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Specific Radial Positions of Centromeres of Human Chromosomes X, 1, and 19 Remain Unchanged in Chromatin-Depleted Nuclei of Primary Human Fibroblasts: Evidence for the Organizing Role of the Nuclear Matrix

Natalia V. Petrova,[1] Olga V. Iarovaia,[1] Valentin A. Verbovoy,[2] and Sergey V. Razin[1]*

[1]Laboratory of Structural and Functional Organization of Chromosomes, Institute of Gene Biology RAS, Vavilov Street 34/5, 119334 Moscow, Russia
[2]Graphics & Media laboratory, Department of Computational Mathematics and Cybernetics, Lomonosov Moscow State University, 119899 Moscow, Russia

Abstract Radial positions of centromeres of human chromosomes X, 1, and 19 were determined in the nuclei of primary fibroblasts before and after removal of 60%–80% of chromatin. It has been demonstrated that the specific radial positions of these centromeres (more central for the chromosome 19 centromere and more peripheral for the centromeres of chromosomes 1 and X) remain unchanged in chromatin-depleted nuclei. Additional digestion of nuclear RNA did not influence this specific distribution. These results strongly suggest that the characteristic organization of interphase chromosomes is supported by the proteinous nuclear matrix and is not maintained by simple repulsing of negatively charged chromosomes. J. Cell. Biochem. 96: 850–857, 2005. © 2005 Wiley-Liss, Inc.

Key words: chromosomal territories; radial positions; centromeres; nuclear matrix; alphoid satellite

It is well established that in interphase nuclei individual chromosomes occupy limited non-overlapping areas known as chromosomal territories [Schardin et al., 1985; Cremer et al., 1993, 2000; Zirbel et al., 1993; Cremer and Cremer, 2001; Razin et al., 2004a]. Chromosomal territories are separated by the so-called interchromatin domain compartment (ICD). In other studies it was demonstrated that each interphase chromosome occupied a characteristic radial position in the nucleus [Boyle et al., 2001; Lukasova et al., 2002; Taslerova et al., 2003]. Thus, gene-dense chromosomes are typically located close to the nucleus center while gene-poor chromosomes are characterized by peripheral localization [Croft et al., 1999].

It was proposed that simple electrostatic repulsion between negatively charged chromosomal territories was sufficient to keep them at a certain distance, i.e., to form ICD [Cremer et al., 1993, 2000; Zirbel et al., 1993]. An alternative hypothesis suggests that the whole nuclear architecture, including chromosomal territories and ICD, is supported by the nuclear matrix [Razin and Gromova, 1995; Cremer et al., 2000]. To make a choice between these two possibilities, we decided to examine if the nuclear distribution of interphase chromosomes changes drastically upon removal of the major portion of chromatin. We have compared the relative positions of the centromeres of chromosomes X, 1, and 19 in cells and in DNA/chromatin-depleted nuclei of primary human fibroblasts and found that the removal of a greater part of chromatin and also of nuclear

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*Correspondence to: Sergey V. Razin, Institute of Gene Biology RAS., Vavilov Street 34/5, 119334 Moscow, Russia. E-mail: sergey.v.razin@usa.net

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RNA does not affect the radial positions of the above-mentioned centromeres.

MATERIALS AND METHODS

Cell Culture

The culture of primary human fibroblasts was received from the Institute of Medical Genetics of the Russian Academy of Medical Sciences. The cells were grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum.

Preparation of In Situ Nuclear Matrices

Cells grown on microscopic slides were first lysed (10 min, 4°C) in a buffer containing 10 mM Pipes (pH 7.8), 100 mM NaCl, 3 mM $MgCl_2$, 1 mM $CuSO_4$, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), 300 mM sucrose, 0.5% (w/v) Triton X-100. When DNase II was used for digestion of nuclear DNA, the cells were washed with a buffer containing 100 mM sodium acetate (pH 5.0), 20 mM NaCl, 1 mM $MgSO_4$, 0.2 mM PMSF and incubated (30 min, 37°C) in the same buffer supplemented with DNAse II (50 μg/ml). When DNase I was used for digestion of nuclear DNA, the cells were washed in a buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM PMSF. Treatment with DNase I (1 μg/ml) was carried out in the same buffer for 10 min at 37°C. In some experiments RNase A (100 μg/ml) was added along with either DNase I or DNase II. After digestion and washing with TM buffer (50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.1 mM $CuSO_4$, 0.2 mM PMSF), the samples were incubated (30 min at room temperature) in TM buffer containing additionally 0.5 M NaCl. After washing with TM buffer and PBS, the samples were fixed in 4% solution of paraformaldehyde. Further treatment was the same as in the case of non-extracted cells.

Visualization of Centromeres of Chromosomes 1, 19, and X Using Fluorescence In Situ Hybridization (FISH)

The probes for alphoid satellites specific for human chromosomes X, 1, and 19 were kindly given by Yurov et al. [1996]. In addition, the fragment of alphoid satellite specific of chromosome X was PCR-amplified from genomic DNA. The following oligonucleotide primers were used for PCR-amplification: 5′-ataactgaacggaaagcaaa-3′ and 5′-ctgtgaagataaagcgaaaa-3′. These primers were based on the nucleotide sequence of alphoid satellite repeat of human X chromosome (Gene Bank accession number X02418). FISH was carried out as described previously [Iarovaia et al., 2004] with slight modifications aimed to adapt the procedure to cells growing in monolayer. The cells or nuclear matrices attached to microscopic slides were fixed with 4% paraformaldehyde, treated with pepsin (0.01% in 10 mM HCl), post-fixed with 1% paraformaldehyde, and rinsed sequentially in 70%, 80%, and 96% ethanol. To denature DNA the slides were incubated in 70% formamide-2× SSC solution for 5 min at 74°C, dehydrated in cold 70%, 80%, and 96% ethanol and air-dried.

The hybridization probes were labeled with biotin-16-dUTP using a random-prime labeling kit (Roche, Switzerland). The hybridization mixture contained (in a final volume of 10 μl) 50% (v/v) formamide, 2× SSC, 10% dextran-sulfate, 0.1% Tween-20, 10 μg of yeast tRNA, and 25–50 ng of labeled probe. Before hybridization, the mixture was incubated for 10 min at 74°C to denature DNA. Hybridization was carried out overnight at 40–45°C. After hybridization, the samples were washed twice in 50% formamide-2× SSC at 43–48°C for 20 min. The biotinylated probes were visualized using anti-biotin monoclonal antibodies conjugated with Alexa 488 (Molecular Probes) with subsequent signal amplification using an Alexa 488 signal amplification kit for mouse antibodies (Molecular Probes). In all cases the DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The results were examined under a fluorescence microscope DMR/HC5 (Leica) equipped with an objective HCX PZ Fluotar 100×/1.3 and recorded using a CCD camera DC 350 F (Leica).

Computer-Assisted Analysis of Microscopic Images

The microscopic images were analyzed using a special computer program which permitted making a sequential treatment of two photographs of one and the same field taken with filters allowing DAPI-stained nuclei (nuclear matrices) and Alexa 488-stained hybridization signals to be distinguished. The system of recognition makes it possible to work with photographs in bmp and jpg formats and employs only the luminosity component of an image for analysis. In pre-treatment of an image the adaptive methods of filtration based on

standard algorithms (Gauss Blurring, High Pass Filtering, Median Denoising) are used. Recognition is made in several passes (depending on the quality of input images) considering a priori knowledge about the structure of images of cell nuclei. At the first step of the analysis only the low-frequency component of a photograph of cell nuclei obtained from the initial image by means of Gauss blurring is studied. The next step is the analysis of the image treated with a filter having the properties analogous to High Pass filter (Photo Shop). At this stage initial determination of the nuclear borders is made. Then the nuclear borders are defined more exactly according to the data on the degree of difference of the neighboring points in the image. The outlined nuclear borders are smoothed off by median filtration. Then the coordinates of the mass center are calculated for each nucleus.

On the photograph with hybridization signals the low-frequency luminous peaks are distinguished and their centers with the highest total luminance of a group of points are found. The values of luminance are chosen in accordance with general luminosity of the image. The iterative method of marking out from the highest luminance to the lowest one permits both bright and relatively dark signals to be marked out. According to the data on the shape and positions of nuclei (nuclear matrices) obtained from the first image the correspondence of hybridization signals to a particular nucleus is determined and vectors from the nuclear mass center to the hybridization signals are constructed for each nucleus. The vectors for each hybridization signal are extended to the nuclear border and the percent ratio of the first vector to the second one is calculated. The results are presented in tabulated form in Microsoft Exel format.

RESULTS

Comparison of Radial Positions of Centromeres of Chromosomes X and 19 in Cells and in In Situ Nuclear Matrices

In the first set of experiments the nuclear localization of centromeres of chromosomes X and 19 was studied. These chromosomes were chosen because X chromosome occupies a preferentially peripheral position in the nuclei of primary fibroblasts while chromosome 19 occupies a preferentially central position [Croft et al., 1999; Boyle et al., 2001]. Within chromosomal territories centromeres are located at a maximal distance from the nuclear center [Taslerova et al., 2003]. In order to find out whether the radial positions of centromeres of chromosomes X and 19 are characterized by a similar difference as the radial positions of the whole chromosomal territories, we first analyzed the distribution of these centromeres in the nuclei of primary human fibroblasts. The centromeres of chromosomes 19 and X were visualized using fluorescence in situ hybridization (FISH) with biotinylated probes recognizing alphoid satellites specific for these chromosomes (Fig. 1D,E). The distribution of signals was analyzed on flattened samples as it

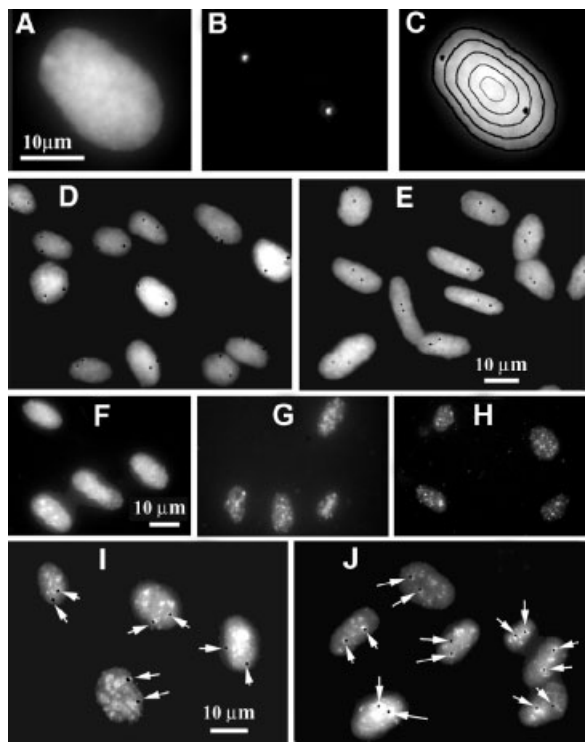
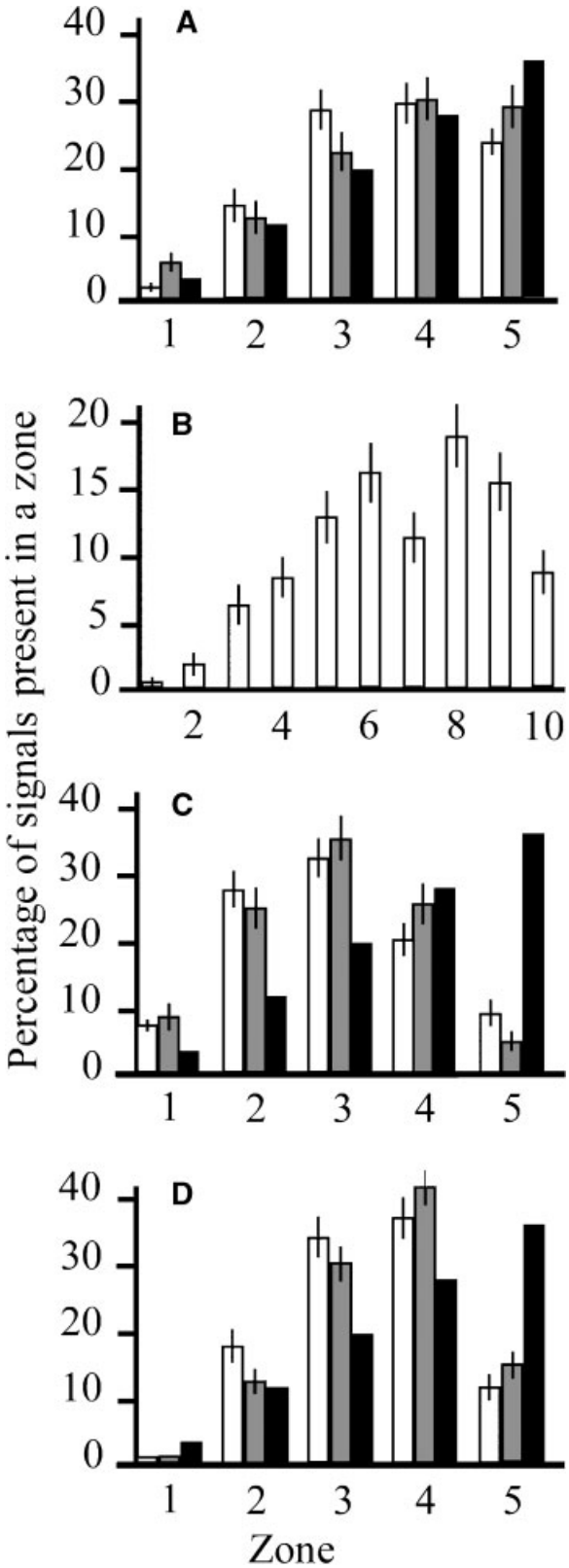


Fig. 1. Visualization of centromeres in nuclei and nuclear matrices and analysis of partitioning of observed signals between five nuclear shells. **A–C**: Experimental approach used to determine the distribution of signals (A) cell stained with 4′,6-diamidino-2-phenylindole (DAPI); (B) immunostaining of a biotinylated probe hybridized to the alphoid satellite of X chromosome; (C) superimposition of A and B and partitioning of the cell into concentric layers. **D**, **E**: Typical images of nuclei with stained centromeres of X chromosome (D) and chromosome 19 (E); hybridization signals are seen as black spots over light nuclei counterstained with DAPI. **F–H**: Kinetics of chromatin removal upon treatment of the nuclei with increasing (5, 25, and 75 μg/ml) amounts of DNase II (DAPI staining of DNA remaining in the nuclei after 0.5 M NaCl extraction). **I**, **J**: Typical images of nuclear matrices with stained centromeres of X chromosome (I) and chromosome 19 (J); hybridization signals are indicated by white arrows.



was reported previously that to a first approximation this kind of analysis gave reliable information about nuclear positions of chromosomal territories, especially in the relatively flat and long nuclei of primary fibroblasts [Croft et al., 1999; Boyle et al., 2001]. The nuclear area was divided into five concentric shells with the inner borders located at distances equal to 20%, 40%, 60%, and 80% of the distance from the nuclear center to the border of the nucleus (Fig. 1C). The positions of hybridization signals in respect to these shells were determined as shown in Figure 1A–C. The distribution of signals among the shells in 150 arbitrarily selected cells was established using a specially developed computer program (see "Materials and Methods"). The results are represented as diagrams showing the percentage of signals present in each shell (Fig. 2A,C). It is evident that the distribution of centromeres of both chromosomes X and 19 is not random, although the difference from random distribution is much more prominent in case of chromosome 19 centromeres. Furthermore, the centromeres of chromosome 19 occupied more central positions in nuclei as compared to the centromeres of X chromosome (Fig. 2A,C). The average radial positions for centromeres of chromosomes 19 and X constitute respectively 50.4% and 60.9% of the nuclear radius. However, the distribution of X chromosome centromeres was clearly bimodal. It was especially evident when the distribution of the signals between 10 concentric shells with the inner borders located at distances equal to 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% of the distance from the nuclear center to the border of the nucleus was analyzed (Fig. 2B). It looks like about half of the X chromosome centromeres occupy nearly the same position as centromeres of the chromosome 19, while the other half is located close to the nuclear border. This bimodality possibly reflects the fact that active and

Fig. 2. Distribution within the nuclear space in non-treated cells and chromatin-depleted nuclei of centromeres of X chromosome (**A**, **B**), chromosome 19 (**C**), and chromosome 1 (**D**). Open rectangles show the observed partitioning of signals in non-treated cells among 5 distance zones (A, C, D) or among 10 distance zones (B). Gray rectangles show the partitioning of signals between five distance zones in nuclear matrices. SSD is shown by vertical bars. Closed rectangles show expected partitioning of signals among the zones when the distribution of signals is random. Note that the areas of the zones increase in the direction from the nuclear center to the nuclear periphery.

inactive X chromosome copies have different radial positions in nuclei. The data presented in Figure 2 agree in principle with previously published results [Croft et al., 1999; Boyle et al., 2001]. To find out if the maintenance of the above spatial arrangement depends on the presence of huge masses of chromatin in each chromosome (i.e., if an electrostatic repulsion between chromosomal territories is essential to maintain the characteristic difference in the radial positions of centromeres of chromosomes 19 and X), the analysis was repeated with "in situ" nuclear matrices. The latter were prepared using DNase II or DNase I treatment and mild salt extraction (0.5 M NaCl) of permeabilized cells attached to microscope slides.

Mild salt extraction (0.5 M NaCl) does not remove core histones, although it removes histone H1. Thus the nuclear matrix-bound DNA remains organized in nucleosomes but not in 30 nm chromatin fibrils. After extensive DNAse treatment of permeabilized nuclei 60%–80% of DNA was removed by 0.5 M NaCl extraction, as indicated by a decrease in DAPI staining (Fig. 1F–H). Nevertheless, the nuclei retained their normal shape and dimensions. In such in situ nuclear matrices the centromeres of chromosomes X and 19 could be easily visualized using the corresponding alphoid satellite probes (Fig. 1I,J). The radial positions of these centromeres were determined in 150 arbitrarily chosen in situ nuclear matrices in the same fashion as in the experiment with non-extracted nuclei. The results (Fig. 2A,C) demonstrate that the centromeres of chromosome 19 retained a more central position as compared to the centromeres of X chromosome. The choice of DNase used for DNA cleavage in the course of nuclear matrix preparation (DNase I or DNase II) had no detectable effect on the distribution of the centromeres under study in the in situ nuclear matrices (not shown). It was concluded that the nuclear positions of centromeres of chromosomes X and 19 are retained in the nuclear matrix. To check if this conclusion is true of other chromosomes, all the above-described experiments were repeated with the probe for the centromere of chromosome 1. Again, no significant relocation of this centromere was observed in nuclear matrices as compared to non-extracted nuclei. Importantly, the observed distribution of chromosome 1 centromeres was clearly different from the random one (Fig. 2D).

Extensive Digestion of Nuclear RNA Does Not Influence the Radial Positions of Centromeres in Chromatin-Depleted Nuclei

It was reported previously that RNA plays an important role in maintaining the nuclear architecture and that nuclear matrix integrity depends on the preservation of RNA in the course of chromatin removal [Nickerson et al., 1989, 2001; Belgrader et al., 1991; Barboro et al., 2003]. Furthermore, it was reported that residual chromosomal territories observed in the nuclear matrices were disrupted after RNase A treatment and high salt extraction [Ma et al., 1999]. In order to find out whether, indeed, nuclear RNA plays an essential role in supporting the specific radial arrangement of chromosomal territories, the experiments described in the previous section were repeated with in situ nuclear matrices obtained using double digestion with DNase I or II and RNase A. In a preliminary experiment it was demonstrated that after RNase A treatment under conditions used in our experiments more than 90% of nuclear RNA became acid-soluble and was released from nuclear matrices. The results shown in Figure 3 demonstrate that the specific radial positions in nuclear matrices of centromeres of chromosomes 1 and 19 remain unchanged after digestion of nuclear RNA.

Alphoid Satellites of Human Chromosomes X and 19 do not Contain Strong MARs

One possible explanation of our results is that centromeres themselves rather than whole chromosomal territories are retained on the nuclear matrix. To analyze this possibility we checked whether the alphoid satellite repeats of chromosomes X and 19 used as probes in this study contain MAR elements. The experiments were carried out essentially as was originally described by Garrard and coauthors [Cockerill and Garrard, 1986; Cockerill et al., 1987]. The results of these experiments are presented in Figure 4. It is clear that none of the satellite probes tested in these experiments behaved as MAR. Both probes were washed of the nuclear matrices in the presence of about the same amount of a non-specific competitor as the control fragment of vector DNA. In contrast, the bona fide MAR from the *Drosophila* histone gene cluster [Mirkovitch et al., 1984; Cockerill and Garrard, 1986] remained in nuclear

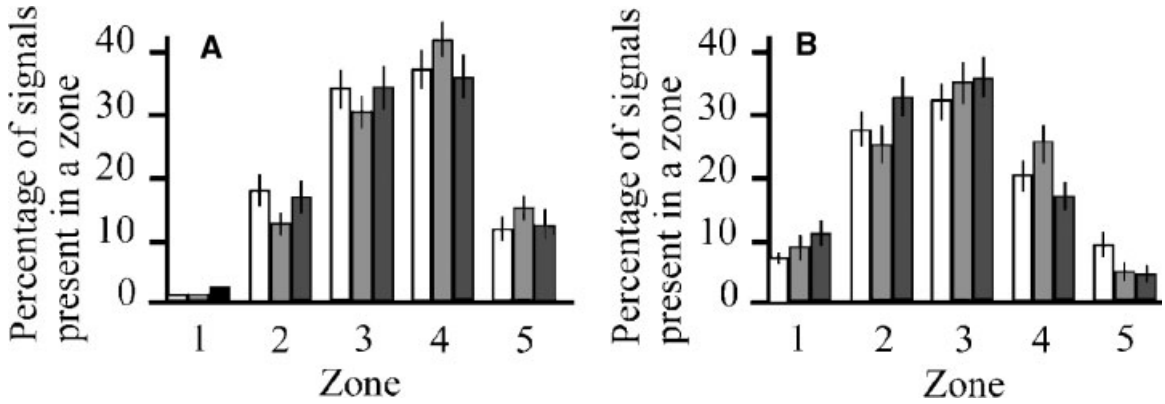


Fig. 3. Partitioning of centromeres of chromosomes 1 (**A**) and 19 (**B**) between five distance zones in non-treated cell nuclei (open rectangles) in nuclear matrices obtained using DNase II treatment (light gray rectangles) and in nuclear matrices obtained using DNase II and RNase A treatment (dark gray rectangles). SSD is shown by vertical bars.

matrices even when the concentration of competitor DNA was increased 50-fold.

DISCUSSION

Although it is known that in interphase nuclei chromosomes occupy characteristic radial positions, the mechanisms supporting this organization are poorly understood. Here, we have demonstrated that the relative radial positions of the centromeric regions of human chromosomes 1, 19, and X are maintained in the absence of a vast amount of chromatin. Consequently, electrostatic repulsion between chromosomal territories can hardly be an essential factor stabilizing the organization of chromosomes in interphase nuclei. It is important to underline that we used mild salt extraction (0.5 M NaCl) to remove the major portion of chromatin cleaved by DNase I or II. The rest of chromosomal DNA remained organized into nucleosomes as the nucleosomal core particles are not disrupted under these conditions. Correspondingly they possessed about the same electrostatic potential as non-extracted chromosomal territories. Our data strongly suggest that the specific radial positions of centromeres of the chromosomes under study are maintained due to the interaction of either the centromeres themselves or whole chromosomal territories with the underlying nuclear structure, i.e., with the nuclear matrix. "Nuclear matrix" is an operationally defined term. It represents a residual nuclear structure retaining the shape and some morphological features of the nucleus after extraction of chromatin and RNA [Berezney and Coffey, 1977]. The nuclear matrix constitutes a structural milieu for the functional processes taking place in the cell nuclei [Berezney et al., 1995]. In a previous study of Ma et al. [1999] it was demonstrated that chromosomal territories remain compact in nuclear matrices. However, the radial positions

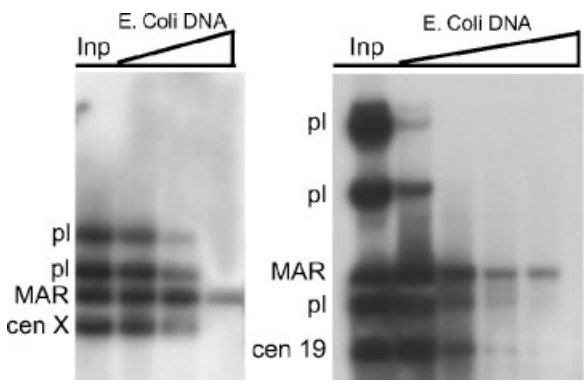


Fig. 4. MAR-binding assay of fragments of alphoid satellites of chromosomes X and 19. The assay was carried out essentially as described [Cockerill and Garrard, 1986]. In two separate experiments a fragment of the alphoid satellite from human chromosome X (cen X, 1,400 bp) and a fragment of the alphoid satellite of human chromosome 19 (cen 19, 670 bp) were mixed with 1,300 bp (experiment with chromosome 19 alphoid satellite) or 1,788 bp (experiment with X chromosome alphoid satellite) DNA fragments containing MAR from *Drosophila* histone genes (MAR) [Mirkovitch et al., 1984; Cockerill and Garrard, 1986] and with two or more fragments of plasmid DNA (pl). The mixture (input, Inp) was incubated with high salt-extracted nuclear matrices in the presence of different (0–1 mg/ml) amounts of competitor *E. coli* DNA. After several washings, the matrix-bound DNA fragments were isolated and analyzed by agarose gel electrophoresis. Note that the ''cen X'' and ''cen 19'' DNA fragments as well as the fragments of plasmid DNA were washed off the nuclear matrices in the presence of about the same amount of the competitor.

of residual chromosomal territories observed in nuclear matrices were not analyzed. Croft et al. [1999] have reported that chromosome 19 is more tightly associated with the nuclear matrix as compared to peripherally located chromosome 18. Our data are in principal agreement with this conclusion. It is, however, important to underlain that the above conclusion about different mode of interaction with the nuclear matrix of chromosomes 19 and 18 was made basing on the observed degree of extension of both chromosomal territories into the crown of DNA loops in high salt extracted nuclei, and quantitative analysis was not carried out. The important advantage of our study is that a number of cells and nuclear matrices were analyzed and statistical criteria were used to justify the conclusions.

In some of recent publications the nuclear RNA is considered as an integral part of the nuclear matrix [Nickerson et al., 1989, 2001; Belgrader et al., 1991; Barboro et al., 2003; Razin et al., 2004b]. In particular, it was reported that residual chromosomal territories were disrupted in nuclear matrices treated with RNase A [Ma et al., 1999]. We have shown that digestion of nuclear RNA with RNase A does not affect the radial positions of the centromeres under study in in situ nuclear matrices. It should be, however, pointed out that we used 0.5 M NaCl extraction to solubilize cleaved-off chromatin from in situ nuclear matrices while Ma et al. [1999] used 2 M NaCl extraction. The most important result of our experiments is, however, that in no case the specific nuclear distribution of the centromeric regions of the chromosomes under study became random. Thus, the interaction of chromosomal territories and/or centromeres themselves with the proteinous part of the nuclear matrix supports the observed non-random positioning of the centromeres. As demonstrated in additional experiments, the X- and 19-chromosome-specific alphoid satellites do not possess the properties of MARs. When the distribution on nuclear halos of the X-chromosome-specific alphoid satellite repeats was studied, most of these repeats were found in the crown of DNA loops [Iarovaia et al., 2004]. Thus, no experimental evidence supports the supposition that centromeres themselves are attached to the nuclear matrix. It is more probable that it is the arrangement of whole chromosomal territories rather than of centromeres only which is maintained by the interaction of the chromosomes with the nuclear matrix. It would be perhaps even more correct to state that interphase chromosomes are integrated into the nuclear matrix as the internal nuclear matrix and the scaffold of metaphase chromosomes have many common proteins, such as DNA topoisomerase II [Berrios et al., 1985; Earnshaw et al., 1985].

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