodium citrate (SSC), 0.02% (v/v) Tween-20 (Pierce). (1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium citrate.) Next, the cells were pelleted and resuspended in 20 μl 50% (v/v) deionized formamide, 2 $\times$ SSC, 0.02% Tween-20.

The biotinylated DNA probe (0.1 μg) was dried under vacuum in a SpeedVac Concentrator (Savant Instruments; Farmingdale, N.Y.) and dissolved in 10 μl deionized formamide. After denaturation (10 min, 80°C), 10 μl of 2 $\times$ hybridization mixture [4 $\times$ SSC, 4 $\mu\text{g}/\text{ml}$ bovine serum albumin (BSA), 0.4 g/ml dextran sulfate] was added to the probe. Next, the cell suspension and the DNA probe were mixed and allowed to hybridize for 18 h at 37°C . Ex-

cess of probe was washed away in $2\times$ SSC, 0.02% Tween-20 (30 min, 37°C).

Prior to the tubulin labeling, the cells were preincubated in PBS containing 1% (w/v) BSA (PBS/BSA) (45 min, RT). Incubation for 30 min with a murine monoclonal antibody to β -tubulin (Boehringer), diluted 1: 50 in PBS/BSA, was followed by two washes in PBS/BSA. The hybridized probe was labeled with Avidin-FITC (fluorescein isothiocyanate) DCS Grade (Vector; Burlingame, Calif.) by incubating the cells for 90 min at RT with Avidin-FITC diluted 1: 1000 in PBS/BSA. After one wash in $4\times$ SSC, 5% (w/v) non-fat dry milk, 5% (v/v) normal goat serum, 0.05% Triton X-100 for 20 min at RT and a second wash in PBS for 15 h at 4°C , the cells were incubated for 1 h at RT with FITC conjugated goat polyclonal antibody to murine Ig-G light chains (Tago; Burlingame, Calif.) diluted 1: 50 in PBS/BSA. Finally, the cells were resuspended in VectaShield (Vector) containing 0.5 $\mu\text{g}/\text{ml}$ PI after three washes in PBS.

Confocal scanning microscopy. A Bio-Rad MRC 600 CSLM (Bio-Rad; Hertfordshire, UK) was used to record 35 optical X-Y sections of each cell. A $63\times/1.3$ oil objective (Leica; Heidelberg, Germany) was used and the zoom factor was set at 6 (1 pixel, 81 nm). Each optical section (170×170 pixels) was separated by 300 nm in the Z-direction. Measurements were performed with 1% (± 0.1 mW, adjusted by a gray filter) laser output of the 10 mW 488 nm argon line.

Cells were recorded in simultaneous two-color detection mode. The FITC fluorescence was separated from the PI fluorescence by a standard A2 filter block. Both detection pinholes were set at scale mark 4.

3D image analysis. Analysis of the cells was performed on a Hewlett Packard/Apollo 425 series workstation (Hewlett Packard; Palo Alto, Calif.) with the interactive software program IMA3D. This software program was designed especially for interactive determination of positions of spots in 3D confocal data sets, and transformation to a nominated 3D coordinate system (Montijn et al. 1994; Houtsmuller et al. manuscript in preparation).

To indicate the position of the centromeres a 3D cursor was used. The cursor was present in three windows on the computer screen. The central window displayed a front view (XY-projection) of the 3D confocal data set. A top view (XZ-projection) and a side view (YZ-projection) were displayed on two sides of the central window. The position of the centromeres could be accurately indicated by the user, by moving the cursor to the XY-position of the centromere spots in the front view, and to the Z-position in the side views. The 3D coordinates were stored by pressing a user defined key.

The dimensions of the nuclei were also determined using the 3D cursor. First, the cursor was positioned at the center of the nucleus. Second, the X-, Y- and Z-axes were elongated such that they reached the nuclear boundary. The length of the three axes (in voxels) was stored with the positions of the centromeres. In order to compare the data from different cells, the nuclear dimensions were transformed to a nominated orthogonal coordinate system with the nuclear center at $(x, y, z)=(0, 0, 0)$ and the nuclear boundary at 1. The position of the centrosome was used to rotate the coordinate system such that the centrosome was on the positive Z-axis. Finally, the following distances were calculated: centromere-nuclear center, centromere-centromere and centromere-centrosome.

Statistical analysis. The nuclear radius was divided into ten classes. Next, each measured distance between the nuclear center and a centromere was categorized into the appropriate class. For the categorization of the intercentromeric distances and distances between the centrosome and a centromere, the nuclear diameter was divided into ten classes. The Kolmogorov-Smirnov Test (Kolmogorov 1933; Young 1977) was used to test the hypothesis that the measured distances were a population of random distances in a sphere. Cumulative values of the distributions were calculated and the

maximum deviation ($\delta_{\max}$) was determined. The level of significance taken was $\alpha=5\%$. The solution c of the equation $P(A\leq c)=1-\alpha$ was determined and the hypothesis, that both samples were taken from the same population, was rejected if $\delta_{\max}>c$.

Results

Flow cytometric analysis and sorting of lymphocytes

The position within the cell cycle was determined on the basis of the DNA content by means of flow cytometry. The PHA stimulated sample was first analyzed on the percentage of PI negative (viable) cells after culturing. Only the PI negative cells (52%) were sorted (Fig. 1A). The DNA histogram of the viable cells was characteristic for activated/cycling cells, showing cells in all stages (G_0/G_1 , S, G_2 and mitosis) of the cell cycle. The first

Fig. 1. A Histogram of propidium iodide (PI) stained lymphocytes after culturing with phytohemagglutinin (PHA) for 65 h. PI negative cells (gray area), characteristic for viable cells, were sorted. B DNA (Hoechst) histograms of nonstimulated human lymphocytes (thin line) and viable PHA stimulated lymphocytes (bold line). The single peak, shown for the nonstimulated cells, represents noncycling cells in G_0/G_1 stage of the interphase. The DNA histogram of the viable stimulated cells is characteristic for cycling cells, representing cells in G_0/G_1 , S, G_2 stages of the interphase and cells in mitosis. The gray area indicates the sorted fraction of cells, enriched for cells in the G_1 stage

Fig. 2A, B. Projection of 35 optical sections of a human peripheral blood lymphocyte, recorded by confocal scanning laser microscopy (CSLM) in simultaneous two-color detection mode. **A** Image of the propidium iodide (PI) stained nucleus, and **B** three fluorescein isothiocyanate (FITC) stained regions, representing the centrosome (arrow) and the two chromosome 11 centromeres. In each figure the central window displays a front view (XY-projection), a top view (XZ-projection) and a side view (ZY-projection). The three-dimensional (3D) cursor (displayed in all views) can be moved in the X-, Y- and Z-direction to indicate and record the coordinates of the objects to be measured. The three axes of the cursor were elongated to transform the nuclear dimensions to a nominated orthogonal coordinate system with the nuclear center at $(x, y, z)=(0, 0, 0)$ and the boundary at 1. Bars represent 5 μm

peak of the DNA histogram represented the section containing cells in G_1 stage (Fig. 1B). Only cells from the left side of the first peak were sorted (25% of the viable cells) to diminish strongly the possibility that the sorted sample would contain cells in early S stage. The DNA

histogram of the nonstimulated cells showed a single peak, characteristic of cells in G_0 stage (resting/noncycling; Fig. 1B).

Simultaneous labeling of centromeres of chromosome 11 and the centrosome in lymphocytes in suspension

Experiments on the accuracy of resuspension of the cells after each centrifugation step, revealed that a suspension of single cells increased the probability of a high percentage of double labeled cells (data not shown). Both the tubulin (not necessarily the centrosome) and the centromeres could be detected in 94% of the cells. Cells in which the centrosome was obscured by microtubuli were not considered for further analysis (16% of the double labeled cells). Eventually, both the centrosome and the centromeres could be detected in 78% of the cells. The centrosome could easily be recognized as a bright green fluorescent spot located outside the PI stained area. Since the centromeres were also labeled with FITC, the localization of the fluorescent spot (outside or within the nucleus) was used to distinguish between centrosome and centromeres, respectively (Fig. 2).

Distances between the nuclear center and the centromeres of chromosome 11

The position of the centromeres relative to the nuclear center was analyzed in 83 G_0 cells and in 107 G_1 cells. A model that describes a random distribution of two points in a sphere, predicts that in 26% of the cells the distance between the center and the centromere is greater than 0.9 length units. We found that in 31% of the G_0 cells, the distance between the center and the centromere was greater than 0.9 length units. Statistical analysis revealed a significant deviation from the random distribution ($\delta_{\text{max}}=0.120$; $c=0.106$) (Fig. 3A), i.e. a larger proportion of the centromeres occurred at the nuclear periphery than in a random situation.

A still greater difference between a random distribution and our data was found after analysis of cells in G_1 stage ($\delta_{\text{max}}=0.283$; $c=0.093$). Clearly, more cells were found with the centromeres located at the periphery of the nucleus (56%) than would be expected in the case of a random distribution of centromeres (26%) (Fig. 3A). In G_1 cells the centromeres are more peripherally localized than in G_0 cells. These results thus indicate that the centromeres of chromosome 11 move toward the nuclear membrane as the cell goes from the G_0 into the G_1 stage of the cell cycle. As the centromeres are located at their periphery of the nucleus, they thus appeared to be arranged in a shell underlying the nuclear membrane.

Distances between the two centromeres of chromosome 11

The distance between the two homolog centromeres, the intercentromeric distance, was used as a second param-

Fig. 3A–C. Percentage of G_0 (white bars) and G_1 lymphocytes (gray bars) as a function of the distance between the nuclear center and the centromeres of chromosome 11 (A), the two homolog centromeres (B), and the centrosome and the centromeres (C). The lines represent the random distributions of the corresponding

distances in a sphere (●) or in a shell of 3/10 of the nuclear radius (▲). The distances are normalized to a nuclear radius of 1.0. The inset shows the nucleus with two centromeres (small dots) and centrosome (large dot); C, nuclear center

ter of the centromere distribution. The distribution of the intercentromeric distances indicates whether the position of one centromere is dependent on the position of its homolog. As described above, both cell samples showed a non-random distribution of the centromeres within the nucleus, although this was much more clear in G_1 cells.

The less distinct preference for a peripheral location in G_0 cells (Fig. 3A) was reflected in the distribution of the intercentromeric distances (Fig. 3B). Statistical analysis of this second parameter revealed no deviation from a random distribution in G_0 cells ($\delta_{\max}=0.065$; $c=0.145$), whereas a small difference was found in the G_1 cells ($\delta_{\max}=0.136$; $c=0.131$). Considering a shell-like arrangement of the centromeres, the distribution of the intercentromeric distances in G_1 cells was compared with the distribution in a mathematical model that describes a random distribution of two points in a concentric shell of a sphere (A.B. Houtsmuller, unpublished results). These results showed that the centromere distribution in G_1 cells is best described by a model of a random distribution of centromeres relative to each other, located in a shell of about 3/10 of the nuclear radius ($\delta_{\max}=0.050$; $c=0.131$).

Distances between the centrosome and the centromeres of chromosome 11

Although the centromeres were clearly randomly distributed relative to each other at the periphery of the nucleus, the position of a centromere may still depend on the position of the centrosome. By taking the centrosome as a second point of reference, one axis of the coordinate system was fixed. Therefore, the coordinate system was rotated such that the centrosome could be regarded as the 'north pole' of the cell (see Materials and methods).

The distribution of the distances measured between the centrosome and the centromeres in G_0 cells showed no difference from a model that describes this distribu-

tion for randomly distributed centromeres in a sphere ($\delta_{\max}=0.069$; $c=0.106$) (Fig. 3C). Apparently, in G_0 cells the centromere position was independent of the position of the centrosome.

In 43% of the G_1 cells, the centromeres were located more than 1.4 units from the centrosome, whereas the expected percentage of cells (in the case of a random distribution in a sphere) was 25%. This appeared to be a significant deviation ($\delta_{\max}=0.182$; $c=0.093$), and can be explained by the mainly peripheral location of the centromeres in G_1 cells. Therefore, a second model was developed. This model described the random distribution of the centrosome-centromere distances in a shell. Comparison of our data with this model revealed a random arrangement of the centromeres relative to the centrosome ($\delta_{\max}=0.023$; $c=0.093$).

Another parameter to be derived from these data, was the absolute difference between the two centromere-centrosome distances. This parameter shows whether one centromere is always close to the centrosome while its homolog is always far from the centrosome. Statistical analysis revealed no significant deviation between G_0 and G_1 cells, and no difference between the distribution of this parameter in our study and that of a random model (data not shown). We therefore conclude that, during G_0 and G_1 stage of the cell cycle the position of these centromeres is not dependent on the position of the centrosome.

Discussion

The position of centromeres of chromosome 11 was investigated in human lymphocytes, during G_0 and G_1 stage of the interphase. Measurements were performed relative to three areas: the nuclear center, the homolog of the centromere and the position of the centrosome. Using the centrosome as a reference point, it was possible to assign a top and a bottom to the nucleus. Moreover,

the possibility of a positional relationship between the centrosome and the centromeres during interphase could now be investigated.

To preserve the *in vivo* situation as much as possible, experiments were carried out on whole cells in suspension, rather than on isolated nuclei. The applied fixation procedure has been proven maximally to preserve the morphology of the cells and nuclear structures for the purpose of DNA topographical studies (Manuelidis 1984; Van Dekken et al. 1990; Ferguson and Ward 1992). A software package designed for the semi-automatic measurement of 3D distances in a CSLM data set increased the accuracy and speed of the analysis procedure (Montijn et al. 1994; Houtsmuller et al. manuscript in preparation).

Results on the distribution of the center-centromere distances indicated that the centromeres were located at the nuclear periphery (the distance is more than 0.9 length units from the center) in a majority of the cells. If compared with a random distribution of centromeres in a sphere, both the nonstimulated and the PHA stimulated sample contained an excess of cells with centromeres located at the nuclear periphery (5% and 30%, respectively). It is known that only a certain percentage (30%–60%) of lymphocytes is activated by PHA to progress from G_0 into G_1 stage (Darzynkiewicz et al. 1979; Giaretti et al. 1985). The percentage of activated cells varies with the amount of PHA and the sensitivity of the donor's cells to the stimulus (Cotner et al. 1983). Not all the cells from the PHA stimulated sample contained centromeres located at the nuclear periphery. This may reflect that transition from G_0 to G_1 was accomplished in only 30% of the sorted cells, leaving 70% still in G_0 stage. The statistically justified 5% excess (31% vs 26%) of nonstimulated cells with peripherally located centromeres, might indicate that this sample contained 5% G_1 cells.

The results mentioned above, lead to the hypothesis that the centromeres of chromosome 11 are randomly distributed in G_0 stage lymphocyte nuclei and move toward the nuclear membrane upon entering the G_1 stage (Fig. 4). Several studies have indicated movement of centromeres during interphase (Manuelidis 1985; Weimer et al. 1992). However, results on centromere movement of specific chromosomes relative to early differentiation, have, to the best of our knowledge, not been published before.

Fig. 4. Schematic representation of the relocation of centromeres (dots) upon PHA stimulation of human lymphocytes. Note that, so far, the analysis has been carried out for chromosome 11 only

It has been reported that DNA replication starts at discrete sites in the interior of the nucleus and that specific replication patterns can be recognized in early, mid and late S stage. As the cell cycle proceeds, the replication sites move toward peripherally located chromatin (Nakayasu and Berezney 1989; Fox et al. 1991; Manders et al. 1992). A plausible thought is that the centromeres of chromosome 11 move toward the nuclear membrane during G_0 – G_1 transition, allowing initiation of DNA replication in the interior of the nucleus. The attachment of the centromeres to the nuclear membrane could be facilitated by specific binding sites. This idea is consistent with the finding that the nuclear membrane appeared to be essential for the initiation of DNA replication in G_1 cells (Dingwall and Laskey 1992; Leno et al. 1992). Also, interactions between the nuclear envelope and a small fraction of the chromatin have been reported (Paddy et al. 1990), which is in favor of the idea that centromeres are bound to the nuclear membrane. At the nuclear periphery, as such, the centromeres of chromosome 11 were randomly distributed relative to each other, as has also been found for centromeres of chromosome 1 by Van Dekken et al. (1990). The peripheral location of the centromeres in G_1 stage, could indicate that centromere replication occurs in a later period of the interphase, as has been shown to occur in nuclei of *Allium cepa* (Fussell 1975) and human fibroblasts (Bartholdi 1991). In addition, the peripheral position of G_1 centromeres might help to obtain a proper insight in the (yet undefined) topography of replicating chromosomes. By contrast, a more interior location of chromosome 11 centromeres during G_2 stage has been reported by Ferguson and Ward (1992). Together with our findings these results indicate that after replication, the centromeres move toward the nuclear interior. Apparently, the more interior location of the centromeres during G_2 stage resembles the centromere location during G_0 stage, i.e., a random position. The peripheral position does not seem to be necessary during these stages since there is no DNA replication.

Using β -tubulin specific antibodies in combination with a centromere specific DNA probe, it was possible to distinguish both the centrosome and the centromeres in 78% of the cells. An accurate resuspension of the cells during the labeling steps, appeared to be an important factor for a high detection efficiency of both targets. The use of a recently isolated centrosome specific antibody (Chevrier et al. 1992), would probably contribute to a still higher detection efficiency of the centrosome. Our immuno-labeling procedure was, in contrast to most other double labeling procedures, carried out on fixed cells, after the *in situ* hybridization. Obviously, the antigenicity of the tubulin was not lost after the *in situ* hybridization, enabling the omission of a second fixation step. When the centrosome was thus used as a point of reference, the data reflected the difference in centromere location between the G_0 and G_1 stages, as mentioned above. A significant topographical relationship between the centrosome and the centromeres, however, was not found. Apparently, the centrosome did not play an important role in the orientation of chromosome 11 centromeres.

meres, during the G₀ and G₁ stages of interphase. Considering the relationship between centrosome and centromeres during mitosis, it would be interesting to determine in what stage of the cell cycle the centrosome begins to influence the location of the centromeres.

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References

- Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD (1989) In: Molecular biology of the cell, second edition. Garland Publishing, New York London, pp 762–790
- Arnoldus EPJ, Peters ACB, Bots GTAM, Raap AK, van der Ploeg M (1989) Somatic pairing of chromosome 1 centromeres in interphase nuclei of human cerebellum. *Hum Genet* 83: 231–234
- Bartholdi MF (1991) Nuclear distribution of centromeres during the cell cycle of human diploid fibroblasts. *J Cell Sci* 99: 255–263
- Brinkley BR, Brenner SL, Hall JM, Tousson A, Balczon RD, Valdivia MM (1986) Arrangements of kinetochores in mouse cells during meiosis and spermiogenesis. *Chromosoma* 94: 309–317
- Chevrier V, Komesli S, Schmit A, Vantard M, Lambert A, Job D (1992) A monoclonal antibody, raised against mammalian centrosomes and screened by recognition of plant microtubule organizing centers, identifies a pericentriolar component in different cell types. *J Cell Sci* 101: 823–835
- Comings DE (1968) The rationale for an ordered arrangement of chromatin in the interphase nucleus. *Am J Hum Genet* 20: 440–460
- Cotner T, Williams JM, Christenson L, Shapiro HM, Strom TB, Strominger JL (1983) Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J Exp Med* 157: 461–473
- Darzynkiewicz Z, Traganos F, Andreeff M, Sharpless T, Melamed MR (1979) Different sensitivity of chromatin to acid denaturation in quiescent and cycling cells as revealed by flow cytometry. *J Histochem Cytochem* 27: 478–485
- Dingwall C, Laskey R (1992) The nuclear membrane. *Science* 258: 942–947
- Ferguson M, Ward DC (1992) Cell cycle dependent chromosomal movement in premitotic human T-lymphocyte nuclei. *Chromosoma* 101: 557–565
- Fox MH, Arndt-Jovin DJ, Jovin TM, Baumann PH, Robert-Nicoud M (1991) Spatial and temporal distribution of DNA replication sites localized by immunofluorescence and confocal microscopy in mouse fibroblasts. *J Cell Sci* 99: 247–253
- Funabiki H, Hagan I, Uzawa S, Yangida M (1993) Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J Cell Biol* 121: 961–976
- Fussell CP (1975) The position of interphase chromosomes and late replicating DNA in centromere and telomere regions of *Allium cepa* L. *Chromosoma* 50: 201–210
- Giaretti W, Abmayr W, Dörmer P, Santi L (1985) The G₀ in equilibrium with G₁ transitions of human lymphocytes as monitored by quantitative ¹⁴C-uridine autoradiography and high-resolution image analysis. *Cytometry* 6: 219–225
- Hadlaczy Gy, Went M, Ringertz NR (1986) Direct evidence for the non-random localization of mammalian chromosomes in the interphase nucleus. *Exp Cell Res* 167: 1–15
- Hulspas R, Bauman JGJ (1992) The use of fluorescent in situ hybridization for the analysis of nuclear architecture by confocal microscopy. *Cell Biol Int Rep* 16: 739–747
- Jackson DA (1991) Structure-function relationships in eukaryotic nuclei. *BioEssays* 13: 1–10
- Jorgensen AL, Bostock CJ, Bak AL (1986) Chromosome-specific subfamilies within human alphoid repetitive DNA. *J Mol Biol* 187: 185–196
- Kolmogorov AN (1933) Sulla determinazione empirica di una legge di distribuzione. *G Ist Ital Attuari* 4: 83–91
- Lawrence JB, Singer R (1985) Quantitative analysis of in situ hybridization methods for the detection of actin gene expression. *Nucleic Acids Res* 13: 1777–1799
- Leno GH, Downes CS, Laskey RA (1992) The nuclear membrane prevents replication of human G₂ nuclei but not G₁ nuclei in *Xenopus* egg extract. *Cell* 69: 151–158
- Lichter P, Boyle AL, Cremer T, Ward DC (1991) Analysis of genes and chromosomes by nonisotopic in situ hybridization. *GATA* 8: 24–35
- Manders EMM, Stap J, Brakenhoff GJ, Van Driel R, Aten JA (1992) Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. *J Cell Sci* 103: 857–862
- Manuelidis L (1984) Different central nervous system cell types display distinct and nonrandom arrangements of satellite DNA sequences. *Proc Natl Acad Sci USA* 81: 3123–3127
- Manuelidis L (1985) Indications of centromere movement during interphase and differentiation. *Ann NY Acad Sci* 450: 205–221
- Manuelidis L (1990) A view of interphase chromosomes. *Science* 250: 1533–1540
- Montijn MB, Houtsmuller AB, Oud JL, Nanninga N (1994) The spatial localization of 18S rRNA genes, in relation to the descent of the cells, in the root cortex of *Petunia hybrida*. *J Cell Sci* 107: 457–467
- Nakayasu H, Berezney R (1989) Mapping replication sites in the eucaryotic cell nucleus. *J Cell Biol* 108: 1–11
- Paddy MR, Belmont AS, Saumweber H, Agard DA, Sedat JW (1990) Interphase nuclear envelope lamins form a discontinuous network that interacts with only a fraction of the chromatin in the nuclear periphery. *Cell* 62: 89–106
- Schulman I, Bloom KS (1991) Centromeres: An integrated protein/DNA complex required for chromosome movement. *Annu Rev Cell Biol* 7: 311–336
- Van Dekken H, van Rotterdam A, Jonker RR, van der Voort HTM, Brakenhoff GJ, Bauman JGJ (1990) Spatial topography of a pericentromeric region (1Q12) in hemopoietic cells studied by in situ hybridization and confocal microscopy. *Cytometry* 11: 570–578
- Van den Engh GJ, Stokdijk W (1989) Parallel processing data acquisition system for multilaser flow cytometry and cell sorting. *Cytometry* 10: 282–293
- Waye JS, Creeper LA, Willard HF (1987) Organization and evolution of alpha satellite DNA from human chromosome 11. *Chromosoma* 95: 182–188
- Weimer R, Haaf T, Krüger J, Poot M, Schmid M (1992) Characterization of centromere arrangements and test for random distribution in G₀, G₁, S, G₂, G₁['], and early S' phase in human lymphocytes. *Hum Genet* 88: 673–682
- Willard HF (1985) Chromosome-specific organization of human alpha satellite DNA. *Am J Hum Genet* 137: 524–532
- Young IT (1977) Proof without prejudice: use of the kolmogorov-smirnov test for the analysis of histograms from flow systems and other sources. *J Histochem Cytochem* 25: 935–941