Volume ratios of painted chromosome territories 5, 7 and X in female human cell nuclei studied with confocal laser microscopy and the Cavalieri estimator

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Abstract. Chromosome territories 5, 7 and X were painted in female human amniotic fluid or fibroblast cell nuclei with chromosome specific DNA library probes. For probe detection the fluorochromes FITC or TRITC were applied. Using confocal laser scanning fluorescence microscopy ten to twenty light optical sections of FITC or TRITC images were registered with equal spacing (approximately 400 nm) for each of 131 nuclei showing well separated homologous chromosome territories. Chromosome territory areas were segmented applying a range of gray value thresholds to each section. For each gray value threshold the territory volumes for a given pair of homologs were computed by means of the Cavalieri estimator. The larger volume was divided by the smaller one to obtain a volume ratio. Between 9 and 26 territory volume ratios were calculated for a pair of homologs in a given nucleus. This approach yielded the following results: (i) the range of volume ratios was very similar for both autosome and X-chromosome territories and (ii) most volume ratios (> 70%) were between 1 and 1.5. The same results were obtained for cells fixed with 4% buffered formaldehyde or methanol/acetic acid indicating that volume ratio measurements are relatively robust against shrinkage effects induced by the latter type of fixation. These similarities of volume ratios argue against the view that the genetic inactivation of one X-chromosome territory in female cell nuclei should be correlated with a decrease of its volume. Such a decrease was expected in case of a strong overall condensation of this chromosome as compared to other chromosomes.

Keywords: Confocal microscopy, 3-D imaging, gray value thresholding, chromosome territories, cell nucleus, fluorescence in situ hybridization, chromosome painting.

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1. Introduction

In situ hybridization with human genomic DNA has made it possible to visualize entire human chromosomes directly in somatic human x Chinese hamster or human x mouse hybrid cell nuclei (Manuelidis 1985, Schardin et al 1985, Pinkel et al 1986). Surprisingly, not only human autosomes but also an active human X-chromosome observed in such hybrid cell nuclei were organized as rather compact territories (Schardin et al 1985). Later, chromosome specific DNA libraries established from flow sorted human chromosomes (Davies et al 1981, Cremer C et al 1984, Van Dilla and Deaven 1990, Collins 1991) were used to ‘paint’ entire human chromosomes directly in human cell nuclei. These studies confirmed the existence of chromosome territories in nuclei of both normal and tumor cells (Pinkel et al 1988, Lichter et al 1988, Cremer T et al 1988, 1993). Since then, the territorial organization of chromosomes has been demonstrated in nuclei from numerous animal and plant species (Heslop-Harrison and Bennett 1990). Recently, a model has been proposed predicting that chromosome territories play an essential role in the functional compartmentalization of the cell nucleus (Zirbel et al 1993, Cremer T et al 1993).

It has been assumed that the inactive X-chromosome forms a very compact territory in the cell nucleus known as the Barr-body (Barr and Bertram 1949), while the active X-chromosome is strongly decondensed (Comings 1968, Alberts et al 1994). To test this view we have used a confocal scanning laser fluorescence microscope (CSLFM) to obtain light optical serial sections from FITC-painted X-chromosome territories in female human amniotic fluid cell nuclei. In a previous study (Bischoff et al 1993) a fixed gray value threshold was interactively chosen for a given nucleus. After segmentation and computation of chromosome territory areas and nuclear areas the nuclear volume and the X-territory volumes were computed from the stack of equally spaced optical sections (Cavalieri estimator). To minimize some of the problems connected with the measurement of absolute volumes (see section 4), volume ratios were calculated for the two territories segmented in a given nucleus. These data suggested that differences in the volumes of the active and inactive X-chromosome territories were small.

The choice of the gray value threshold could have affected the outcome of absolute and relative volume measurements. Further experiments were therefore undertaken to measure volume ratios for the two X-chromosome territories in female amniotic fluid cell nuclei using a wide range of thresholding conditions. The same procedure was applied to reinvestigate the experimental data set used by Bischoff et al (1993). For a direct comparison of X-territory volume ratios with the volume ratios obtained for an autosome of similar size, X-territories and chromosome 7 territories were simultaneously painted using a differently labelled chromosome 7 specific alphoid DNA probe for unequivocal identification. Volume ratios were also determined for the territories of FITC or TRITC painted chromosome 5 homologs in fibroblast cell nuclei.

2. Materials and methods

2.1. Cell material

Human fibroblast and amniotic fluid cell cultures with normal female karyotypes (46, XX) were subcultured (approximately 1:6) on glass slides. After three days growing subcultures were fixed with methanol/acetic acid (3:1; v/v) and stored in 70% ethanol until use. Alternatively, subcultures were fixed for five minutes with buffered formalin (4% formaldehyde solution (Merck) in PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 6.5 mM Na2HPO4; pH 7.0)). After formalin fixation, slides were twice treated with Triton X-100/Saponin (0.1% each, 5 min), washed in 0.1 M Tris-HCl (pH 7.2), equilibrated in 20% glycerol in PBS for 20 min. They were then freeze-thawed by briefly dipping three times in liquid nitrogen, and stored at 4°C in PBS containing 0.04% sodium azide until use. Prior to in situ hybridization, cells were digested with pepsin in 0.01 M HCl, pH 2.0, at 37°C. The effect was observed with a standard light microscope. Digestion was allowed to proceed until the cytoplasm was largely removed. Postfixation was carried out for 10 min with 1% formaldehyde solution buffered in PBS.

2.2. DNA probes and probe labelling

Chromosome specific plasmid libraries (Collins et al 1991) established from sorted human chromosomes 5 (pBS5), 7 (pBS7) and X (pBSX) were a gift from Dr J Gray (University of California, San Francisco). Total DNA from these libraries was prepared and nick-translated with Bio-11-dUTP (Sigma) or Dig-11-dUTP (Boehringer Mannheim) (for details see Lichter and
Volume ratios between chromosome territories

Cremer 1992). The chromosome 7 specific alphoid DNA probe p7tl (Waye et al 1987) was a gift from Dr H F Willard (The Western Case University). Nick-translation of the p7tl probe was performed with Dig-11-dUTP.

2.3. Chromosomal in situ suppression (CISS-) hybridization of chromosome territories 5, 7 and X

CISS hybridization was carried out as described (Lichter et al 1988, Lichter and Cremer 1992) with the following modifications. Dehydration of the nuclei by an ethanol series was omitted and any air drying was carefully avoided throughout the whole procedure. For visualization of the biotinylated probes avidin conjugated to FITC (Vector) was used. Dig-11-dUTP labelled probes were detected by indirect immunofluorescence with mouse anti-digoxin (Sigma) and goat anti-mouse Ig- TRITC (tetramethylrhodamine isothiocyanate) antibodies (Sigma). In one experiment (No. 1) the nuclei were counterstained with propidium iodide (PI). The slides were mounted in PBS/glycerol (1:9; v/v) containing 0.1% 1,4-phenylenediaminedihydrochloride as antifade (Serva). In one experiment performed with human amniotic fluid cell nuclei chromosome 7 and chromosome X homologs were simultaneously painted with FITC. The chromosome 7 territories were distinguished from the X- chromosome territories by the additional hybridization of the Dig-11-dUTP-labelled chromosome 7 specific alphoid DNA probe detected with TRITC conjugated antibodies. Counterstaining with PI was omitted in this two-color FISH experiment.

2.4. 3-D recording of chromosome territories and cell nuclei

3-D recording was performed using a confocal scanning laser fluorescence microscope (CSLFM) described in detail elsewhere (Stelzer et al 1986). PI and FITC were simultaneously excited by an argon ion laser at 488 nm. For TRITC excitation a helium neon laser at 543 nm was used. Using a Zeiss planapochromat, oil immersion objective 100x/NA 1.3, the lateral resolution (x-, y-axes) was determined to be 250 nm, while the resolution along the z-axis was approximately 500 nm (Stelzer 1990, Stelzer et al 1991). The fluorescence emission signals of FITC-painted chromosome territories and PI-stained cell nuclei were recorded simultaneously, separated by appropriate filters and dichroic mirrors, and registered by two photomultipliers followed by two separate amplification systems. The amplification gains were individually set for a medium nuclear section and adjusted independently for the PI/TRITC- and FITC-channels. To obtain a better signal-to-noise ratio, each line was scanned 16 times and averaged. For each optical section two fluorescence images of 512x512 pixels were registered by digitization with an accuracy of 8 bit. The distances between two adjacent sections was approximately 400 nm (Bischoff et al 1993). For each nucleus, one stackcomprising 10 to 20 optical sections was obtained for FITC- or TRITC-painted chromosome territories. In case of PI-counterstaining nuclear sections were recorded as a second-stack of Pi-images.

2.5. Image analysis

Image analysis was done on a PC (80386 or 80486) using a modified software program written in Turbo C++ (Bischoff et al 1993). The main feature of this program is a threshold algorithm applied to the pixel gray values of the image of each registered optical section. The pixel gray value zero stands for maximum fluorescence signal intensity, while gray value 255 defines nuclear areas with zero intensity. Accordingly, a lower gray value threshold only counts the more intensive pixels resulting in a smaller volume estimate (figure 1). For example, a threshold of 10 means that only the most intense pixels with gray values between 0 and 10 contribute to the segmented area. Using the magnification factors known from the instrumental setup, the sum of all pixels with gray values below a given threshold (see section 3 for definition of a plausible threshold range) was used to compute section areas (µm²). Slice volumes (µm³) were obtained by multiplication of the section areas with the distance between two subsequent sections. The volumes of the FITC or TRITC painted chromosome territories, as well as the total volumes of PI-stained nuclei were determined by summing up the segmented slice volumes.

3. Results

Composite chromosome specific DNA library probes for chromosomes 5, 7 and X provide a strong and largely homogeneous painting of the respective metaphase chromosomes from pter to qter. Only the pericentromeric heterochromatin may show little painting due to the suppression of hybridization by the excess of cot 1-DNA fraction contained in the hybridization mixture (Lich-
Volume ratios between chromosome territories

The following notation for X-chromosome territories was used: Xa = active X-Chromosome, Xi = inactive X-Chromosome, XL = territory with larger segmented volume, XS = territory with smaller segmented volume. X1, X2 arbitrarily assigned X-Chromosome territories for volume calculations. As expected the absolute nuclear volumes (figures 2(a), 3(a), 4(a)), the absolute volumes of the two X-territories (figures 2(b), 3(b), 4(b)) and the relative volumes of the X-territories calculated as percentage of the total nuclear volume (figures 2(c), 3(c), 4(c)) increased with increasing thresholds. For each threshold the larger volume was divided by the smaller one to obtain a chromosome territory volume ratio (figures 2(d), 3(d), 4(d)). Distinct differences were noted with regard to the effect of threshold changes on volume ratio measurements. In figure 2(d), the observed volume ratios are very similar (±10%) for the whole range of chosen thresholds ('constant type'); in figure 3(d) ratios show a monotonous increase with decreasing thresholds ('monotone type'); the case presented in figure 4(d) indicates that the X-territory showing the larger volume at high threshold became the smaller territory at a low threshold ('interchange type'). In table 1 the 131 nuclei evaluated in the present experiments are classified with regard to their dependence of volume ratios on threshold. The three types exemplified in figures 2 - 4 were observed in all experiments. The relative frequencies of the three types observed for given chromosome varied considerably in different experiments. In the large majority of nuclei with painted X-chromosome territories showing the constant type, the volume ratios were between 1 and 1.5 (11/14). The same was true for all nuclei with painted autosome territories, i.e. 5 and 7, showing the constant type (44/44). In nuclei showing the monotone or interchange type, the range of volume ratios was more pronounced, although only about 5% of all nuclei showed volume ratios > 3.

Figure 5(a-c) shows the percentage of chromosome territory volume ratios which exceeds a given chromosome territory volume ratio for the constant (a) monotone (b) and interchange type (c). Figure 5(d) summarizes these percentages for all three types. Notably, a very similar frequency dependence was noted for chromosome 5, 7 and X-territories. For both autosome and X-chromosome territories more than 70% of all volume ratios were 1.5 (figure 6). Only a few per cent of the ratios exceeded a value 2.0.
Figure 1. Threshold dependent representation of the X-chromosome territory and the cell nucleus in one optical section. The higher the threshold of the gray values (in this example 150-220) the more pixels contribute to the segmented areas.

Table 1. Number of nuclei showing a constant (compare figure 3), monotone (compare figure 4) and interchange (compare figure 5) type of territory volume ratio. The last column gives the total number of different thresholds applied. Three different types of chromosomes in 5 different experiments were evaluated (XX= female nuclei with labelled X-chromosomes; 5,7 = labelled autosomes; FITC, TRITC = fluorescence dye used for visualization of painted chromosome territories; ME = methanol/acetic acid fixation; FO = formaldehyde fixation).

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Cell type</th>
<th>Constant</th>
<th>Monotone</th>
<th>Interchange</th>
<th>Investigated nuclei</th>
<th>Total number of ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XX FITC FO amniotic fluid</td>
<td>4</td>
<td>8</td>
<td>21</td>
<td>33</td>
<td>304</td>
</tr>
<tr>
<td>2</td>
<td>XX FITC FO2 amniotic fluid</td>
<td>10</td>
<td>7</td>
<td>3</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>2</td>
<td>7 FITC FO amniotic fluid</td>
<td>6</td>
<td>5</td>
<td>9</td>
<td>20</td>
<td>396</td>
</tr>
<tr>
<td>3</td>
<td>5 FITC FO fibroblast</td>
<td>11</td>
<td>2</td>
<td>7</td>
<td>20</td>
<td>400</td>
</tr>
<tr>
<td>4</td>
<td>5 FITC ME fibroblast</td>
<td>21</td>
<td>15</td>
<td>5</td>
<td>41</td>
<td>952</td>
</tr>
<tr>
<td>5</td>
<td>5 TRITC ME fibroblast</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>17</td>
<td>340</td>
</tr>
</tbody>
</table>
Figure 2. Nuclear and X-territory volume measurements in a human amniotic fluid cell nucleus (No. XX16) applying a range of thresholds. (a) Threshold dependent variation of the nuclear volume; (b) variation of the volumes of the two X-territories; (c) ratio of each territory volume (X1, X2) to the nuclear volume; (d) volume ratio of the larger chromosome territory to the smaller chromosome territory. Independent from the threshold chosen the ratio was the same (± 10%), i.e. constant type. The variation in absolute volumes for all three types was similar.
Figure 3. Nuclear and X-territory volume measurements in a female human amniotic fluid cell nucleus (No. XX19) applying a range of thresholds. (a) Threshold dependent variation of the nuclear volume; (b) variation of the volumes of the two X-territories; (c) ratio of each territory volume (X1, X2) to the nuclear volume; (d) volume ratio of the larger chromosome territory to the smaller chromosome territory. With a decreasing threshold the ratio increases continuously, i.e. monotone type.
Figure 4. Nuclear and X-territory volume measurements in a female human amniotic fluid cell nucleus (No. XX18) applying a range of thresholds. (a) Threshold dependent variation of the nuclear volume; (b) variation of the volumes of the two X-territories; (c) ratio of each territory volume (X1, X2) to the nuclear volume; (d) ratio of the larger chromosome territory to the smaller chromosome territory. At a threshold of 190 the larger territory became the smaller one and vice versa, i.e. interchange type.
Figure 5. Frequencies of the estimated chromosome territory volume ratios calculated for a range of plausible thresholds (Ordinate: percentage of all volume ratios higher than the abscissa value). Frequencies are given (a) for nuclei with constant type threshold patterns of XL/XS ratios, (b) for nuclei with monotone type threshold patterns, (c) for nuclei with interchange type threshold patterns, (d) for the combined frequencies in (a-c). For example (figure 6(d)), in 78% of all 304 thresholds used to evaluate experiment No. 1 (XX FITC FO'), the volume ratio XLYXS was larger than 1.1; in 23% of all 304 thresholds used, the ratio XL/XS was larger than 1.5. Different colored columns indicate different painted chromosome territories, fixation or probe labelling methods (for details compare table 1). Ratios above 1.5 are mostly due to the monotone type.
4. Discussion

Laser confocal serial sections of chromosome territories 5,7 and X painted in female human cell nuclei were used to test for possible differences in the volume ratios of the active and inactive X-territory in comparison to the volume ratios of homologous autosome territories. For segmentation of chromosome territories and nuclear borders, thresholds were varied systematically through a plausible range. In the present experiments it was not known which of the two X-territories represented Xa and Xi, respectively. To obtain an upper estimate of the Xa/Xi volume ratio, the larger volume XL was divided by the smaller one XS. The threshold dependence of volume ratios observed for homologous territories were surprisingly similar for the X-chromosome and the two autosomes. These data do not support a simple relationship between territory volume and genetic activity of the two X-chromosomes in female cell nuclei. If differences in condensation play a decisive role with respect to the active and inactive X-chromosome they must be more subtle (Walker et al 1991, Bischoff et al 1993, Cremer T et al 1993).

The results of absolute and relative chromosome territory volumes determined by the present approach have to be assessed with several limitations in mind. One major concern relates to changes of nuclear morphology and three-dimensional chromosome territory structure due to the fixation and in situ hybridization procedures employed. To keep such effects as small as possible, a collapse of the nuclear structure due to air drying was carefully avoided during all steps of the fluorescence in situ hybridization procedure. Evidence obtained by Nomarski interference microscopy suggests that the three-dimensional size and shape of nuclei from living cells could be maintained after fixation with 4% buffered formalin within linear deviations of a few percent of the dimensions prior to fixation. Notably, the shape and positioning of nucleoli showed also no apparent change. Under these conditions overall linear deviations of the three-dimensional nuclear shape were kept below 10% (Paaz, Stelzer, Cremer T, Cremer C, unpublished results). In other experiments, kinetochore antibodies were applied to study the relative positions of these structures in formalin fixed amniotic fluid cell nuclei. The 2-D-distribution pattern of the kinetochore signals obtained before and after FISH with an alphoid consensus DNA probe was apparently the same, allowing an exact overlay of the signal positions (Cremer T et al 1993). We conclude from these experiments that the extent of DNA crosslinking induced by fixation with buffered formalin is sufficient to maintain the three-dimensional nuclear structure to a very considerable extent, even in case of the removal of nuclear proteins by limited pepsin digestion. Volume ratios of chromosome 5 territories obtained in fibroblast nuclei both after fixation with buffered formalin and methanol/acetic acid were very similar indicating that the more pronounced shrinkage effects expected by the latter type of fixation affected both territories of a given chromosome in a similar way. These results further support our expectation that our attempts to measure volume ratios between chromosome territories were not rendered futile by profound changes during fixation and in situ hybridization.

Some fluorescence background due to non-specific binding of fluorescent compounds used for signal detection could not be avoided. Accordingly, too high thresholds resulted in a merging of territory and background regions. The internal structure of painted chromosome territories did not appear to be homogenous. At low thresholds segmentation was restricted to the more intensely painted internal territory regions. If the apparently smaller territory segmented at a high threshold contained more intensely painted regions than the larger territory, the latter territory should become the smaller one at decreasing thresholds. It cannot be decided whether inhomogeneities in the painting of a chromosome territory resulting in an interchange type of volume ratios reflect differences in the accessibility of the probe to certain chromatin domains due to fixation artifacts or in vivo differences. A pronounced variability of the number of nuclei showing a constant, monotone and interchange type of chromosome territory volume ratios was observed in different experiments both for X-chromosome and autosome territories.

The edges of chromosome territories appear often fuzzy. The possibility needs to be considered that DNA loops bearing genes expanded considera-

Figure 6. Frequency (%) of the estimated chromosome territory volume ratios between 1 and 1.5. The different columns indicate the different experiments (see table 1)
bly from a Xa-territory core, while such an expansion may be less pronounced or absent in Xi-territories. If so, the applied threshold algorithm may have only registered the compact core of the Xa-territory obscuring differences between Xa- and Xi-territory volumes. FISH-experiments yielding painting of X-chromosome territories in one color and signals of subregional X-specific probes in another are presently underway to test this possibility.

Absolute volume measurements by 3-D imaging critically depend on the z-axis resolution of the applied microscope and the accurate determination of the true distances between subsequent light optical sections. Recently, Visser et al (1992) and Hell et al (1993) have shown that the focal position of the laser beam in the z-axis does not simply reflect the respective vertical movement of the object stage, but is moved over a smaller distance depending on the refractive index of the object. This may lead to an overestimate of the distance of certain optical sections. Thus the upper and lower part of an image may contribute to the calculated volume values in a different way. Furthermore, mismatches in refractive indices between the specimen, its surrounding medium and the optical set-up may affect critically the intensity and axial resolution of the confocal images. For the conditions used, i.e. height of nuclei in the order of 6 µm, high aperture oil immersion objective and glycerol embedding of the specimens, it has been calculated using an electromagnetic approach that changes in intensity and both axial and lateral resolution should be less than 10% (Hell et al 1993).

The registration of the fluorescence of the nuclei and the chromosome territories via two independent detector and amplification systems are also factors which have an influence on the absolute values, especially because the registration parameters, e.g. amplification gains of the detection systems, had to be determined for each nucleus interactively. In any case, we expect that optical and registration effects should influence volume measurements of both X-territories in the same direction and thus have a comparatively small effect on volume ratios.

In conclusion, results of volume ratio measurements based on the Cavalieri estimator argue against the view that the genetic inactivation of one X-chromosome territory in female cell nuclei should be correlated with a substantial decrease of its volume. Such a decrease was expected in case of a strong overall condensation of this chromosome as compared to other chromosomes. The same conclusion was recently obtained using a completely different approach for volume estimation based on a three-dimensional Voronoi tessellation procedure (Eils et al 1995).

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Volume ratios between chromosome territories

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