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Spatial Arrangement of Macro-, Midi-, and Microchromosomes in Transcriptionally Active Nuclei of Growing Oocytes in Birds of the Order Galliformes

A. V. Maslova and A. V. Krasikova*

Laboratory of Chromosome Structure and Function, St. Petersburg State University, St. Petersburg, Russia

**e-mail: spbchromas@gmail.com*

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Abstract—Three-dimensional genome organization in the cell nucleus reflects its functional state and is one of the regulatory levels of gene expression. Thus, the extensive exploration of the relationship between the spatial organization of the genome and its functioning is very important. In this work, the three-dimensional genome organization in growing oocytes of Galliform birds was analyzed in detail. Avian oocytes have giant transcriptionally active nuclei that are distinct from somatic interphase nuclei in their almost complete lack of structural constraints on chromosome decondensation. The radial distribution of three groups of chromosomes with different sizes and gene densities in the nuclei of chicken and Japanese quail oocytes was analyzed by confocal laser scanning microscopy followed by 3D reconstruction. The chromosome position relative to the nuclear center was estimated by analyzing its localization in certain radial nuclear zones and directly measuring the distance from the nuclear center to the terminal regions and center of chromosome gravity. It was shown that, in transcriptionally active nuclei of avian oocytes, chromosomes are localized at a significant distance from the nuclear envelope; gene-rich microchromosomes are localized mainly on the periphery of the region occupied by the whole chromosome set, rather than in the nuclear center. Therefore, the radial distribution of lampbrush chromosomes in oocyte nuclei differs from the ordered spatial arrangement of chromosomes in the interphase nucleus with gene-rich chromosome territories being located at the nuclear center and gene-poor territories positioned at the nuclear periphery. By visualizing 3D-preserved lampbrush chromosomes in the intact nucleus, we confirmed the presence of repulsion forces between lateral loops of lampbrush half-bivalents and the lack of interactions between heterochromatic segments of different bivalents at the lampbrush stage of oogenesis.

Keywords: avian oogenesis, cell nucleus, domestic animals, genome architecture, lampbrush chromosomes, telomeres, transcription, three-dimensional reconstruction

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Abbreviations used: CLSM, confocal lazer scanning microscopy; LB, lampbrush; FISH, fluorescence in situ hybridization

Nuclear architecture is one of the levels in the hierarchy of mechanisms that regulate genome functioning (van Driel et al., 2003; Razin, 2006; Razin et al., 2007). Spatial chromosome arrangement in the nucleus is frequently applied to characterize the relationships between large-scale genome structure and its functioning (Lanctôt et al., 2007; Mateos-Langerak et al., 2007).

It is generally accepted that, in an interphase nucleus, chromosomes occupy certain nonoverlapping territories (Cremer et al., 2000; Albiez et al., 2006). In somatic nuclei, the location of a chromosome near the nuclear center usually correlates with its gene density and their transcriptional activity. Gene-rich chromosome territories are typically observed in the central nuclear area whereas chromosomes with a smaller number of encoding genes are localized on the nuclear periphery (Croft et al., 1999; Boyle et al., 2001; Kupper et al., 2007). The radial distribution of chromosome territories in somatic cells is evolutionarily conserved (Tanabe et al., 2002). An analysis of three-dimensional (3D) distribution of chromosome territories in proliferating and quiescent human fibroblasts showed that radial distribution of chromosomes also depended on their size: in quiescent fibroblasts, regardless of the density of encoding sequences, small chromosomes were located closer to the nuclear center than large chromosomes (Bolzer et al., 2005).

The chromosome territory is a dynamic nuclear compartment. The positions of chromosomes relative to one another vary significantly in the cell population. It is proposed that a typical chromosome landscape for the interphase nucleus is established at the early G1 stage of the cell cycle and presumably mirrors the chromosome distribution in the mitotic metaphase

(Sun et al., 2000; Habermann et al., 2001; Bolzer et al., 2005). Dynamic behavior is also evident in changes in the organization of chromosome territories and changes in the position of chromosome segments or particular loci relative to the visible territory border after external action, during differentiation, and in diseases (Volpi et al., 2000; Mahy et al., 2002; Stadler et al., 2004).

Despite the numerous discoveries in this field, the interrelation between spatial chromosome arrangement and the regulation of genome functioning remains obscure. Here, the main obstacles are the limited experimental approaches for manipulation with functional compartmentalization of the nucleus (van Driel et al., 2003). In addition, it is very difficult to take into account all of the structural and functional relationships inside the nucleus that are postulated by the model of the self-organization of nuclear compartments (Misteli, 2007). In this sense, it seems very fruitful to study the nuclei of differentiating cells. Cell differentiation is accompanied by the regular switching of gene expression and, therefore, can be considered as an appropriate model to study the regulation of genome functioning at the level of the genome architecture (Bartova and Kozubek, 2006; Schofer and Weipoltshammer, 2008).

It is well known that gametogenesis is accompanied by changes in the spatial chromosome arrangement (see, e.g., Solovei et al., 1998; Foster et al., 2005). It has recently been shown that, in pig spermatogenesis, during the primordial spermatocyte differentiation into spermatides, sex chromosomes are repositioned from the periphery to the inner region (Foster et al., 2005). The exploration of the chromosome arrangement during gametogenesis is the first step toward elucidating the mechanisms of genome reorganization and regulation at the early stages of embryo development. Therefore, the pattern of chromosome topology and the mechanism of its modification in meiocytes development require in-depth study.

In this work, avian growing oocytes (Galliformes order) were used as a model to study the spatial organization of the genome in germ cells. Advantages of the model are as follows. At the diplotene stage of the first meiotic division, growing avian oocytes are characterized by high transcriptional activity (Gaginskaya et al., 2009). At this stage, homologous chromosomes that form bivalents have typical lampbrush (LB) shapes. The length of LB chromosomes is on average 30 times larger then that of the corresponding metaphase chromosomes (Chelysheva et al., 1990; Khutinaeva et al., 1989; Rodionov and Chechik, 2002; Saifitdinova et al., 2003; Derjusheva et al., 2003), whereas the nucleus size in the growing oocyte is dozens of times higher than in somatic cells. In these giant chromosomes, numerous transcription units are localized on lateral loops with lengths reaching dozen of microns (Callan, 1986; Morgan, 2002; Gaginskaya et al, 2009). An examination of LB chromosomes allows one to elucidate the fine details of chromosome organization due to their partially decondensed state and facilitates the understanding of the chromatin structure in transcriptionally active nuclei. This model is also convenient to study genome architecture because avian growing oocytes lack both chromosomal and amplified extrachromosomal nucleoli (Gaginskaya and Gruzova, 1969, 1975).

Representatives of the order Galliformes, such as domestic chicken (*Gallus gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*), are important agricultural species and are also widely applied in developmental biology and virology as research models. Karyotypes of these species are morphologically differentiated; the chromosome set is composed of six pairs of macrochromosomes including *Z* and W sex chromosomes, five pairs of midichromosomes and 28 pairs of microchromosomes (Burt, 2002). Microchromosomes are GC-rich, contain numerous CpG islands, replicate in the early S-phase of the cell cycle, and contain the greatest part of genes. It was estimated that microchromosomes of the domestic chicken contain 25% of the genome and more than 45% of genes (Rodionov, 1996; Smith et al., 2000; Burt, 2002; ICGSC, 2004). This makes this object very appropriate to study the nuclear distribution of chromosomes of various sizes; furthermore, it is possible to identify gene-enriched chromosomes morphologically. Here, a detailed analysis has been performed for the first time of the 3D arrangement of macro-, midi-, and microchromosomes in the nuclei of growing oocytes at the LB stage in sexually mature females of the domestic chicken and Japanese quail.

MATERIALS AND METHODS

Preparation of intact nuclei from growing oocytes. Oocyte nuclei from the domestic chicken (*Gallus gallus domesticus*) and Japanese quail (*Coturnix coturnix japonica*) ($2n = 78$) were isolated according to the standard technique (Solovei et al., 1993). Oocytes 0.5–1.5 mm in diameter were placed into 5 : 1 + phosphates medium (83.0 mM KCl, 17.0 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM dithiothreitol). Nuclei were isolated with thin tungsten needles under a Leica MZ16 stereomicroscope. Isolated nuclei were purified of yolk and stained for 5 min with 0.07 μM NA-specific fluorescent dye Sytox Green (Molecular Probes) added to the 5 : 1 + phosphate medium (Krasikova et al., 2009). Then, intact nuclei were transferred by the tip into 18 × 18 × 2 mm well filled with the same medium. The well was made by attaching the camera 18 × 18 × 2 mm with a 6-mm hole to a 24 × 50 × (0.16–0.19)-mm slide (Deckgläser, Menzel-glaser) with a paraffin–vaseline (1 : 1) mixture. Stained nuclei (32 from Japanese quail oocytes, 28 from chicken oocytes) were analyzed by confocal lazer scanning microscopy.

Confocal laser scanning microcopy (CLSM) was performed with a Leica TCS SP5 microscope based on a Leica DMI 6000 CS inverted microscope. Objects were examined by the XYZ scanning technique using an argon laser (496 nm), HC PL APO 20× objective and LAS AF software (Leica-Microsystems, Germany). Confocal images were obtained with a 1024 × 1024-pixels scanning format and 400–700-Hz scanning frequency by applying confocal zoom and software to reduce noise. Optical stacks were obtained with a 1.3–1.5-μm step along the z axis. A view of the nucleus was reconstructed from serial optical sections with Max projection option in LAS AF software. For further processing, the data were converted from *.lif into *.tiff format.

Analysis of chromosome position relative to nuclear center. To clearly visualize single chromosomes inside the nucleus, 3D images were processed with Imaris 5.0.1 (Bitplane, AG) and ImageJ software. To improve the image quality of nuclear 3D reconstruction, we applied "Gaussian Blur" and "Background Remover" automatic program filters. The radius of the nuclear area occupied by the chromosomes was calculated by the difference between z coordinates of the first ($z1$) and the last ($z2$) sections with detected fluorescent signal from chromosomes, i.e., $R = \frac{(z1 - z2)}{2}$.

The Isosurface software module, which is implemented for the construction of 3D surfaces around objects with particular fluorescence intensities, was applied for the 3D graphic reconstruction of isosurfaces in oocyte nucleus (germinal vesicle) (Figs. 1a, 1b). Every chromosome in the set was made identifiable in 3D reconstructions of the nuclear isosurface by changing the signal intensity threshold parameters applied for the isosurface construction (Fig. 1b). The lowest signals corresponding to the background fluorescence were cut off. The application of "Split" allowed one to divide the entire nuclear isosurface into single objects corresponding to individual bivalents; therefore, it became possible to perform their independent morphometric analysis (Fig. 1c).

Macro-, midi-, and microbivalents were sorted visually using the "Sort" option. The "Color" option allowed one to label 39 bivalents with various colors (Fig. 1d). The coordinates of the gravity center of the body outlined by isosurface constructed around the bivalent were determined using Imaris 5.0.1 software ("Statistics" option). The distance from the nuclear center to the center of gravity of the body was calculated according to the formula $L = \sqrt{(x1 - x2)^2 + (y1 - y2)^2 + (z1 - z2)^2}$, where ($x1$; $y1$; $z1$) are coordinates of the nucleus geometric center and ($x2$; $y2$; $z2$) are the coordinates of the center of gravity of the body (Roberts et al., 2005).

The estimation of the nuclear center is the first step in spatial measurements. Because the oocyte nucleus has a spherical shape, the central section has the maximal diameter. To estimate the lateral coordinates of the center, the circle was inscribed along the nuclear contour on the central section of the z axis and the automatically determined center (with Corel Draw 12.0.0.458 software) was tagged with a 2 × 2 pixel label. Taking into account the physical characteristics of confocal images (actual voxel size), the real coordinate values of the nuclear center ($x1$; $y1$; $z1$) were obtained by importing serial optical sections with a labeled central section into the Imaris program.

The deconvolution of confocal images was performed by blind 3D deconvolution (by applying the adaptive point spread function and physical parameters of image capture, namely, voxel size, numerical aperture of objective, refraction index, maximum of fluorochrome emission spectra) using AutoDeblur v.9.3 and AutoVizualize v.9.3 software (AutoQuant Imaging, Inc.).

RESULTS

Spatial Organization of Lampbrush Chromosomes in Intact Nuclei

The application of confocal microscopy allowed us to confirm previously suggested ideas on the functional organization of the genome at the LB stage and to provide new data on the architecture and compartmentalization of avian germinal vesicles. Nuclei isolated by microsurgery from growing oocytes of domestic chicken and Japanese quail were stained with Sytox green fluorochrome and scanned with laser confocal microscopy. It was clearly seen on the confocal images that, in the germinal vesicle, chromosomes lack any connection with the nuclear envelope (Figs. 2a, 2c). Chromosomes are located in the germinal vesicle center and are separated from the nuclear envelope with a wide nucleoplasm zone (Figs. 2a, 2c). Telomere regions of LB chromosomes are not associated with the inner membrane of the nuclear envelope or nuclear lamina. An analysis of optical stacks in serial confocal images confirmed that, at LB stage, the chicken and Japanese quail chromosomes had no contact with one another; chromosome subdomains did not produce chromocenters typical of the interphase nuclei of somatic cells (Figs. 2a, 2c).

The approach we used to study the structure of LB chromosomes in nuclei with structural integrity made it possible to reduce artifacts common for the microsurgical isolation of LB chromosomes from germinal vesicles and to describe LB chromosomes in intact unstrained nuclei in detail for the first time. Three-dimensional images of LB chromosomes show that their lateral loops are spread in all directions from chromosome axes and chromosomes resemble household tube brushes (Fig. 3a). Chromosomal lateral loops that repel each other make bivalents with two terminal chiasmata ellipsoid or ringlike (Fig. 3c). In the case of bivalents with more chiasmata, planes that cross parts of the

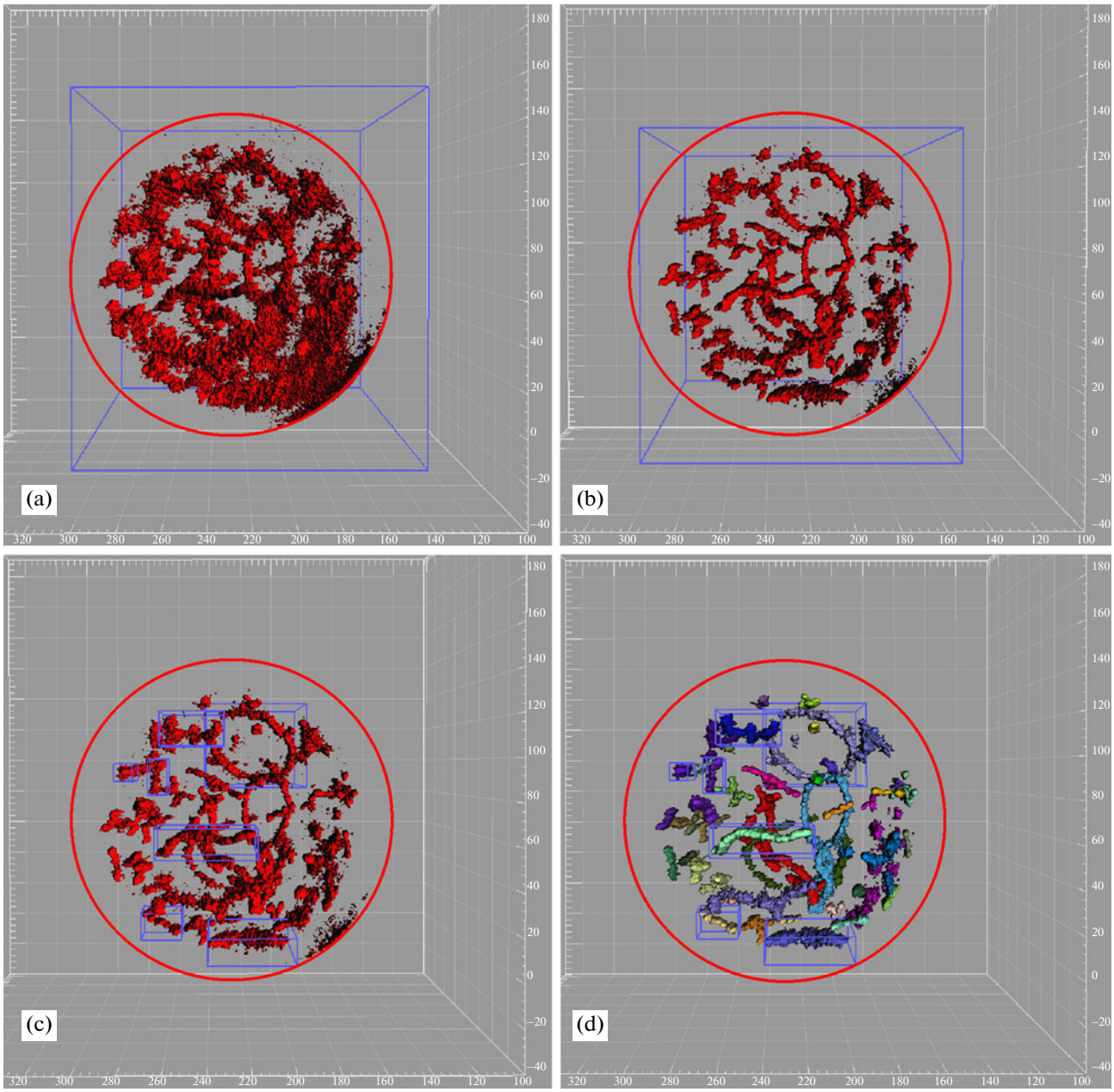

Fig. 1. Consecutive steps of 3D reconstruction of Japanese quail oocyte nucleus from optical stack obtained by confocal laser scanning microscopy. (a) Construction of nuclear isosurface with the distinct level of fluorescent signal; (b) empiric adjustment of the lower threshold value which allows visualization of individual bivalents on the nuclear isosurface; (c) splitting of the nuclear isosurface into distinct objects that correspond to individual bivalents; (d) nuclear reconstruction with 39 meiotic bivalents that were given individual pseudocolors. *Red circles* indicate nuclear borders; *blue frames* indicate examples of isosurfaces.

homologues limited by adjacent chiasmata are located at an angle to one another (Fig. 3a). Interestingly, so-called "double-loop bridges", which are regions of sister chromatids corresponding to lateral loops that are not connected at base (Morgan, 2002), are visible in some bivalents inside of the intact nucleus as loops that extend out of one chromomere and enter the neighboring chromomere (Figs. 3b, 3c).

Position of Macro-, Midi-, and Microchromosomes Relative to Nucleus Center

Two indices are usually applied for the characteristics of the spatial arrangement of the chromosome in the interphase nucleus, i.e., the radial position of the chromosome territory and position of a particular chromosome relative to other chromosomes of the karyotype. Here, we have analyzed the radial chromosome distribution inside of germinal vesicles because it

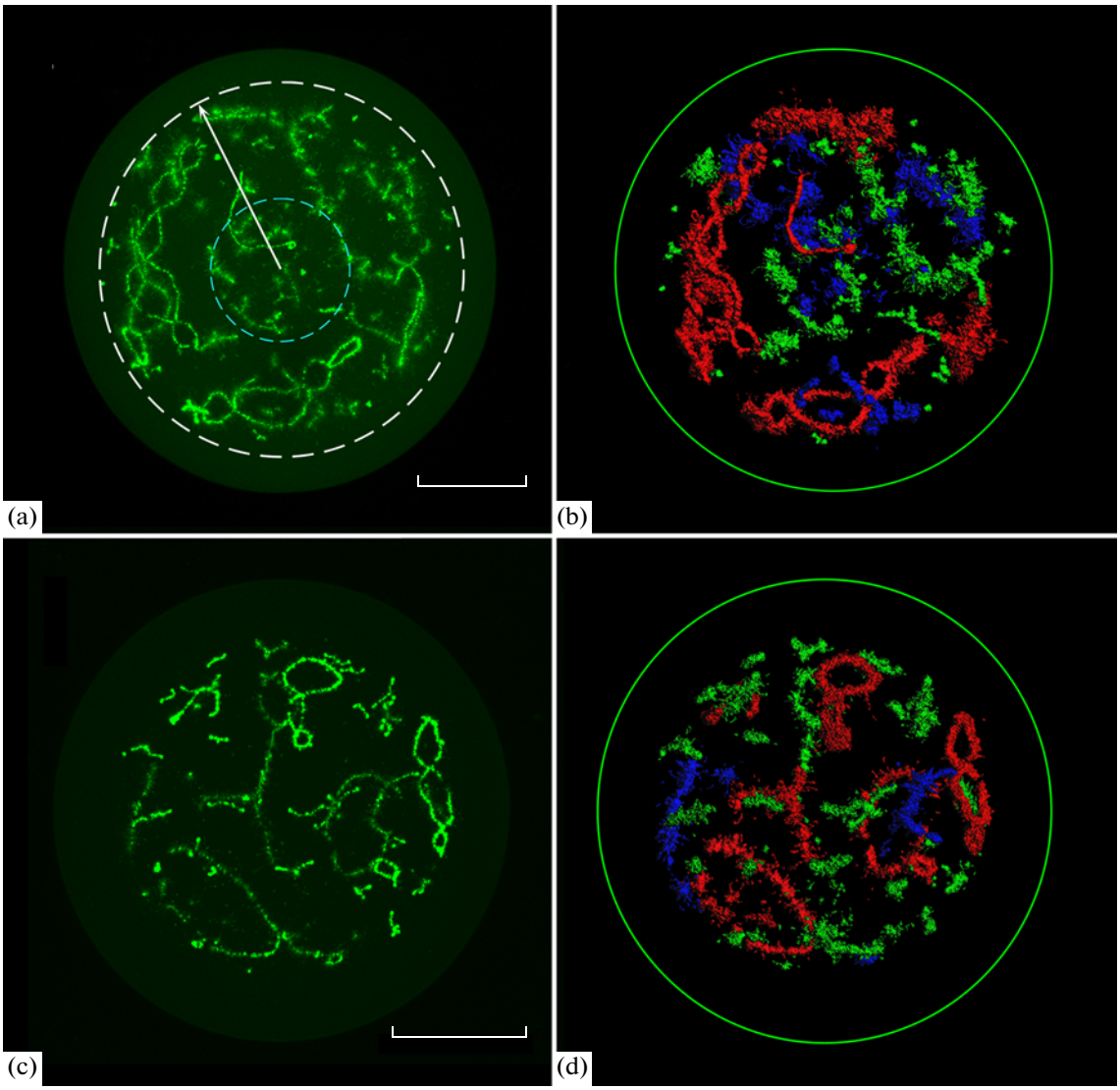

Fig. 2. XY projections of nuclei from chicken (a, b) and Japanese quail (c, d) oocytes.
(a, c) Maximum projections of chicken (a) and quail (c) oocyte nuclei stained with NA-specific dye Sytox Green. *Green circles* indicate nuclear borders; *dashed circles* indicate borders of nuclear zones where chromosomes were counted. (b, d) Projections of 3D reconstructions of the same nuclei with macro-, midi-, and microchromosome isosurfaces pseudocolored with red, blue and green, respectively. Scale bars are 50 μm.

comprehensively characterizes the large-scale architecture of the oocyte genome.

It is obvious that the combination of various methods is the most informative for the analysis of the radial chromosome distribution. After 3D nuclear reconstruction and the estimation of the coordinates of the nuclear center, the spatial distribution of the complete set of chromosomes in avian germinal vesicles was evaluated in three ways as follows:

1. counting macro-, midi-, and microchromosomes in three areas provisionally marked on 3D nuclear reconstruction;

2. measuring the distance from the nuclear center to bivalent telomere regions with a 3D micrometer during the examination of the optical section;

3. calculation of the distance from the nuclear geometric center to the center of gravity of the body outlined by an isosurface constructed around individual bivalents. The results of each of these approaches are presented below.

Counting Bivalents of Different Morphological Groups in Three Zones Visually Recognized in Nuclear 3D Reconstructions

In interphase nuclei, the position of chromosome territories that occupy a large volume and interact tightly with each other is difficult to analyze without applying specific methods of chromosome identification. However, an analysis of the radial distribution of

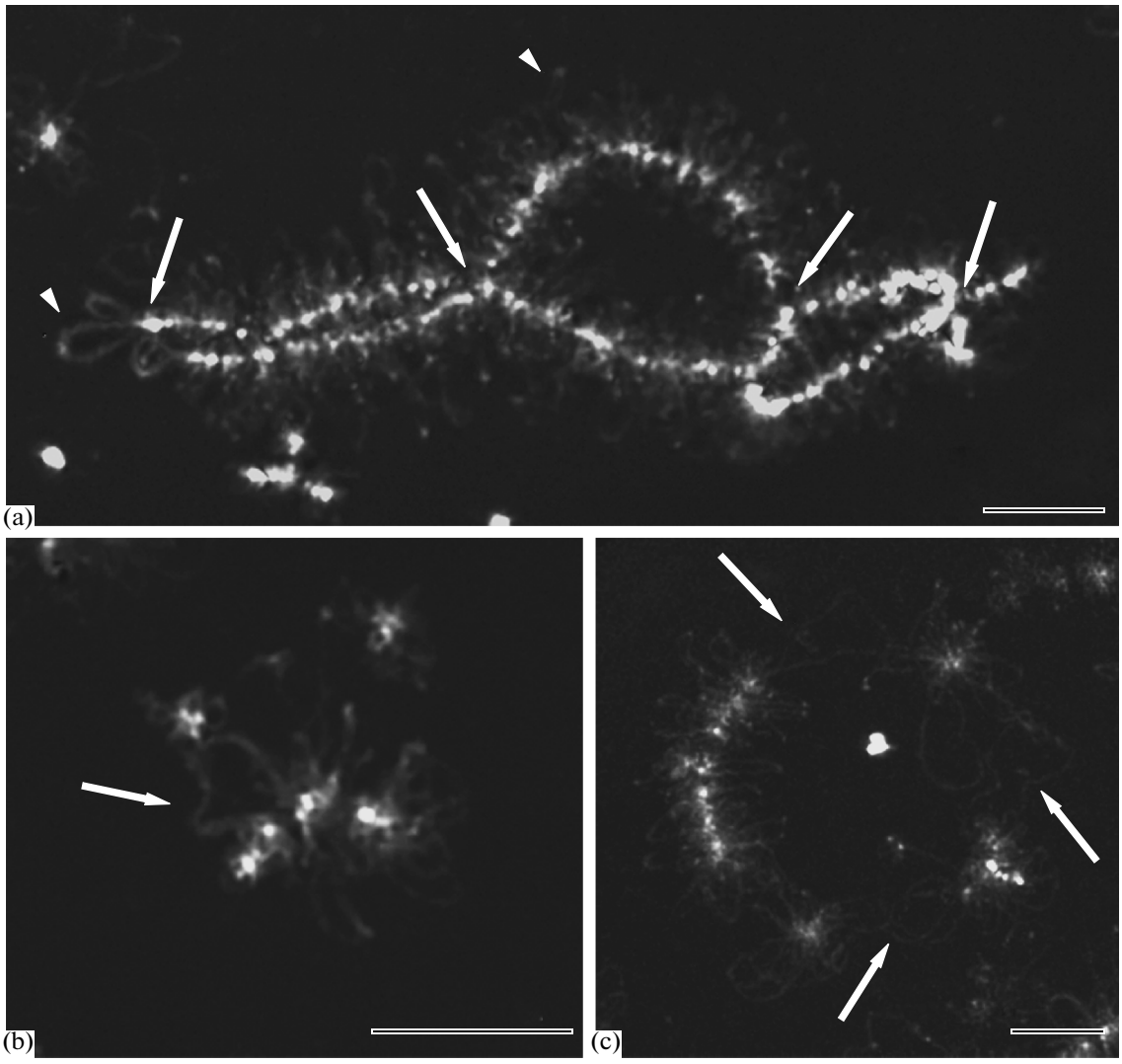

Fig. 3. Chicken lampbrush chromosomes in intact nucleus.
(a) Lampbrush bivalent 3, maximum XY projection view of 72 optical sections after signal deconvolution. *Long arrows* indicate chiasmata; *arrowheads* point to certain lateral loops. (b, c) Double loop bridges (*arrows*) on chicken lampbrush chromosomes; (b) a microchromosome, a maximum projection of 22 optical sections after deconvolution; (c) a midichromosome, a maximum projection of 54 optical sections after deconvolution. Scale bars are 10 μm.

well-defined, giant LB chromosomes that are separate from one another is possible by visually observing the 3D nuclear reconstruction. The established correspondence of chicken meiotic bivalents to mitotic chromosomes (Derjusheva et al., 2003; Krasikova et al., 2006), as well as availability of the constructed cytological maps of chicken and quail LB macrochromosomes (Schmid et al., 2005; Galkina et al., 2006), make it possible to identify the particular LB chromosomes of these species without a laborious procedure of fluorescence in situ hybridization (FISH) with chromosome-specific probes.

The total number of bivalents in both chickens and quails is 38 autosome bivalents and one sex bivalent (ZW). In most cases, macro-, midi-, and microbivalents were distinguished by their sizes. In addition, the high level of resolution of nuclear confocal images allows us to identify single macro- and midichromosomes according to their unique chromomere-loop pattern and landmark structures (Fig. 3). Examples of 3D reconstructions of chicken and Japanese quail oocyte nuclei with macro-, midi-, and microchromosomes labeled with red, blue, and green pseudocolors, correspondingly, are presented in Figs. 2b, 2d.

For a visual analysis of the chromosome arrangement in chicken and Japanese quail germinal vesicles, 3D reconstructions of optical sections were partitioned into 3 concentric zones, i.e., central (area limited by a sphere with a radius equal to 1/3 of the nuclear radius), peripheral (area under the nuclear envelope that is limited by two spheres, where the radius of the external sphere is equal to the nuclear

radius and the radius of the internal sphere is equal to the radius of the area with detected fluorescent objects, i.e., chromosomes), and intermediate (the area between central and peripheral zones). Figure 2a depicts the zone ratio on the maximal XY projection of the chicken germinal vesicle. Each bivalent was attributed to a particular zone in the oocyte nucleus.

LB chromosomes do not contact with the nuclear envelope, therefore, peripheral zone of oocyte nucleus is chromosome-free nucleoplasm (Figs. 2a, 2c). At the oocyte growth stage, when the most active transcription takes place on LB lateral loops and chromosomes occupy the greater part of the nuclear volume, the diameter of the area occupied by chromosomes increases in proportion to the enlarged diameter of the nucleus (Fig. 5). However, the pattern of LB chromosome arrangement inside the nuclear area they occupied does not significantly change at this time.

LB chromosomes occupy the central and intermediate nuclear zones, but their arrangement is not uniform. Counting of macro-, midi-, and microchromosomes in these distinct zones showed that, in chicken and quail germinal vesicles, most microbivalents (as a rule, more than 20) are localized close to the external border of the intermediate zone. In the central zone, the number of microchromosomes varied from one to seven; occasionally, no chromosomes were found in this area. The total number of macro- and midichromosomes in the karyotypes of both species is about three times smaller than the number of microchromosomes. Furthermore, they were also distributed unevenly between labeled nuclear zones; i.e., large bivalents were found in both the intermediate and central zones with the least numbers in the central zone (Fig. 2).

Analysis of Radial Distribution of Terminal Chromomeres of Chromosomes

A visual assessment of the distribution of bivalents that belong to various groups in nuclei has several disadvantages. For example, some bivalents have sizes close to the radius of the nuclear area occupied by the chromosomes; therefore, if a bivalent is located at an angle to the spherical surface, it is difficult to attribute it to a particular zone. In this case, other, usually subjective, criteria should be used.

To define the pattern of chromosome arrangement in avian oocytes more precisely, the radial positions of telomere regions of the chromosome in the germinal vesicles of female quails were examined. The LB chromosomes of quails are characterized by a lack of large terminal loops. The distance from the labeled nuclear center to the optical section with the most intensive fluorescence of the chromosome terminal chromomere was measured. It should be noted that, as a rule, the homologous terminal chromomeres in each bivalent are well defined. However, in the case of terminal chiasmata when terminal chromomeres of homologous chromosomes become undistinguished, these colocalized terminal choromomeres were considered to be a single object. Histograms that illustrate the distribution of terminal regions of macro-, midi-, and microchromosomes in the nucleus of a quail oocyte are presented in Figs. 4b, 4d, 4f.

To compare the results obtained for nuclei of various sizes and measurement standardizations, we calculated the ratio of the distance from the nuclear center to the terminal chromomere and the radius of the nuclear area occupied with chromosomes (R) (Fig. 2a). The requirement for the relative values is determined by the variations in the ratio of the radius of the area occupied by chromosomes to the nuclear radius in the oocyte at the LB stage; it varies from 0.65 to 0.82 in chickens (mean value is 0.73 ± 0.05) and from 0.65 to 0.80 in Japanese quails (mean value 0.74 ± 0.05) (calculated from the results presented in Fig. 5).

It was found that the majority of chromosome terminal chromomeres was localized in the nuclear volume from 0.8R to 1R, i.e., in the intermediate zone and its external border. 56.5–72.7% of macrobivalent terminal chromomeres, 78.9–85% of midibivalent terminal chromomeres, and 78.0–82.6% of microbivalent terminal chromomeres are concentrated in this area. As a whole, the pattern of the arrangement of telomere regions of differently sized chromosomes in various radial zones of germinal vesicles corresponds to the pattern of bivalent distribution obtained by visual counting them on 3D reconstructions.

Despite the obvious advantages of accurate measurements of the positions of telomere regions in the chromosome, the radial location of the chromosome should be evaluated, not only based on the estimated spatial position of particular chromosome segments, but also by the position of other chromosome regions. There is another approach to defining the spatial object coordinates in the nucleus that is helpful in resolving this problem.

Analysis of Radial Arrangement of Bivalent Gravity Centers

The method is based on computer 3D reconstructions and the creation of an isosurface, followed by splitting these isosurfaces into separate surface objects corresponding to bivalents (see Materials and Methods) (Fig. 1). During the segmentation of confocal images, the contours of the isosurface are determined for all bivalents based on the adjusted threshold of fluorescence intensity (Figs. 1a, 1b). The spatial coordinates of the center of gravity of a body bordered by an isosurface constructed around a bivalent depend on the shape of the object (Roberts et al., 2005). The histograms of the radial positions of the centers of gravity of macro-, midi-, and microbivalents relative to the estimated nuclear center are presented for the same quail germinal vesicle (Figs. 4a, 4c, 4e) as in the case

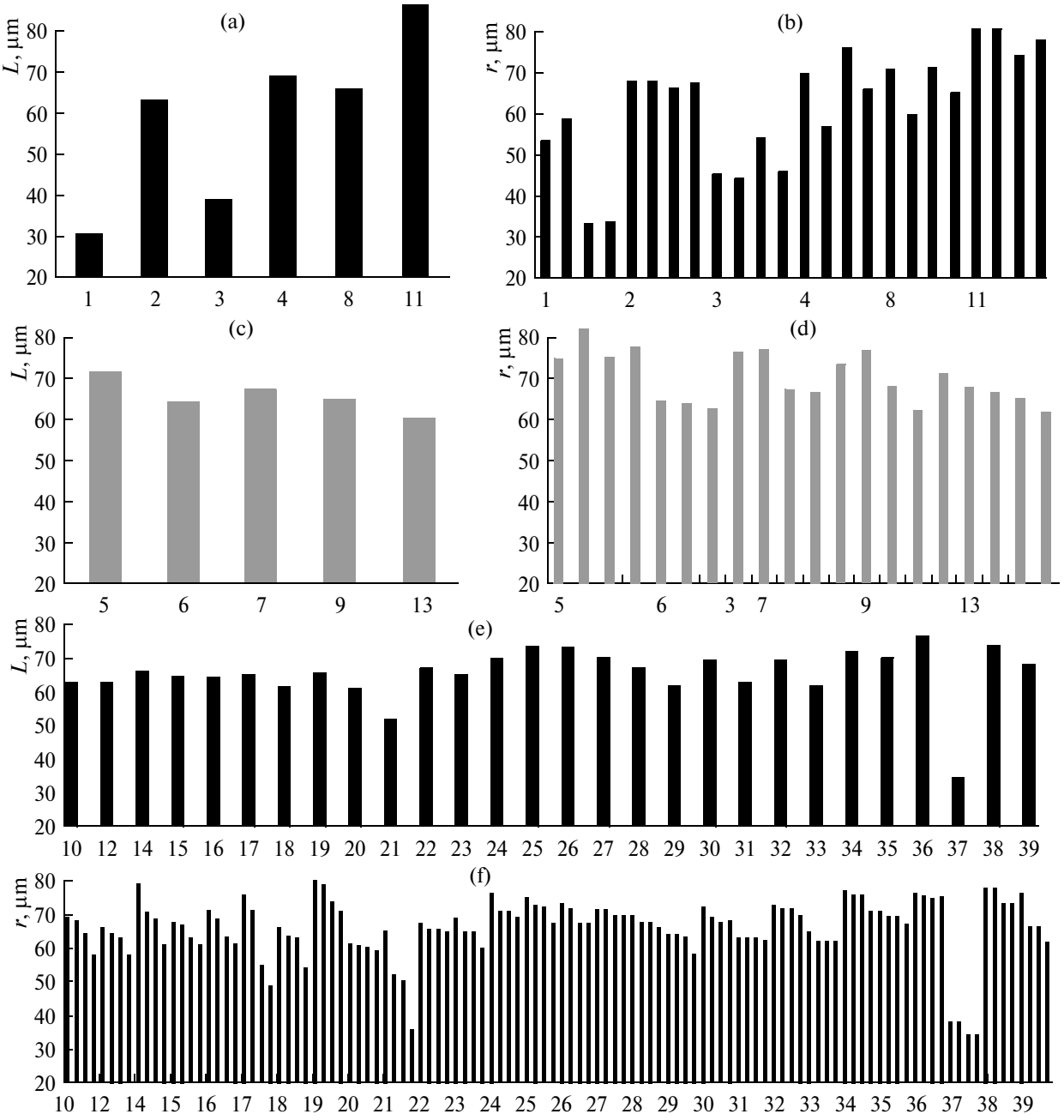

Fig. 4. Radial distribution of chromosomes in nucleus from Japanese quail oocyte. (a, c, e) histograms of centers of gravity positions of bodies bordered by isosurfaces corresponding to macro-, midi-, and microchromosomes, respectively; (b, d, f) histograms of terminal region positions of macro-, midi-, and microchromosomes, respectively. Numbers of meiotic bivalents isosurfaces attributed after computer-assisted 3D reconstruction of the nucleus are plotted on the X line (from 1 to 39). Distances from the center of the nucleus to the center of gravity of body bordered by isosurface (L) and terminal regions of the bivalent (r) are plotted on the Y line. General view of the 3D reconstruction of this nucleus from quail oocyte is shown on Fig.1 (panel d). Nuclear radius is 86 μm.

of the distribution of chromosome terminal chromomeres (Figs. 3b, 3d, 3f). The histogram profiles of the radial positions of chromosome telomere regions and the centers of gravity of bodies bordered with isosurfaces for the same germinal vesicle are almost identical. In general, in bivalents with more peripheral position of centers of gravity, one or both pairs of terminal chromomeres are located close to the nuclear periphery with one pair of bivalent terminal chromomeres being frequently positioned at the same distance from the center as the second pair (Figs. 4b, 4d, 4f). Very rarely, terminal chromomeres of bivalents may be located in different radial zones of the nucleus

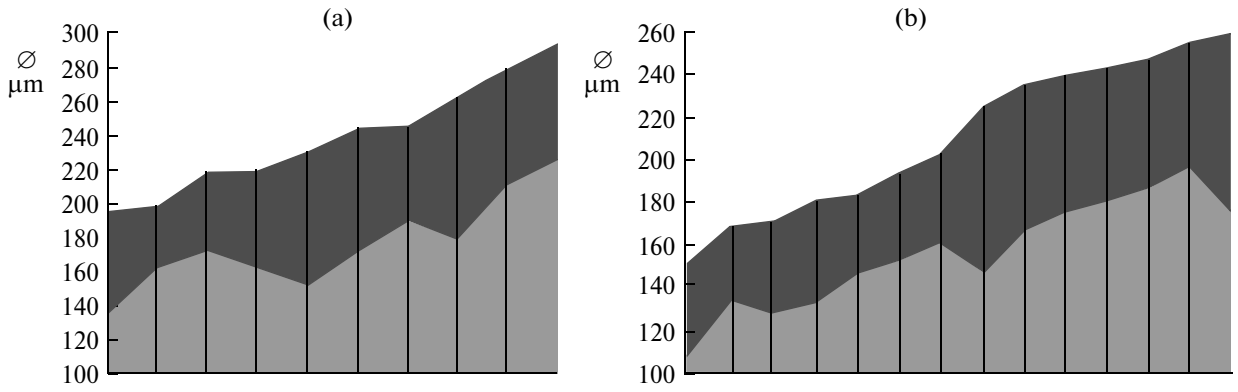

Fig. 5. Relation between oocyte nucleus diameter and diameter of nuclear volume occupied by chromosomes at the lampbrush stage in chicken (a) and quail (b).

Graphs are plotted basing on measurements of 10 chicken isolated nuclei (a) and 14 quail isolated nuclei (b). Upper border of dark area shows the influence of nuclear diameter from oocyte growth stage; upper border of the light area shows the influence of the diameter of volume occupied by chromosomes in the same nuclei from the oocyte growth stage.

(see, e.g., the bivalent with isosurface number 1 in Fig. 4b).

It was found that the centers of gravity of bodies that correspond to almost all macrobivalents (5.5 ± 0.6 from 6), including sex chromosomes, and almost all midibivalents (4.5 ± 0.6 from 5) are in the nuclear area from 0.4 to 1R. The centers of gravity of bodies outlined by isosurfaces constructed around microbivalents are mostly localized in the nuclear area from 0.8 to 1R. On average, 24.7 ± 2.2 microbivalent centers of gravity are found in this area. Close to the nuclear center, microbivalents are found more rarely (4.2 ± 1.6 microbivalents, in average). The least number of macro-, midi-, and microchromosomes are positioned in the central nuclear zone of 0.1–0.4R. The total number of bivalent centers of gravity in this area varies from 0 to 2.

Summarizing the data on radial arrangement of variously sized LB chromosomes in germinal vesicles of chicken and Japanese quail oocytes obtained by three different methods of bivalent location relative the nuclear center it can be concluded that there is a general principle of spatial chromosome organization in avian oocytes. In transcriptionally active avian oocytes, chromosomes are arranged in the certain nuclear area at a significant distance from the nuclear envelope. Gene-rich microchromosomes are localized mainly on the periphery of the area occupied by a complete chromosome set, rather than in the nuclear center. On the other hand, macro- and midichromosomes frequently occurred in the intermediate zone may also be found in the central zone of the oocyte nucleus.

DISCUSSION

Amphibian and avian LB chromosomes are classical objects of cytological, molecular, and cytogenetic studies (Morgan, 2002; Gaginskaya et al., 2009). Most studies are performed on LB chromosomes isolated from oocyte nuclei and the dispersed on slides and fixed (Morgan, 2008; Gall and Wu, 2010). Here, using CLSM and the digital processing of optical stacks of stained germinal vesicles (Ronneberger et al, 2008), for the first time, we have obtained 3D images of single LB chromosomes in the intact nucleus, i.e., in their natural microenvironment. This has allowed us to describe their spatial morphology in detail and, more specifically, to show that LB chromosomes have double-loop bridges.

Double loop bridges were first revealed in amphibian LB chromosomes and considered to be an artifact of mechanical chromosome stretching, which resulted from manipulations with the nuclear envelope and content. Moreover, in experiments with chromosome extension, the induced generation of double bridges was observed. These data significantly impacted the understanding the structure and structural integrity of meiotic bivalents (Callan, 1986; Morgan, 2002). However, in addition to the description of mechanically generated double bridges, there was evidence of natural LB double bridges, e.g., in specific regions in LB chromosomes of crested newt *Triturus cristatus cristatus* namely, giant fused loops (Callan, 1986). The author suggested that lumpy matrix with elastic structure was accumulated in these giant fused loops, preventing chromatin associations at their base.

We found that, in intact, unstrained nuclei of avian oocytes, double bridges were also present in simple lateral loops. These double-loop bridges are formed more often in specific regions of avian LB. For example, on isolated chicken LB chromosomes they were frequently observed in the middle of long arm of chromosome 4 (Chelysheva et al., 1990; Schmid et al., 2005). It was lately proposed that appearance of double bridges in LB was due to specific distribution in

chromosomes axes of SMC (structural maintenance of chromosomes) proteins. The proteins are components of condensin and cohesin complexes and are engaged in chromatin organization in chromomeres (Krasikova et al, 2005; Gaginskaya et al., 2009).

Some features of the spatial morphology of an LB chromosome in germinal vesicles can be explained based on the polymer model of meiotic chromosomes (Marko and Siggia, 1997). This model considers the physical properties of LB chromosomes which have the form of relatively short polymer filaments bound to a central polymer axis. Based on the modeled LB structure, which takes into consideration the rigidity of bending and the radius and axial tension between brushes at various experimental parameters, the authors predicted that chromatin pressure, i.e., the repulsion force between half-bivalent lateral loops (brushes), led to bivalent regions between adjacent chiasmata take on an oval shape (Marko, Siggia, 1997). Repulsion forces direct these regions planes mutually perpendicularly such that the loops were minimally overlapped. These features of bivalent spatial organization at the LB stage we indeed observed in isolated nuclei of avian oocytes. The polymer model did not indicate the forces that act between whole bivalents (Marko and Siggia, 1997). However, the pattern of chromosome arrangement observed in the oocyte nucleoplasm, which provides a minimal possibility of interactions between individual LB chromosomes, indirectly indicates the repulsion forces between whole bivalents. For these reasons, as a rule, the central nuclear zone in oocytes of the examined species contains a minimal number of bivalents.

The nuclear volume occupied by LB chromosome and its lateral loops could be defined as the LB chromosome territory. Theoretically, in its fine structure, chromosome territories in interphase nuclei are represented by compact chromatin domains composed of about 1 Mbp (Stadler et al., 2004; Albiez et al., 2006; Cremer et al., 2006). Transcriptionally active chromatin, which, as assumed, has the appearance of elongated loops, is located on the surface of compact 1-Mbp domains at sites where the chromatin is in contact with nuclear interchromatin space. The size and estimated DNA content in structural domains of chromosome territories is comparable to the size of LB chromosome chromomeres. Indeed, the average amount of DNA per chromomere in chicken LB macrochromosomes is 1.5–2.0 Mbp (Galkina et al., 2006). Because the LB chromomere and 1-Mbp domains in chromosome territories are presumably identical, it seems quite reasonable to suggest that the chromomere-loop model of chromosome organization can be propagated from the LB to interphase chromosomes. The apparent chromomere loop morphology of the LB chromosomes seems to be defined by the significantly larger size of transcription units (Morgan, 2002; Gaginskaya et al., 2009).

In this work, we used a combination of several quantitative approaches to approximate the position of the chromosome relative to the geometric center of the nucleus and determined the radial genome organization in nuclei of chicken and Japanese quail oocytes during the period of high transcription activity (at the stage of oocyte large growth). A comparison of the data on telomere regions, centers of gravity of the bodies, and the whole bivalent distribution in the nuclear space indicates the random arrangement of macro-, midi-, and microchromosomes inside of the area occupied by chromosomes in chicken and Japanese quail germinal vesicles. Random distribution of gene-poor macrochromosomes and gene-rich microchromosomes differs from the ordered radial arrangement of chromosome territories in interphase nuclei of somatic cells. In fact, it was shown that, in the nuclei of chicken neurons and proliferating fibroblasts, the group of 19 microchromosomes occupies the central nuclear area, whereas the territories of large (chromosomes 1–5 and Z) and medium-sized (chromosomes 6–10) chromosomes with a smaller number of genes are located mostly at the nuclear periphery (Habermann et al., 2001). It was also demonstrated that, in nuclei of chicken somatic cells, early replicating chromosome segments tend to be located close to the nuclear center, whereas late replicating regions are more frequently associated with the nuclear periphery, as was observed in mammalians. There is some evidence that confirms the evolutionary conservatism of the distribution of chromosome territories in Neognathae birds depending on the chromosome size (Skinner et al., 2009) and content of the GC pair (Federico et al., 2005). Since chicken and Japanese quail karyotypes are very similar, it can be expected that the arrangement of macro, midi-, and microchromosomes in the interphase nuclei of both species will be the similar.

In the discussed problem, it is interesting to note that, according to a computer model that simulates the probabilistic distribution of chicken chromosome territories, the opposite distribution pattern of chromosome territories in the elliptic nucleus common for fibroblasts was observed, i.e., most microchromosomes were located at the nuclear periphery (Habermann et al., 2001). The observed microbivalent arrangement in chicken germinal vesicles was in total compliance with the modeled distribution only taking into account geometric limitations.

The spatial organization of the genome in the interphase nuclei at the level of chromosome territories and their domains conforms to the general principle of the functional compartmentalization of the cell nucleus (Sadoni et al., 1999; O'Brien et al., 2003). According to the self-organization model, nuclear compartments are highly dynamic functional assemblies controlled by intranuclear processes. The microenvironment of these complexes enhances, synchronizes, and regulates their functions. The common concept on the

nuclear structure and function within the frame of the model may be revised. It may be proposed that the 3D stationary state of the genome results from many factors, including the permanent interaction of the genomic DNA with molecules in the nucleoplasm, the self-organization and disassembly of nuclear domains and their functional interactions, and the mobility of nuclear structures (O'Brien et al., 2003; Misteli, 2007). Complex interactions between multiple factors defines the position of chromosome territory in the nucleus; however, the radial organization of the genome is not responsible for any function (van Driel et al., 2003; Fedorova and Zink, 2008).

A previtellogenic oocyte is an example of a terminally differentiated, highly specialized cell. The main feature of the genome functioning at this stage is its high transcriptional activity. The disordered random radial arrangement of LB chromosomes we observed in this trancriptionally active nucleus probably results from the lack of structural limitations that determine the regular organization of chromosome territories in the interphase nuclei.

The lack of chromosome associations with the nuclear periphery, as well as the lack of chromocenters that integrate heterochromatin regions of nonhomologous chromosomes, is common for oocytes at the LB stage. For this reason, close neighbors of a bivalent are random. Conversely, in interphase nuclei, chromosome interactions with the nuclear lamina and each other are ensured by regions of constitutive heterochromatin, which generate a repressive nuclear compartment. It should be stressed that satellite DNA sequences that form chromosome heterochromatin regions exhibit high transcriptional activity at the LB stage. Indeed, a significant portion of DNA sequences transcribed at avian LB chromosomes are pericenromeric, subtelomeric, interspersed tandem repeats, and noncoding spacers, although the transcription of encoding sequences was also registered (Gaginskaya et al., 2009). The length and density of transcription units in gene-poor LB macrochromosomes is a little higher than in gene-rich LB-microchromosomes, except for the sex chromosome W, which retains a somewhat condensed state in the oocyte nucleus (Solovei et al., 1993).

Random chromosome distribution we observed is probably determined also by huge size of the germinal vesicle and volume of free nucleoplasm. Under these conditions, LB chromosomes with a chromomere-loop morphology acquire the most optimal spatial configuration with minimal interactions of adjacent bivalents and their lateral loops. This unique nuclear architecture in oocytes in some animal species may have functional significance for the maturation and future fertilization of the oocyte, as well as zygote formation.

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