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ORIGINAL ARTICLE

Eva Bártová · Stanislav Kozubek · Pavla Jirsová
Michal Kozubek · Emilie Lukášová
Magdalena Skalníková · Alena Cafourková
Irena Koutná · Renata Paseková

Higher-order chromatin structure of human granulocytes

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Abstract The structural organisation of chromatin in eukaryotes plays an important role in a number of biological processes. Our results provide a comprehensive insight into the nuclear topography of human peripheral blood granulocytes, mainly neutrophils. The nuclei of granulocytes are characterised by a segmented shape consisting of two to five lobes that are in many cases connected by a thin DNA-containing filament. The segregation of chromosomes into the nuclear lobes was studied using fluorescence in situ hybridisation (FISH). We were able to distinguish different topographic types of granulocytes on the basis of the pattern of segregation. Five topographic types were detected using dual-colour FISH in two-lobed nuclei. The segregation of four sets of genetic structures could be studied with the aid of repeated FISH and a large number of topographic types were observed. In all these experiments a non-random distribution of chromosomes into nuclear lobes was found. The painting of a single type of chromosome in two-lobed nuclei showed the prevalence of symmetric topographic types (on average in 65.5% of cases) with significant variations among individual chromosomes. The results of analysis of five topographic types (defined by two chromosomes in two-lobed nuclei) showed that the symmetric topographic types for both chromosomes are significantly more frequent than predicted. Repeated hybridisation experiments confirmed that the occurrence of certain patterns of chromosome segregation is much higher than that predicted from the combination of probabilities. The frequency of symmetric topographic types for chromosome domains was systematically higher than for genes located on these chromosomes. It appears that the prevalence of symmetric segregation patterns is more probable for large objects such as chromosome domains than for genes located on chromatin loops extending outwards from the surface of the domain defined by specific chromosome paints. This means that one chromosome domain may occur in different lobes of granulocytic nuclei. This observation is supported by the fact that both genes and centromeres were observed on filaments joining different lobes. For all chromosomes, the distances between the membrane and fluorescence gravity centre of the chromosome were measured and correlated with the segregation patterns. A higher percentage of symmetric topographic types was found in those chromosomes that were located closer to the nuclear membrane. Nuclear positioning of all genetic elements in granulocytic nuclei was studied in two-dimensional projection; however, the results were verified using three-dimensional analysis.

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E. Bártová · S. Kozubek (✉) · P. Jirsová · E. Lukášová
A. Cafourková · I. Koutná
Institute of Biophysics,
Academy of Sciences of the Czech Republic,
Královopolská 135, 612 65 Brno, Czech Republic
e-mail: kozubek@ibp.cz

M. Kozubek · M. Skalníková · R. Paseková
Faculty of Informatics, Masaryk University,
Botanická 68a, 602 00 Brno, Czech Republic

Introduction

Changes in nuclear architecture are related to many biological processes in eukaryotic cells (Hendrich and Willard 1995; Karpen and Allshire 1997; Francastel et al. 1999; Hennig 1999; Meyer 1999). Nuclear events such as replication, transcription, recombination and repair (Spector 1993; Zirbel et al. 1993; Dolling et al. 1997; S. Kozubek et al. 1997; Verschure et al. 1999) and cellular processes such as the cell cycle and cell differentiation are also accompanied by structural changes in chromatin (Manuelidis 1985, 1990; Ferguson and Ward 1992; Ceccarelli and Cionini 1993; Vourc'h et al. 1993; Hulspas et al. 1994; Ferreira et al. 1997; Bártová et al. 2000a, b; Leitch 2000). On the other hand, a higher-order chromatin arrangement influences induction of chromosome translocations and consequently cancer induction (Lukášová et al. 1997; S. Kozubek et al. 1999).

Genetic inactivation of chromosomal loci in eukaryotic cells is correlated with a greater compactness of nuclear chromatin (Grigoryev and Woodcock 1998; Bártová et al. 2000a), which is highly condensed in terminally differentiated, quiescent cells (Edwards 1994). Therefore, the process responsible for the production of human blood cells, known as haematopoiesis, provides a convenient experimental system to study the inter-connection between chromatin condensation and down-regulation of the transcriptional activity of the mature cells.

Granulocytes (neutrophils, basophils and eosinophils) represent an example of terminally differentiated cells with the highest possible condensation of chromatin (Alberts et al. 1994; Grigoryev and Woodcock 1998). The heterochromatic layer, representing the transcriptionally inactive part of the chromatin, is located at the periphery of the granulocytic nucleus (Edwards 1994; for review see Francastel et al. 2000). In spite of a high degree of chromatin condensation, some genetic loci are actively transcribed (Alberts et al. 1994).

The purpose of the present study is to focus on the higher-order chromatin structure of human peripheral blood granulocytes, mainly neutrophils, and provide basic information on the nuclear topography of a segmented cell population. A number of experiments revealing the regularities of higher-order chromatin structure have been done using spherical cell nuclei such as lymphocytes or the oval nuclei of fibroblasts (T. Cremer et al. 1993; Ferreira et al. 1997; S. Kozubek et al. 1997, 1999; Croft et al. 1999; Sadoni et al. 1999; Skalníková et al. 2000); however, restricted information is available concerning higher-order chromatin structure in segmented granulocytes (Sanchez et al. 1997; Grigoryev and Woodcock 1998; Sanchez and Wangh 1999; Alcobia et al. 2000). It was found that cell nuclei are compartmentalised in spherical cells; chromatin in interphase cell nuclei is arranged in spatially separated chromosome territories (Manuelidis 1985; Lichter et al. 1988; Leitch et al. 1990; T. Cremer et al. 1993; C. Cremer et al. 1996; Zinket al. 1998; Münkel et al. 1999). Higher-order compartments of nuclear chromatin have been defined according to replication timing, transcriptional activity and information content (Ferreira et al. 1997; Sadoni et al. 1999). We have found that the *Abl*, *Bcr* and *p53* genes are located mostly in the interior of cell nuclei, especially in comparison with the corresponding centromeric sequences located near the nuclear periphery. Such observations are in accordance with the definition of the higher-order compartments (S. Kozubek et al. 1997, 1999; Bártová et al. 2000a; Skalníková et al. 2000).

Our experiments on the higher-order chromatin structure of human granulocytes, mainly the neutrophils, represent a continuation of the observations of Sanchez et al. (1997) and Sanchez and Wangh (1999). These authors focused their studies on the nuclear location of chromosomes X and Y and the degree of condensation of chromosomes 2 and 18. They not only described the location of the X and Y chromosomes on nuclear appendages, but also found peripheral positioning of centromeres and suggested a new model of the mechanisms governing nuclear filament formation in neutrophils. In addition, the presence of different chromocentres (spatial associations of centromeric heterochromatin), "myeloid" (in monocytes and granulocytes) and "lymphoid", was detected by Alcobia et al. (2000).

In this article we look at the nuclear positioning of the *Abl*, *Bcr*, C-*myc* and *p53* genes, and the centromeric regions of chromosomes 3, 6, 7, 8, 9 and 17, including the location of all human chromosome territories in the two-lobed nuclei of granulocytes, mainly neutrophils. Five topographic types in two-lobed nuclei were distinguished in dual-colour fluorescence in situ hybridisation (FISH) experiments. For a single type of chromosome and two-lobed nuclei, we observed the prevalence of symmetric patterns of chromosome segregation. In parallel, the membrane-to-chromosome distances were measured for all chromosomes and the average values were calculated. A higher percentage of symmetric topographic types was observed in chromosomes that were located closer to the nuclear membrane. In several cases the two-dimensional (2D) results were confirmed using three-dimensional (3D) analysis of paraformaldehyde-fixed cells.

The peripheral relocation of many chromatin elements observed during granulopoiesis (Bártová et al. 2000a, b) was also found in the experiments presented in this paper.

In rare cases, the *Abl*, *Bcr*, C-*myc* and *p53* genes and the tested centromeres, 3, 6, 9 and 17, were located in the granulocytic filaments. In addition, we found that chromosomes may extend through a filament, which is illustrated for chromosomes 3 and 17 detected using 3D analysis. Our studies contribute to the knowledge of the higher-order chromatin structure of segmented human peripheral blood neutrophils whose chromatin arrangement is still poorly understood.

Materials and methods

Fractionation and isolation of human peripheral blood neutrophils

Dextran solution was added to heparinised human peripheral blood. The samples were kept at room temperature for 20–60 min to allow the sedimentation of red cells. Leukocyte-rich plasma on the top of the red cell fraction was carefully collected and centrifuged at 300 *g* at room temperature for 8 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml of PBS. A layer of Ficoll-Hypaque solution (5 ml) was carefully placed on the cell suspension. The cells were centrifuged at 400 *g* for 30 min at 4°C. A buffy coat containing lymphocytes and monocytes and appearing as a white, cloudy band between the plasma and Ficoll-Hypaque was collected. The granulocytic fraction under the Ficoll-Hypaque layer was fixed for FISH experiments.

Cell cultivation and differentiation

The human leukaemic promyelocytic cell line HL-60 was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). The cells were cultivated in RPMI-1640 medium, supplemented with 10% fetal calf serum (Pan Systems, Germany), at 37°C in a humidified atmosphere containing 5%

CO_2 up to 25 passages. The leukaemic cells at an initial cell density of 2×10^5 cells/ml were treated with the differentiating agent dimethyl sulfoxide (DMSO, 1.25%; Sigma). Undifferentiated HL-60 cells were used as a control population of progenitor blood cells.

Fluorescence in situ hybridisation

Cytological preparationswere made using cell fixation by standard cytogenetic procedures (methanol:acetic acid mix, 3:1) or using paraformaldehyde. Paraformaldehyde (three dimensionally) fixed cells were analysed in order to verify the results obtained from two dimensionally fixed cells. We avoid the dehydration of the nuclei to preserve the native 3D structure. The cells were fixed with buffered 4% paraformaldehyde (5 min), washed twice in PBS and permeabilised using 0.1 M HCl (10 min), then the samples were treated with 0.5% Triton X-100 and 0.2% saponin (10 min), and finally, equilibration was performed in 20% glycerol in PBS (20 min) and the cells were frozen in liquid nitrogen. The target DNA in the cells was denatured in 70% formamide in PBS, pH 7.0 (3 min). The DNA probe for unique sequences of the *Abl*, *Bcr*, C-*myc* and *p53* genes and probe for alpha-satellite DNA sequences of the centromeric regions of chromosomes 3, 6, 7, 8, 9 and 17 were purchased from Oncor (USA). Total DNA probes (Star-FISH paint box) for all chromosomes were obtained from Cambio (UK). Probe preparation was carried out in accordance with the instructions of the manufacturer. The hybridisation procedure was similar to that described in Kurz et al. (1996) and S. Kozubek et al. (1997). Fast-FISH (Durm et al. 1998) was used for the detection of centromeric regions. This cytogenetic procedure utilises a thermal denaturation step at 96°C and avoids formamide treatment.

Repeated fluorescence in situ hybridisation

The repeated hybridisation method was used to detect the positions of four sets of genetic structures in the same granulocyte nucleus. The preparations were hybridised using the dual-colour *Abl/Bcr* probe and subsequently using a dual-colour probe for the centromeric regions of chromosomes 6 and 17. Both pairs of DNA probes were digoxigenin and biotinyl labelled. The images of both hybridisations were acquired using the relocation feature of FISH 2.0 software, stored electronically and evaluated in pairs (M. Kozubek et al. 1999).

Image acquisition and analysis

The high-resolution cytometer developed in our laboratory (M. Kozubek et al. 1999) comprised a DMRXA (Leica, Germany) microscope [immersion objective 100×, filters for Rhodamine and 4',6-diamidino-2-phenylindole (DAPI)]. A Quantix (Photometrics, Tuscon, Ariz.) CCD camera (pixel size 9.3 µm) and 2×Pentium II 266 MHz computer (256 MB RAM and 2×9.1 GB HDD) were used for 2D analyses. The 3D analyses were performed using a Zeiss Axiovert 100 microscope (Germany) with a Carv confocal unit (Atto Instrument, USA). The images were captured with a fully programmable digital CCD camera Micromax (Princeton Instruments). The final results were obtained from 40 optical sections (axial step 0.3 µm) that were analysed. Both cytometers were computer controlled; acquisition and analysis were performed using FISH 2.0 software. The image analysis procedure has been described elsewhere (see S. Kozubek et al. 1997; Lukášová et al. 1997; M. Kozubek et al. 1999). In short, the nuclei are segmented using thresholding based on the intensity histogram analysis. The genes (centromeres, chromosomes) were found using a modified watershed algorithm. The threshold was calculated using a histogram in a counterstain colour as a local minimum between two maxima corresponding to the background and to the nuclear signal. The boundaries of the nuclei found using this method corresponded well to the actual boundaries detected with anti-lamin B antibodies. In addition, comparative experiments with HL-60 cells and granulocytes were always performed in parallel (the cells were fixed, denatured, hybridised, counterstained and analysed together). Consequently, thresholding was applied in very similar conditions. Experiments were repeated; therefore, the influence of different intensities of FISH signals as well as the influence of thresholding procedures on the results should be to some extent eliminated.

The detection of hybridisation signals can be set by various parameters defined by the user. The user can select, for example, the minimal size of nuclei, their maximal roundness, the minimal height of hybridisation dots, their maximal size, minimal intensity, etc. The positions of individual genes (centromeres, chromosomes) are calculated in relation to the nuclear weight centre in many experiments and all data are stored electronically. Genes, centromeres and chromosomes were represented by fluorescence weight centres. All further calculations were performed in the coordinate system in which the weight centre of the nucleus is placed at the origin.

For the purpose of this paper, a special topographic gene (centromere and chromosome) parameter was computed, namely the membrane-to-gene (centromere, chromosome) distance (MG, MN, MH). This parameter was measured as the Euclidean distance between the given gene (centromere, chromosome) and the nearest point of the nuclear boundary. Such analysis is appropriate for pyknotic nuclei of irregular shape such as neutrophils.

Our analysis of the nuclear location of female sex chromosomes was based on the results published by Mittwoch (1964), who described the drumstick-containing inactive X chromosome of human granulocytes located either as a sessile nodule on the nuclear envelope or outside the neutrophil nuclei to which it is connected with a thin filament. Bearing this in mind, the distances from the membrane for both X chromosomes were determined and the domain located closer to the nuclear envelope was considered to be a drumstick.

Statistical evaluation of the results

The gene-to-gene distance ($\overline{GG}$), the nearest point on the nuclear membrane-to-gene distance (MG), centromere-to-centromere distance (NN), the nearest point on the nuclear membrane-to-centromere distance (MN), chromosome-to-chromosome distance (HH), and membrane-to-chromosome distance (MH) were determined and normalised to cell size (average cellular radius calculated as $\sqrt{\text{Area}/\pi}$). The average cellular radius calculated as $\sqrt{\text{Area}/\pi}$) was used in order to characterise the linear dimensions of cell nuclei. In our experience, the relative quantities (parameters normalised to nuclear radius) are well conserved although the radii of cells may differ. Therefore, for human granulocytes also we introduced quantities normalised to the average cellular radius.

The average values of the above-mentioned topographic parameters were determined from at least three independent experiments in which about 500–1000 nuclei per experiment were analysed in 2D projection and again averaged. In 3D experiments 200–400 nuclei were analysed. For the segregation analyses nuclei with lobes of considerably different volumes were excluded. The comparison of different data sets was done using Student's t-test option of SigmaPlot (Jandel Scientific, Calif.). Standard errors shown in tables are standard deviations of the mean values ($SE=SD/\sqrt{n}$ where n is the number of observations). The inter-connection between the probability of the occurrence of symmetric (non-symmetric) types of chromosome location and membrane-to-chromosome distances was analysed using Linear Regression Options in SigmaPlot 2000 software, including 99% confidence and 99% prediction intervals.

The probabilities of the occurrence of a particular topographic type for a two-lobed nucleus in the case of dual-colour probes can be calculated assuming random segregation of chromosomes into the lobes or non-random segregation (the probabilities for individual chromosomes can be determined independently). Let the probability of symmetric segregation for the i-th chromosome be p_i. Then the probability of the formation of a symmetric topographic

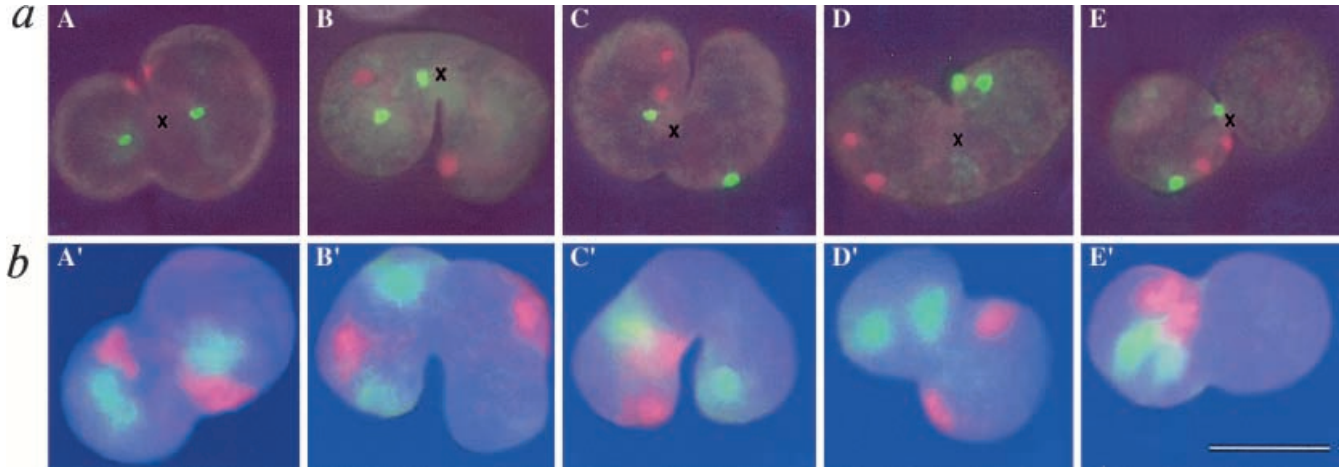

Fig. 1 Five topographic types (*A–E*) of granulocytic nuclei visualised using dual-colour fluorescence in situ hybridisation (FISH) for the centromeric regions of chromosome 3 (*red*) and chromosome 17 (*green*) can be seen in **a**. The *crosses* indicate the constriction between the two lobes of granulocytic nuclei. Similar topographic types (*A'–E'*) were found using total chromosome painting probes for chromosome 14 (*green*) and chromosome 18 (*red*) (**b**). This and subsequent colour figures are presented using Photoshop 5.0 LE. *Bar* represents 2.5 µm

Table 1 Topographic types (%) of neutrophils defined by dual-colour fluorescence in situ hybridisation (FISH) in two-lobed nuclei. The values in brackets are theoretical estimations ±SE calculated from segregation probabilities of individual chromosomes (see Materials and methods); SE=SD/√n where n is the number of observations. (G, green; R, red)

Genetic structures	Type A	Type B	Type C	Type D	Type E
Centromeres 3^R and 17^G	46.1±0.7* (35.2±0.6)	13.5±0.8* (24.3±0.7)	13.1±0.7* (23.9±0.8)	12.8±0.7* (8.3±0.8)	14.5±0.8* (8.3±0.6)
Centromeres 6^R and 9^G	46.5±1.5* (36.3±1.3)	14.7±1.4* (24.9±1.3)	12.6±1.5* (23.0±1.3)	12.0±1.5 (7.9±1.2)	14.2±1.3* (7.9±1.3)
Genes *Bcr*^{R}/*Abl*^{G}	41.1±0.9* (31.8±0.8)	16.6±0.9* (23.3±0.9)	14.1±0.8* (25.9±0.8)	16.0±0.7* (9.5±0.8)	12.2±0.8 (9.5±0.9)
Chromosomes 18^R and 14^G	43.7±1.5* (36.8±1.5)	17.2±1.5* (23.6±1.4)	16.7±1.5* (24.2±1.4)	10.9±1.4 (7.7±1.5)	11.5±1.4 (7.7±1.5)

* Statistically significant for $P \leq 0.05$ (evaluated using Student's t test for independent experiments)

type for both chromosomes is $p_i p_j$, (type A), the probability of the combination of non-symmetric (for one chromosome) and symmetric (for the other) will be $p_i(1-p_j)$ and $p_j(1-p_i)$ (types B and C), and the probability of the formation of non-symmetric type for both chromosomes in the same lobe (opposite lobes) will be $(1-p_i)(1-p_j)/2$ in both cases (types D and E). For the special case in which both probabilities p_i and p_j are 0.5 (random segregation of both chromosomes), the resulting occurrences will be 25% for types A, B, and C and 12.5% for types D and E (Table 1, Fig. 1). The standard errors of p_i and p_j determined from the experiments were used for the calculation of the errors of theoretical values in Table 1. The statistical significance of the differences was evaluated using Student's t test for different independent experiments.

Results

Our experiments investigated the topography of human peripheral blood granulocytes, mainly neutrophils. The two-lobed nuclei were hybridised using dual-colour DNA probes and divided into two parts according to the constrictions between the lobes corresponding to the positions of connecting filaments. We found non-random segregation of genetic elements into the lobes of granulocytes, with symmetric topographic types being more frequent (each element in one lobe).

Five topographic types (types A, B, C, D and E, see Fig. 1a, b) were distinguished using dual-colour hybridisation for several pairs of genetic structures: the *Abl* (on chromosome 9) and *Bcr* (on chromosome 22) genes (not shown); centromeres of chromosomes 3 and 17 (Fig. 1a); centromeres 6 and 9 (not shown); and chromosomes 14 and 18 (Fig. 1b). The majority of values calculated for types A, B, C, D and E (see also Table 1) are significantly different ($P \leq 0.05$) from theoretical predictions obtained on the assumption of independent segregation of genetic elements (see Materials and methods). Comparison of the experimental results with the theoretical values ±SE (Table 1) indicates the prevalence of symmetric types of location of both chromosomes (type A in Fig. 1a, b) and, on the other hand, decreased occurrence of the combination of symmetric and non-symmetric types (B and C in Fig. 1a, b and Table 1). The differences between the experimental and theoretical probabilities of the occurrence of types D and E are also statistically significant in many cases. The prevalence of some

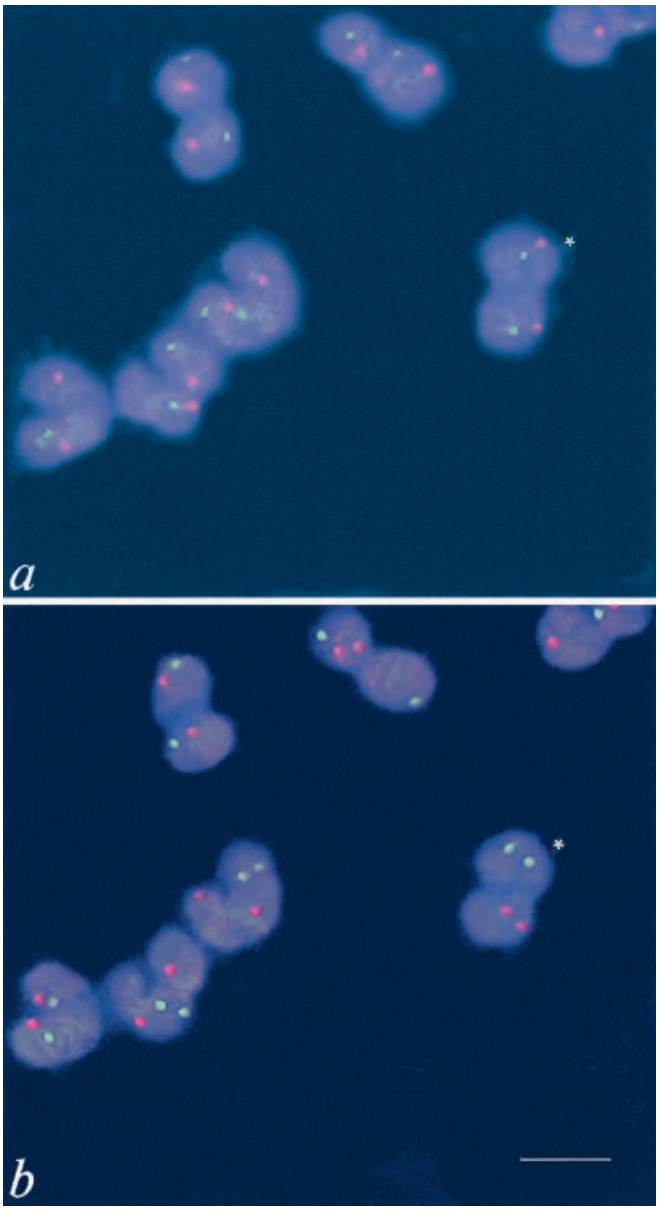

Fig. 2a, b The results of repeated FISH. The *Abl*/*Bcr* (*green/red*, respectively) dual-colour DNA probe was used in the first hybridisation (**a**) and the cen6/cen17 (*red/green*, respectively) dual-colour DNA probe was used in the second hybridisation (**b**). The images were stored electronically and subsequently evaluated simultaneously. The topographic types defined by the two dual-colour FISH experiments are combined to produce a large number of topographic types of higher order. *Bar* represents 5.0 µm

topographic types might reflect the asymmetry of chromosome distribution in the cell nucleus at the moment of chromosome segregation into the lobes.

In the experiments with repeated FISH the location of the *Abl* (on chromosome 9) and *Bcr* (on chromosome 22) genes was determined in the first FISH (Fig. 2a) and the location of centromeric regions of chromosomes 6 and 17 (Fig. 2b) was detected in the second hybridisation. The topographic types from both hybridisations were compared in individual cells usingthe reallocation option of our microscope system. We conclude from this observation that the topographic types from the first hybridisation (see the asterisk indicating type A in Fig. 2a) are not identical with the topographic types from the second FISH in many cases (see the asterisk indicating type D in Fig. 2b). This observation reveals the existence of a large number of topographic types (although not all combinations were found). Therefore, the term "topographic type" seems to be more appropriate than the term "morphological type". For genes, centromeres and chromosomes the symmetric distribution into lobes is more frequent than the non-symmetric one. In addition, for dual-colour experiments, topographic type A (symmetric) (see Table 1) is again much more frequent than predicted from the probabilities obtained for individual genetic elements. These experiments indicate that genetic structures tend to be symmetrically distributed into thelobes of granulocytic nuclei.

Human peripheral blood neutrophils consist of two to five segments that are connected by thin, short DNA-containing filaments. In the case of the dual *Abl*/*Bcr* probes, centromeric 3/17, 6/9 probes and C-*myc* and *p53* gene detection, the genetic structures investigated were occasionally also positioned in the connecting filaments that were clearly visible after DAPI staining. Staining the nuclei of granulocytes using DAPI helps to reveal the boundaries between the nuclear lobes. There are thin filaments (or constrictions) connecting the lobes, which are clearly visible in two-lobed and three-lobed nuclei after DAPI staining. An important observation was that in two dimensionally fixed cells the filaments are oriented horizontally (in parallel with slides) and consequently the lobes are well separated. After the denaturation step of hybridisation the filaments are hardly visible but their position corresponds to the constriction among the lobes. In several cases chromosomes were found to extend through a filament. As an example of a 3D image we present the chromosome 3 domain extended through a filament (see Fig. 3). We have actually found nuclei in which: firstly, the domain of a chromosome passes through the filament and is located partially in two lobes of the nucleus (Fig. 3). Secondly, the fluorescence gravity of the domain is located in a different lobe than a gene molecularly located on the same domain (Fig. 4). In more than 4000 nuclei of neutrophils (analysed for filament conformation) a low percentage of the studied genetic structures were found at the site of constriction, corresponding to a filament clearly visible after DAPI staining: *Abl* (0.37%), *Bcr* (0.58%), C-*myc* (0.6%), *p53* (0.45%), centromere of chromosome 3 (0.23%), centromere 6 (0.025%), centromere 9 (0.15%) and centromere 17 (0.17%). It appears that the presence of genes on the filaments is more frequent than centromeric regions.

In further experiments we systematically analysed the segregation of 19 homologous chromosomes into the lobes of two-lobed nuclei (Table 2). In our dual-colour experiments (Table 1), symmetric location (each chromosome in one nuclear lobe) is presented as types A and B for red FISH signals (Fig. 1a, b and Table 1) or types

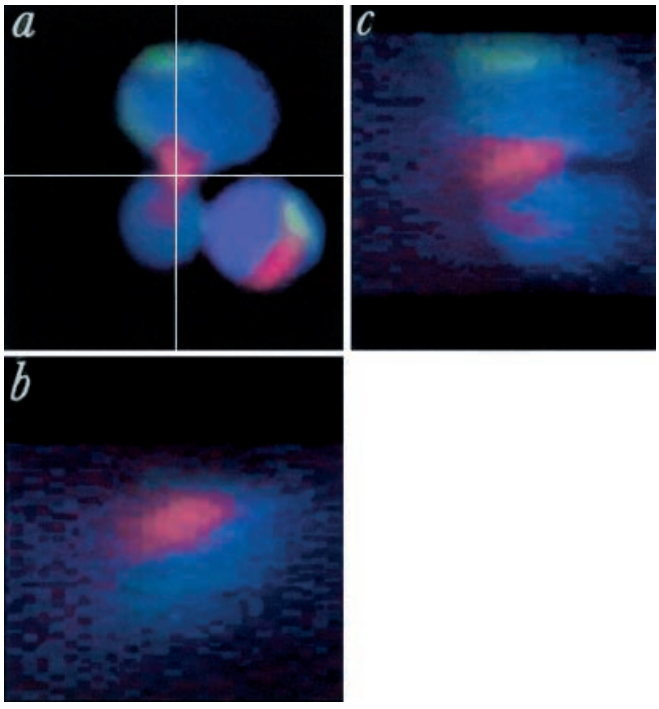

Fig. 3a–c Three-dimensional (3D) image of nuclear location of chromosomes 3 (*red* fluorescent signals) and 17 (*green* fluorescent signals) in human peripheral blood neutrophils. The following projections are presented: *x–y* (**a**), *x–z* (**b**) and *y–z* (**c**). The projections were obtained from 40 sections (axial step 0.3 µm) using a confocal cytometer and FISH 2.0 software. As can be observed, chromosome 3 extends through the filament connecting the granulocytic lobes

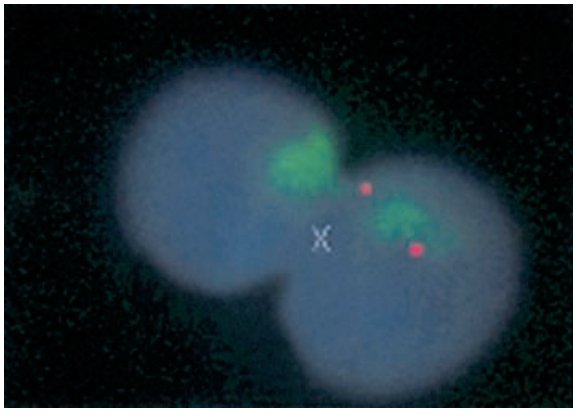

Fig. 4 Nuclear location of chromosome 8 domain and the C-*myc* gene (8q24). The C-*myc* gene is located in a different lobe from the fluorescence gravity centre of its chromosome domain

A and C for green FISH signals (Fig. 1a, b). Non-symmetric location (both chromosomes in one segment) is presented as types C, D and E for red FISH signals or types B, D and E for green FISH signals (Fig. 1a, b and Table 1). On average we found 65.5±1.2% of symmetric cases and 34.5±1.2% of non-symmetric types of chromosome location, and on average 58.8±3.4% of symmetric types and 41.2±3.4% of non-symmetric positioning of

Table 2 Symmetric and non-symmetric location of chromosomes in human neutrophils

Chromosome	Symmetric location (%)	Non-symmetric location (%)
1	62.0±1.1	38.0±1.1
2	67.6±1.9	32.4±1.9
3	59.6±2.1	40.4±2.1
4	72.6±3.5	27.4±3.5
5	70.0±1.5	30.0±1.5
6	61.2±1.8	38.8±1.8
7	70.4±2.9	29.6±2.9
8	73.8±3.8	26.2±3.9
9	61.0±1.2	39.0±1.1
10	67.1±4.1	32.9±4.1
11	68.2±0.9	31.8±0.9
13	67.8±0.9	32.2±0.9
14	60.4±2.0	39.6±2.0
15	61.5±2.6	38.5±2.6
16	75.8±2.0	24.2±2.0
17	59.2±1.5	40.8±1.5
18	60.9±2.9	39.1±2.9
20	60.5±2.0	39.5±2.0
22	64.0±2.7	36.0±2.7

genes like *Abl*, *Bcr*, C-*myc* and *p53*. In parallel, the membrane-to-chromosome fluorescence gravity centre distances were determined in 2D projection and the average values were calculated for each chromosome. Correlation between the frequency of occurrence of the symmetric type of chromosome location and membrane-to-chromosome distance (MH) was found. The data were analysed using the linear regression model (Fig. 5). The empirical values were close to the theoretical regression straight line, which is shown with 99% confidence and prediction intervals (Fig. 5). The correlation coefficient was 0.47, which is significant for 21 *df* at $P \leq 0.05$. These results indicate that the segregation of chromosomes into the nuclear lobes might at least be partially related to other topographic features of chromosomes.

The results of measurement of the nuclear membrane-to-chromosome distances for all human chromosomes are shown in Table 3. The fluorescence gravity centres of chromosomes 15, 17, 19, 21 and 22 were located more centrally and chromosomes 3, 6 and 16 more peripherally. The nuclear location of sex chromosomes was also detected. The average distance of the drumstick-containing X chromosome from the membrane was 15.1±0.7%; the same parameter for the second female X chromosome was 45.7±1.3%. The male X chromosome was located at a distance of 20.6±0.6% from the nuclear membrane, surprisingly closer to the membrane than the second female X chromosome. This could be due to different transcriptional activities of female and male X chromosomes. The nuclear location of the Y chromosome was 23.0±1.6% from the nuclear membrane, which is similar to that of the male X (Table 3).

The topographic results obtained through 2D analyses (Table 3) were also checked by 3D analyses of three dimensionally fixed cells. An example is shownfor chromosomes 3 and 17 in which we also found that the fluo-

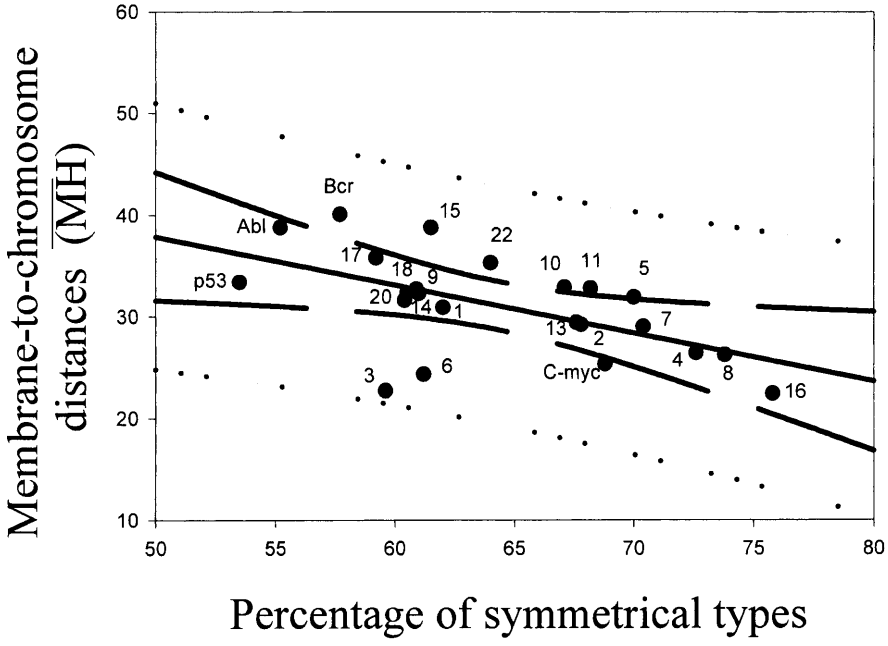

Fig. 5 The relationship between the percentage of symmetric topographic types and the membrane-to-chromosome distance (MH). The linear regression analysis showed a clear correlation between both parameters (correlation coefficient r=0.47 for 21 df, $P \leq 0.05$). The 99% confidence (*long-dashed line*) and 99% prediction intervals (*dotted line*) are shown

Table 3 Nuclear location of chromosomes in human peripheral blood neutrophils

Type of chromosome		Membrane-to-chromosome average distances (MH) (%)
Autosome	1	36.9±0.5
	2	29.4±0.8
	3	22.0±0.7
	4	26.4±0.6
	5	31.9±0.8
	6	23.7±1.9
	7	29.0±0.8
	8	26.2±0.7
	9	32.3±0.6
	10	33.0±1.2
	11	32.8±1.1
	12	27.3±0.8
	13	29.2±0.6
	14	31.6±1.0
	15	38.8±0.9
	16	22.4±1.3
	17	35.8±0.8
	18	32.7±1.0
	19	43.0±1.6
	20	32.3±0.8
	21	39.8±1.7
	22	35.3±1.5
Sex chromosome	Female drumstick (chromosome X)	15.1±0.7
	Female second chromosome X	45.7±1.3
	Male X chromosome	20.6±0.6
	Chromosome Y	23.0±1.6

rescence gravity centre of chromosome 3 is located more peripherally than that of chromosome 17 (see Fig. 6).

In further observations we found that many genetic structures were located closer to the nuclear periphery during granulopoiesis (Table 4). Nuclear repositioning of the *Abl*, *Bcr* and C-*myc* genes was detected in DMSO-differentiated HL-60 leukaemic cells (granulocytic pathway of cell maturation in vitro). Similarly, the genes were located more peripherally in human blood neutrophils in comparison with the undifferentiated HL-60 population. This peripheral reallocation was most pronounced for the C-*myc* gene, which was found (applying both 2D and 3D analyses) to be closer to the nuclear envelope (Table 4). The repositioning of the genetic elements to the nuclear periphery during differentiation was not detected in some cases. We did not observe such movement for centromere 9 in DMSO-differentiated leukaemic cells (Table 5) nor for some chromosomes (Bártová et al. 2000a).

Discussion

The nuclear topography of chromatin structures such as genes, centromeric regions and whole chromosomes has been studied in a variety of experiments (Manuelidis 1985; Lichter et al. 1988; Leitch et al. 1990; T. Cremer et al. 1993; C. Cremeret al. 1996; S. Kozubek et al. 1997; Zink et al. 1998; S. Kozubek et al. 1999; Münkel et al. 1999); however, there is a lack of information available on the higher-order structure of segmented granulocytic nuclei (Sanchez et al. 1997; Grigoryev and Woodcock 1998; Sanchez and Wangh 1999; Alcobia et al. 2000). The granulocytes represent a group of white blood cells that can be divided into three subgroups: neutrophils (40%–60% of white blood cells), basophils (0.5%–1 %) and eosinophils (2%–5%) (Begemann and Rastetter 1989). These cell populations are characterised by segmented nuclei with a low level of transcription as a result of a reduced nucleolus and endoplasmic reticulum (Edwards 1994). Moreover, some neutrophils have a remarkably located condensed, inactive X chromosome.

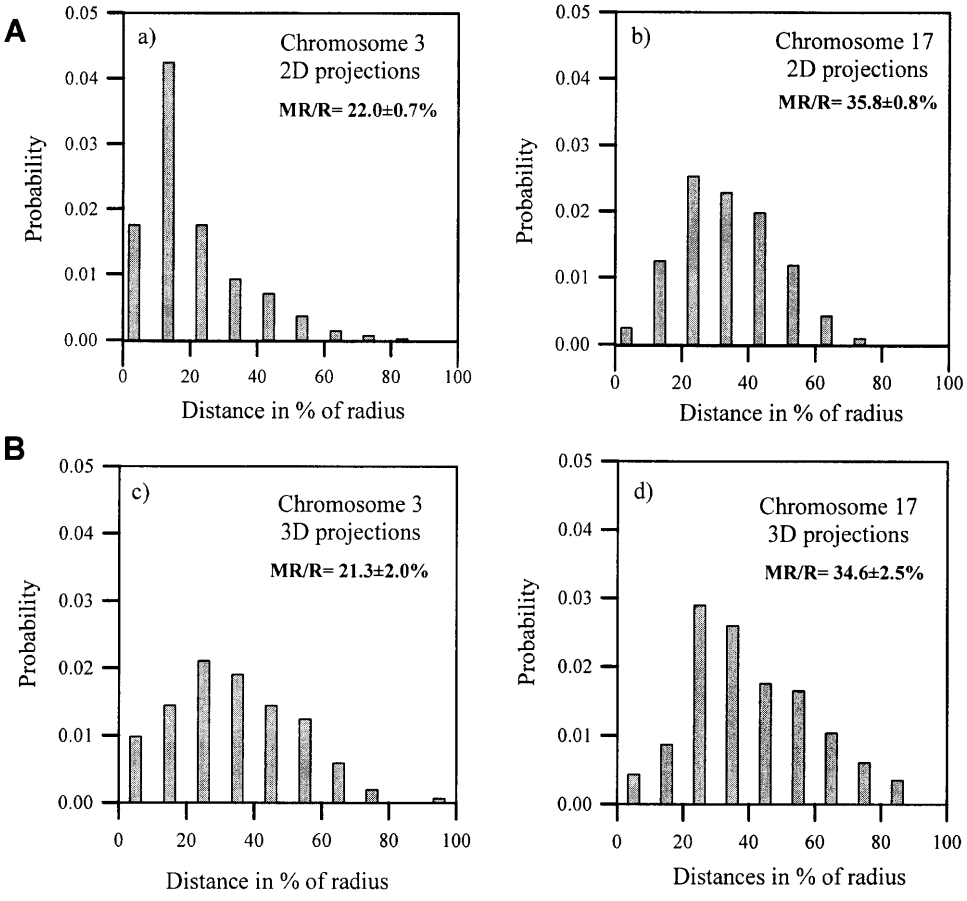

Fig. 6A, B Comparison of the results of 2D and 3D analyses for membrane-to-chromosome distances. **A** The 2D distributions of the membrane-to-chromosome fluorescence gravity centre distances for chromosomes 3 (panel *a*) and 17 (panel *b*) in human peripheral blood granulocytes. **B** The 3D distributions of the membrane-to-chromosome fluorescence gravity centre distances for chromosomes 3 (panel *c*) and 17 (panel *d*). The results of 2D analysis are in accordance with the nuclear location determined by 3D analysis (chromosome 3 is located more peripherally than chromosome 17)

Table 4 The topography of the *Abl*, *Bcr* and C-*myc* genes during in vitro-induced granulopoiesis and in human peripheral blood neutrophils. The table shows the gene-to-gene distances (GG±SE) and the nearest point on the nuclear membrane-to-gene distances (MG±SE) determined in the control HL-60 leukaemic cell population, in differentiated HL-60 cells and in human peripheral blood granulocytes. The average radius of HL-60 control cell nuclei is 7.2±0.14 µm. The average radius of DMSO-differentiated HL-60 cell nuclei is 5.6±0.13 µm and the average radius of neutrophil nuclei is 5.6±0.14 µm

Gene	HL-60 promyelocytic cells (undifferentiated)		HL-60 cells differentiated into neutrophils		Human peripheral blood neutrophils	
	$\overline{(GG)}$ (%)	$\overline{(MG)}$ (%)	$\overline{(GG)}$ (%)	$\overline{(MG)}$ (%)	$\overline{(GG)}$ (%)	$\overline{(MG)}$ (%)
Abl (2D)	77.9±0.9	51.0±1.3	82.3±2.7	47.1±1.6	83.7±0.5*	38.1±1.1*
Bcr (2D)	68.2±0.7	49.3±1.9	80.8±2.6*	40.1±0.2*	73.4±1.8*	40.1±1.2*
C-*myc* (2D)	94.8±1.4	34.3±1.2	100.3±1.1*	24.6±1.1*	102.2±1.6*	26.2±0.9*
C-*myc* (3D)	106.8±1.4	27.2±0.9	–	–	110.9±1.3	21.0±1.4*

* Student's t test was applied for statistical analysis with statistical significance at $P \leq 0.05$

Table 5 The nuclear location of centromeres 7, 8 and 9 during in vitro-induced granulopoiesis and in human peripheral blood neutrophils. The table shows the centromere-to-centromere distances (NN±SE) and the nearest point on the nuclear membrane-to-centromere distances (MN±SE) for the control HL-60 leukaemic cell population, in differentiated HL-60 cells and in human peripheral blood granulocytes. The average radius of HL-60 control cell nuclei is 7.2±0.14 µm. The average radius of DMSO-differentiated HL-60 cell nuclei is 5.6±0.13 µm and the average radius of neutrophil nuclei is 5.6±0.14 µm

Centromere	HL-60 promyelocytic cells (undifferentiated)		HL-60 cells differentiated into neutrophils		Human peripheral blood neutrophils	
	$\overline{(NN)}$ (%)	$\overline{(MN)}$ (%)	$\overline{(NN)}$ (%)	$\overline{(MN)}$ (%)	$\overline{(NN)}$ (%)	$\overline{(NN)}$ (%)
Centromere 7 (2D)	103.9±2.2	24.0±0.8	109.8±3.9	15.8±1.0*	113.7±5.7	22.4±1.4
Centromere 8 (2D)	87.5±0.2	33.9±1.4	112.6±3.6*	19.8±1.0*	99.0±5.3*	24.8±1.4*
Centromere 9 (2D)	91.4±1.7	37.6±0.6	93.8±0.3	36.3±1.1	95.6±1.3	31.3±0.8*

* Student's t test was applied for statistical analysis with statistical significance at $P \leq 0.05$

The drumstick (analogous to the Barr body in epithelial and other tissue cells), an inactivated female X chromosome, can be located outside the nucleus but as a sessile nodule on the membrane (Mittwoch 1964; Alberts at al. 1994). In some neutrophils, the drumstick is connected with the cell nucleus by a thin chromatin filament. This fact indicates that the suppression of transcription can be related not only to chromatin condensation, but also to the relocation of the condensed chromatin structure closer to the nuclear periphery (or even outside the nucleus), which accompanies granulocytic differentiation (Bártová et al. 2000a, b).

Our work provides basic information about the higher-order chromatin structure of segmented human blood granulocytes, mainly neutrophils. We have found that the segregation of chromosomes into the lobes of segmented nuclei leads to a large number of topographic types. Using dual-colour FISH we have defined five topographic types (A–E in Fig. 1a, b) for two-lobed granulocytes. In experiments with repeated hybridisation in which four sets of genetic elements were visualised, 26 types were distinguishable. Some configurations occur frequently, the others do not occur at all. For example, the symmetric configuration of all four sets of genetic elements (*Abl*/*Bcr*/cen6/cen17+*Abl*/*Bcr*/cen6/cen17) (cen6, cen17 are abbreviations for the centromeric regions of the respective chromosomes) in every two lobes is three times more frequent than predicted by the method described in Materials and methods. The configuration (*Abl*/*Abl*/cen17/cen17+*Bcr*/*Bcr*/cen6/cen6) is substantially more frequent (eight occurrences instead of the predicted three). These observations suggest that the segregation of chromosomes into the lobes of segmented nuclei is not random; however, a large number of topographic types is observed.

The centromeres and genes studied in our experiments were also found in the chromatin filaments connecting granulocytic segments, but the incidence was very low (0.025%–0.6% in more than 4000 cells analysed). In contrast to the results of Sanchez et al. (1997) we found that centromeric DNA and genes are located in the filaments connecting neutrophil lobes. An explanation of this discrepancy is that with the aid of Ficoll-Hypaque gradients we were able to obtain large quantities of neutrophils, which is impossible using blood smears (Sanchez et al. 1997). Another explanation is that the location of genetic structures may vary among individuals. According to Sanchez et al. (1997), only a subset of chromosomes participates in filament formation. A model of filament formation and the possible presence of filament control factor was suggested by Sanchez and Wangh (1999). However, the questions remain: under what circumstances is filament formation initiated and what is the reason for granulocytic segmentation. One explanation is that the segmented shape is more advantageous for diapedesis of neutrophils through the epithelium of blood vessels. It is possible that drumstick formation may also control filament assembly during differentiation (Sanchez et al. 1997).

Previous experiments showed that nuclear positioning of chromosomes and other genetic elements is non-random (Hulspas et al. 1994; C. Cremer et al. 1996; Ferreira et al. 1997; S. Kozubek et al. 1997; Skalníková et al. 2000). Therefore, the non-random segregation of chromosomes and other genetic elements into the nuclear lobes seems probable. We found that for genes, centromeres and chromosomes the symmetric distribution into lobes is more frequent than non-symmetric location. In addition, in dual-colour experiments topographic type A (symmetric) (see Table 1) is again found to be much more frequent than predicted from probabilities obtained for individual genetic elements. In our experiments with single-chromosome painting the prevalence of symmetric types (one chromosome in each symmetric part) was observed. The occurrence of the symmetric topographic types was correlated with the nearest distances of the chromosome fluorescence gravity centres to the nuclear membrane. The correlation was fitted using a linear regression model (Fig. 5). This finding suggests that the probability of the occurrence of specific topographic types might be related to some other topographic features, e.g. to the initial positioning of chromosomes at the moment of cell nucleus segmentation.

For chromosomes, symmetric topographic types were detected more frequently than for genes located on the same chromosome. The segregation ratio between symmetric and non-symmetric topographic types was near to the random distribution (1:1) for genes. Our explanation is that chromosome territories are rather extended entities of large volume and consequently tend to segregate into neighbouring lobes of granulocytic nuclei. On the other hand, genes located on large chromatin loops segregate more randomly owing to the possibility that they can extend outwards from the chromosomal domain. This explanation is supported by the results of Volpi et al. (2000), who published the large-scale chromatin organisation of the major histocompatibility complex and other regions of chromosome 6. The authors found that large chromatin loops containing several megabases of DNA were observed extending outwards from the surface of the domain defined by the specific chromosome 6 paint. The frequency with which a genomic region was observed on an external chromatin loop was cell type dependent and appeared to be related to the number of active genes in the region. It is clearly possible that in our experiments we detected the same phenomenon with the genes located on large thin chromatin loops (Sachs et al. 1995) spreading into different lobes of granulocytes.

Our findings lead to a number of important questions: could the non-symmetric types be a disadvantage in granulocyte survival or in ability to participate in phagocytosis? Why are there so many topographic types of chromosome location in granulocytic nuclei and might responses to different pathogens require different topographic types of granulocytes?

The relocation of many genetic structures closer to the nuclear envelope under certain conditions was observed in our experiments. This relocation is even more pronounced if absolute (instead of relative) values are

taken. For example, the average membrane-to-C-*myc* gene distances (to the nearest point on the nuclear membrane) are reduced from 2.4 μm in HL-60 cells to 1.4 μm in DMSO-differentiated cells and 1.5 μm in peripheral blood neutrophils. The differentiated cells are, however, smaller (see legend to Table 4) and therefore normalised distances were used throughout the experiments. The relative parameters are conserved for cells of the same culture with different sizes. The normalised parameters indicate similar reallocationof genetic structures to the nuclear periphery during differentiation. In addition, the complex nuclear shape may also to some extent influence the values of topographic parameters. Therefore, the results should be compared carefully. However, the reduction of membrane-to-locus distances differs for various genetic elements (e.g. centromeres 8 and 9 in Table 5), which means that if some of these changes can be attributed to changes in nuclear shape, the others should represent actual distance reduction.

The literature suggests that a more peripheral location of genetic elements is connected with decreased transcriptional activity of cells (reviewed by Francastel et al. 2000). In addition, gene silencing can be regulated by the centromeric heterochromatin, which forms myeloid chromocentres and is located on the nuclear periphery of human granulocytes (Alcobia et al. 2000). Stable expression of a transgene requires an intact enhancerthat maintains its localisation away from centromeric heterochromatin (Francastel et al. 1999). Nuclear compartmentalisation and gene activity have been discussed by many authors (Kurz et al. 1996; Francastel et al. 2000; Schübeler et al. 2000). Changes in higher-order chromatin structure could be an important aspect of the process of gene silencing that is most extensive during the differentiation of haematopoietic cells (Francastel et al. 2000).

In addition to the observation of chromosome segregation into segments of granulocytic nuclei, the distances between individual chromosomes and distances of all chromosomes from the nuclear membrane were determined with respect to the fluorescence gravity centres of chromosomes. The results are in agreement with those of other authors (Croft et al. 1999; Skalníková et al. 2000), who measured the centre-to-chromosome distances for spherical cells. For example, Croft et al. (1999) found chromosome 19 located in the central region of cell nuclei and chromosome 18 nearer the nuclear membrane. Skalníková et al. (2000) observed a similar nuclear positioning of chromosomes 8, 9, 14, 18 and 22 in comparison with our data. The inner location of chromosomes correlates with a higher content of euchromatin and R-bands on interphase chromosomes. In our experiments we also determined the area of all types of chromosomes and we found a similar phenomena to that described by Sanchez et al. (1997). The sizes of chromosome territories are not proportional to chromosome length. Therefore, it is possible that individual neutrophil chromosomes vary in their degree of compactness and consequently condensation. For example, the longest chromosome 1 (263.0 Mb) has an area of 4.5 μm^2 in neutrophil nuclei, which is similar to the area (4.5 μm^2) of the smaller chromosome 13 (114.0 Mb). The area of the even smaller chromosome 18 (85.0 Mb) was 6.2 μm^2. The length of chromosomes was determined with an NCBI Map Viewer.

The aim of this work was to provide basic information on the higher-order chromatin structure of human granulocytes, mainly neutrophils and thereby extend knowledge about chromatin arrangement in highly condensed nuclei.

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