

Ultrastructural organization of the chromatin elements in chromosomes of the dinoflagellate *Prorocentrum minimum*

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Summary

DNA of the majority of present-day eukaryotes is folded with the aid of highly conserved histone proteins to form nucleosome fibers, which further condense via interactions with histones and non-histone chromatin proteins. Dinoflagellate chromatin is unique among eukaryotes, as their chromosomes lack the histones and remain condensed throughout the cell cycle. Here, we report the results of electron microscopy study of chromatin elements inside the chromosomes of potentially toxic planktonic dinoflagellate *Prorocentrum minimum*. We found that chromatin fibers inside the condensed chromosomes of *P. minimum* form not only arch-like structures, that were repeatedly described for various dinoflagellate species, but also rosette-like complexes with chromatin fibers radiating from its electron-dense center. The effects of histone loss in the evolution of dinoflagellates are discussed within the frames of the hierarchical chromatin folding paradigm.

Key words: protists, dinoflagellates, chromatin, chromosomes, chromatin folding, nucleus, electron microscopy

Introduction

The eukaryotic genomes are represented by giant DNA molecules, which length exceeds the length of mitotic chromosomes and the diameter of interphase nuclei by several orders of magnitude. The compaction of the nuclear DNA is carried out by the formation of the chromatin that is a complex of DNA, proteins and RNA. It is generally accepted that chromatin folding into higher-

order structures occurs in a step-by-step fashion (Kuznetsova and Sheval, 2016). Initially, DNA and histones interactions lead to the formation of 11-nm nucleosome fibers (Olins and Olins, 1974). The chromatin fibers of 30 nm in diameter, that for a long time have been considered the result of nucleosome fiber compaction, were identified in the interphase nuclei and mitotic chromosomes after aldehyde fixation (Finch and Klug, 1976; Woodcock et al., 1984; Grigoryev et al., 2009). However, their

presence in the live cells is now doubtful, since the results of cryoelectron-microscopy studies showed the lack of ordering in the nucleosome distribution within the nucleus (Eltsov et al., 2008; Maeshima et al., 2010; Fussner et al., 2012; Nishino et al., 2012). The so-called chromonema – thick fiber of 100–130 nm in width – which, according to some data, is by itself a chain of globular structures or “chromomeres” is usually considered as the next level of chromatin folding (Sparvoli et al., 1965; Zatsepina et al., 1983; Belmont and Bruce, 1994; Sheval and Polyakov, 2006). Chromonema, in turn, folds into a fiber with a diameter of 200–250 nm that forms a metaphase chromosome (Strukov et al., 2003; Kireeva et al., 2004).

The researchers’ attention to DNA packaging in the nucleus is long-standing. However, until recently folding levels of the DNA have been studied in detail only in several model objects, mostly in cells of mammals. Data on the chromatin folding and structural diversity at different hierarchical levels of genetic material packaging are largely incomplete for other eukaryotes. However, a few published papers on this subject already indicate significant structural diversity in the molecular and structural organization of chromosomes. It was demonstrated that the absence of positively charged amino acids in the N-terminal region of histone H2A that is characteristic of organisms with a small genome might cause a lower density of chromatin compaction in the yeasts (Macadangdang et al., 2014). The topology and character of the chromatin fiber folding in some plants could be changed due to the increase in the size of genomes during evolution (Kuznetsova et al., 2017). However, up to now, the structural features of the chromatin compaction and the underlying molecular mechanisms remain poorly studied for the most nucleus-containing living creatures. Extensive comparative studies of nuclear apparatus in cells of eukaryotes from different phylogenetic groups are needed to clarify the range of structural diversity and possible ways of the evolution of the chromatin formations.

Organisms with a particularly peculiar architecture of the nuclear apparatus, namely dinoflagellates, are of the outstanding interest (Iwamoto et al., 2016). These ecologically essential protists have chromosomes that stay in a condensed state throughout the cell cycle both in the interphase and during the mitosis and demonstrate several unique structural features (Raikov, 1982, 1995). The chromatin fibers isolated from the dinoflagellate chromosomes appear as smooth threads of about 6.5 nm

in diameter (Rizzo and Burghardt, 1980). They are thicker than the 2-nm DNA molecule, but thinner than the typical eukaryotic 11-nm nucleosome fibers, and do not demonstrate the “beads-on-string” organization. The smooth chromatin fiber is considered as the first level of DNA packaging in the absence of 11-nm fiber in the dinoflagellates (Fukuda and Suzaki, 2015). Several models explaining smooth fiber packaging and chromosome architecture have been proposed for these protists. Early models postulated polythene nature of the dinoflagellate chromosomes (Haapala and Soyer, 1973; Raikov, 1982). According to this concept, the dinoflagellate chromosomes consist of a large number of uniform circular fibers assembled into a composite twisted bundle. At present, these ideas have only historical significance since it has been convincingly demonstrated, at least for some dinoflagellates, that their chromosomes contain only one DNA molecule and their genome is haploid (Roberts et al., 1974; Allen et al., 1975). In the recently proposed models, each dinoflagellate chromosome contains one DNA molecule, like a chromosome in the somatic cells of other eukaryotes. Among them, there is a model that describes a thick right-hand coiled toroidal bundle of chromatin filaments, or chromonema (Oakley and Dodge, 1979). Herzog and Soyer (1983) proposed a model according to which dinoflagellate chromosomes possess helical organization. The DNA fibers are compacted hierarchically through six levels of packaging, and Ca^{2+} and Mg^{2+} ions stabilize the resulting structure. Some researchers favor the cholesteric liquid crystal model proposed by Bouligand and Livolant (Bouligand et al., 1968; Rill et al., 1989). In spite of the latter model is being considered the most probable one, the question of how the chromatin in the dinoflagellate nucleus is organized remains unanswered (Fukuda and Suzaki, 2015).

The unique organization of the dinoflagellate chromosome apparatus arises from the absence of typical core histones. Histones have been replaced by specialized positively-charged proteins at early stages of dinoflagellate evolution. Two groups of these proteins were characterized: (1) DVNPs (Dinoflagellate/Viral NucleoProteins) that show a strong homology with proteins of algae viruses from Phycodnaviridae family (Gornik et al., 2012; Irwin et al., 2018) and (2) HLPs (Histone-Like Proteins) that were identified in some dinoflagellate species (Sala-Rovira et al., 1991; Taroncher-Oldenburg and Anderson, 2000; Chudnovsky et al., 2002).

HLPs show sequence similarities with *E. coli* HU protein (Wong et al., 2003; Chan and Wong, 2007). Of interest, the sequences of core histone and histone-modifying proteins were detected in the transcriptome of the dinoflagellate *Lingulodinium* sp. (Roy and Morse, 2012). However, if the translation of these mRNAs really take place, the level of protein synthesis should be low. The proposed roles of these residual histones in dinoflagellates might include a contribution to the DNA repair pathway and transcriptional regulation (Fukuda and Suzaki, 2015).

It appears that the replacement of histones with dinoflagellate-specific proteins has led to a change in the character of chromatin compactization at the lowest packaging levels. At the same time, it remains unclear how these changes affected the chromatin folding at higher levels of chromatin compaction in dinoflagellates. To clarify this issue, in the present work, we have analyzed the ultrastructure of chromosomes in the interphase nuclei of presumably toxic planktonic dinoflagellates *Prorocentrum minimum*.

Material and methods

The culture of the dinoflagellate *Prorocentrum minimum* isolated from the Black Sea was obtained from the collection of the Department of Hydrobiology, Lomonosov Moscow State University, and is currently maintained as the strain PmBS-1 in the protist collection at the Laboratory of Cytology of Unicellular Organisms (Institute of Cytology RAS). The cells used were grown in 17 PSU f/2 medium (Guillard and Ryther, 1962; Kester et al., 1967) without silicate at room temperature, pH 8.2 and 50 µmol photons m⁻² s⁻¹ under a 12 h light : 12 h dark cycle.

For light microscopy, the cells were fixed in 3.7% formaldehyde solution in f/2 medium for 1 h. Pellets were dehydrated in the series of ethanol solutions with increasing alcohol content and embedded in LR White resin (Sigma, USA). The 200-nm thick acrylic resin sections were prepared using LKB III ultramicrotome (LKB, Sweden), mounted on formvar-coated coverslips and stained with 1 µg/ml DAPI solution for 10 min (Sheval, 2018). Coverslips were attached to the microscope slides with Mowiol (Calbiochem, USA) supplemented with 50 µg/ml anti-bleaching agent, DABCO (Sigma, USA) and observed using Axiovert 200M (Carl Zeiss, Germany) inverted microscope equipped with

Plan-Apochromat 100x NA 1.4 oil immersion lens and ORCAII-ERG2 digital camera (Hamamatsu Photonics, Japan). The series of 45 optical sections with the step of 0.20 µm were acquired and subjected to Constrained Iterative deconvolution routine implemented in Carl Zeiss AxioVision 3.5 software (Carl Zeiss Microimaging, Germany) using model PSF.

For the electron microscopy studies, the cells were fixed for 24 h in 2.5% solution of glutaraldehyde prepared using f/2 medium and post-fixed with 1% aqueous osmium tetroxide at +4 °C for 1 h. Cells were dehydrated in the series of increasing ethanol concentrations. At this stage, cells were contrasted with 2% uranyl acetate in 70% ethanol. A 96% ethanol was replaced with acetone followed by infiltration with acetone-epoxy resin mixtures with increasing content of the resin. The cells were then infiltrated with freshly prepared resin and spun down in the 1.5 ml centrifuge tubes. The tubes were refilled with fresh epoxy resin, and the resin was cured at 70°C for 72 h. SPI-pon 812 (SPI Inc., USA) was used for infiltration and embedding.

The blocks were trimmed with a razor blade, and ultrathin sections were prepared using Ultracut E (Reichert Jung, Austria) ultramicrotome with a diamond knife. The nominal thickness of the sections ranged from about 60 to 200 nm as required. Sections were mounted on formvar-coated copper slotgrids. Mounted sections were post-stained with 2% aqueous uranyl acetate for 40 min and lead citrate for 5 min, or were observed without post-staining.

The ultrathin epoxy-resin sections were observed and photographed using JEM-1400 (Jeol, Japan) equipped with goniometer stage operated at 80 kV to 120 kV depending on the section thickness. For stereo pairs, 5 degrees tilt was used.

Alignment of serial sections and stereo pairs was performed using StackReg plug-in (<http://bigwww.epfl.ch/thevenaz/stackreg/>) for ImageJ. Maximum intensity projections of aligned series were prepared using built-in function of ImageJ (NIH).

Stereo pairs were assembled and colorized for viewing with red-cyan glasses using Photoshop (Adobe Inc., USA).

Results and discussion

We used the semithin (~200 nm) sections of cells embedded in hydrophilic acrylic resin for the light microscopy study as described elsewhere

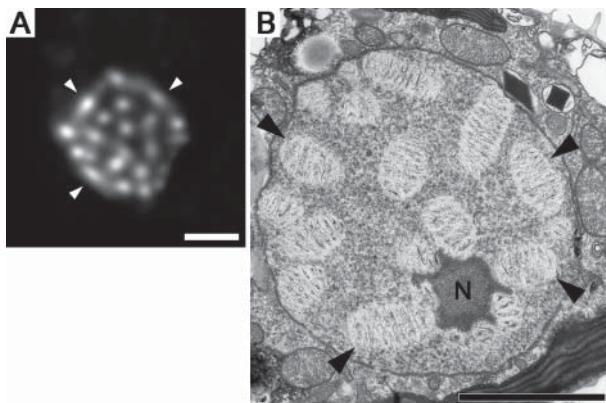


Fig. 1. Condensed chromosomes inside the interphase nuclei of *Prorocentrum minimum*. A – fluorescence microscopy detection of chromosomes with DAPI in semi-thin sections; B – electron microscopy of the interphase nucleus. Abbreviations: N – nucleolus; arrowheads – condensed interphase chromosomes. Scale bars: 2 μm.

(Sheval, 2018). Such sections are thinner than the thickness of the confocal microscope optical slice. Hence, the out-of-focus fluorescence did not affect the resulting images. The *Prorocentrum minimum* interphase nucleus is about 5 μm in diameter and contains rod-shaped, condensed, morphologically similar chromosomes (Fig. 1, A). No variations in fluorescence intensity were noticed along the length of the chromosomes. It was not possible to reliably determine the presence or absence of DNA outside the chromosome bodies using a light microscope.

The separate chromosomes, typical for the dinoflagellate, embedded in the extraordinary electron-dense nucleoplasm, were revealed in the interphase nuclei at low magnifications during electron microscopical study (Fig. 1, B). Details of their organization can vary depending on the cell cycle stage (Baud et al., 2000). Nevertheless, the electron-microscopy study of nuclei at high magnification showed that based on the chromosome organization the vast majority of the interphase *P. minimum* cells fall into two broad types. Cells with sharply different morphology and mitotic cells are beyond the scope of this article.

The chromosomes in the type I cells show the sets of distinct electron-dense disks, oriented orthogonally to the long axis of the chromosome. Each disk, in turn, consists of tight bundles of chromatin fibers that run in the plane of the disk (Fig. 2, A). This longitudinal differentiation of chromosomes is especially visible on relatively thick

sections of about 150 nm (Fig. 2, B). The spacing between adjacent disks does not vary chromosome-to-chromosome and is within 50–60 nm, well below the resolving power of the light microscope.

In the 60 nm transverse sections of chromosomes, the chromatin fibers radiate from the origins on the chromosome periphery and follow the arch-shaped paths (Fig. 2, C). On the transverse sections with a thickness of about 150 nm, chromatin appears as a dense network of chromatin fiber bundles, which tend to become thicker and more closely spaced in the central zone of the chromosome (Fig. 2, D). These bundles become loose towards the periphery of the chromosome as the fibers spread from them and out of the plane of the disk (Fig. 2, D). As a result, the chromosome peripheral zone separates distinctly from the denser central core, but this transition is rather smooth (Fig. 2, B, D).

The transitions from the arch-shaped pattern seen on thin sections into the meshwork observed on the thicker sections can arise from the superposition of two or more disks that are captured within the volume of the observed section.

The space between the adjacent disks (inter-disks) contains loosely arranged fibers. Some of these fibers are cut normal to the section plane and look like dense granules (Fig. 2, A). Electron-dense granules associated with chromatin fibers were considered as a separate structural component of dinoflagellate chromosomes (Spector et al., 1981). Nevertheless, we treat these granules as cross-sections of fibers.

For more detailed characterization of the inter-disks, which look relatively devoid of chromatin on the longitudinal sections and are indistinguishable on the transverse sections of the chromosomes, we analyzed overlays of ultrathin serial longitudinal sections with a thickness of 70 nm (Fig. 3). On the overlaid images, inter-disks look much denser than the disk region since all consecutive serial sections contain transversely cut fibers that have high electron density. This picture arises from the incomplete alignment of the images and doesn't allow to judge about the distribution of the material between disks and inter-disks, but it confirms that the fibers have preferred orientation in the inter-disks. Of note, the inter-disk areas on the overlaid images appear not only sandwiched between the disks but also enveloped in the sheath of fibers that connect disks with each other along the boundaries of the chromosome (Fig. 3, G, G').

In summary, two morphological regions are detected in the interphase chromosomes of the

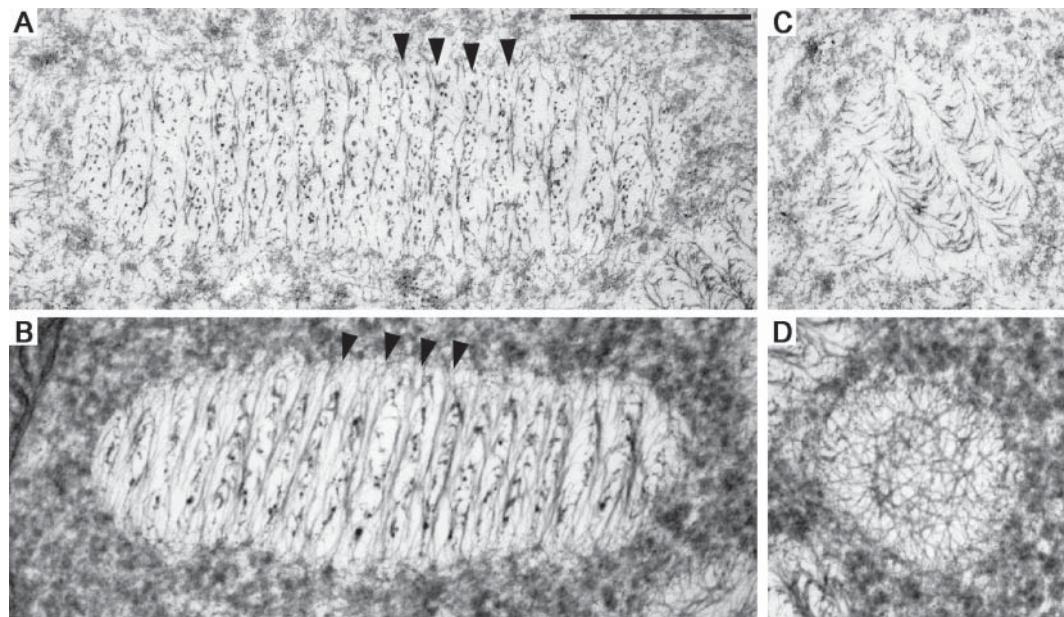


Fig. 2. Ultrastructural organization of the chromosomes with distinct disks attributed to type I interphase nuclei of *P. minimum*. A – longitudinal section of the chromosome (section thickness – 50-60 nm); B – longitudinal section of the chromosome (section thickness – 100-120 nm); C – transverse section of the chromosome (section thickness – 50-60 nm); D – transverse section of the chromosome (section thickness – 10-120 nm). Disks are marked with *arrowheads*. Scale bars: 0.5 μ m.

type I cells: (1) central zone, or the core, formed by chromatin layers with a different orientation of the fibers appearing as disks and inter-disks on the longitudinal sections, and (2) peripheral region containing individual chromatin fibrils that extend from disks and enter the adjacent disks. Due to the high density of nucleoplasm material DNA fibers extending beyond the chromosome boundaries are rarely discerned (Fig. 4, A, B).

In the type II cells, chromosomes lack the clear differentiation between disks and inter-disks (Fig. 5, A, B). Numerous fibers cut at different angles show up on longitudinal sections of these chromosomes (Fig. 5, B). The disks manifest themselves as regularly spaced bundles of chromatin fibers running across the chromosomes perpendicular to the longitudinal chromosome axis from one edge of the chromosome to the other. However, the most prominent feature of the chromosomes in type II cells – the rosette-shaped structures formed by fibers with a diameter of 5-15 nm diverging radially from one center – was observed in the transverse sections of the chromosomes (Fig. 5, C). The tight interlacing of the fibrils forms the dense core of rosettes. These rosettes are scattered throughout the entire volume of the chromosome, as the analysis of the series of consecutive sections revealed (Fig. 5, C-H).

Some cells have chromosomes that demonstrate a mixture of the features of type I and II. In these cells, chromosomes are showing both the arch-shaped and rosette-like arrangements of the chromatin fibers (Fig. 6, A-C). It is likely that the rosettes might be indistinguishable in densely packed disks, especially when section thickness is high. To verify this assumption, we prepared the stereo pairs (red-cyan anaglyphs) from about 150 nm thick sections of nuclei of both type I and type II cells. Longitudinal sections made at a slight angle to the line of sight were used. On the stereo pairs of type I chromosomes the separate tangles of the chromatin fiber, similar to the rosette-like structures described above, become visible within the disks (Fig. 6, D). Alternatively, the study of stereo pairs of the type II chromosomes (Fig. 6, E) allows to detect disk-shaped organization of chromatin in the chromosome “body” more clearly, than in the ultrathin sections (Fig. 6).

The ultrastructural analysis conducted in this work provides evidence that chromatin fibrils can form discrete “rosette-like complexes” in the permanently condensed *P. minimum* chromosomes. These complexes are distinguishable in the ultrathin sections of nuclei of type II cells, where typical dinoflagellate dense chromosomal disks are not prominently manifested. In the cells of type I,

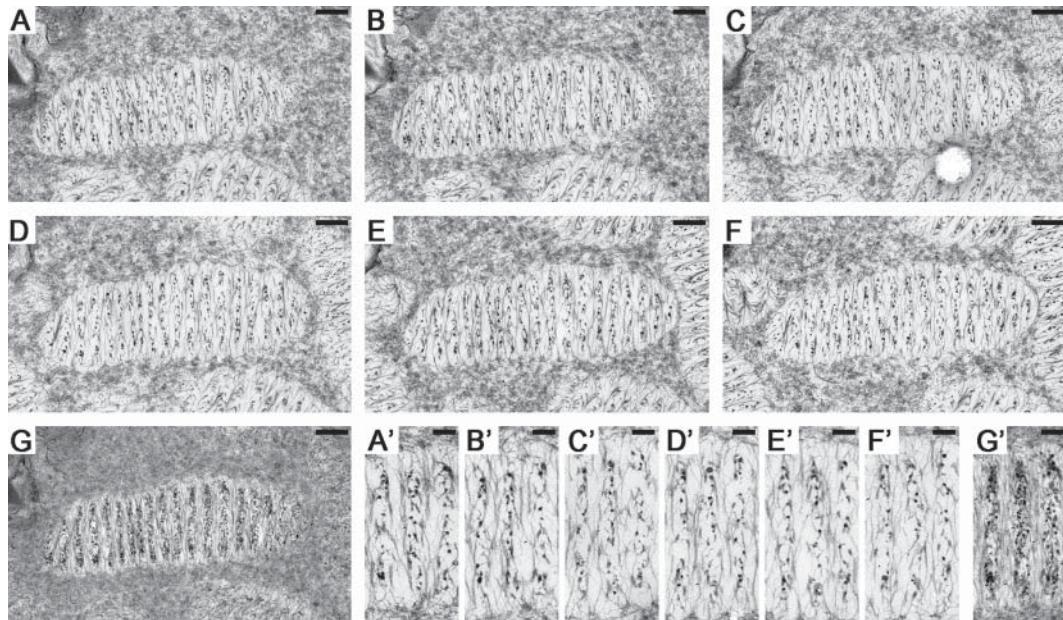


Fig. 3. Serial sections analysis of the *P. minimum* chromosomes of type I. A-F – serial longitudinal sections of the single chromosome; G – overlay (maximum intensity projection) of six serial sections shown on the panels A to F; A'-F' – the enlarged image of the fragment of the chromosome; G' – enlarged fragment of the overlay projected image (A-F). Scale bars: 0.1 μm (A-G) and 0.05 μm (A'-G').

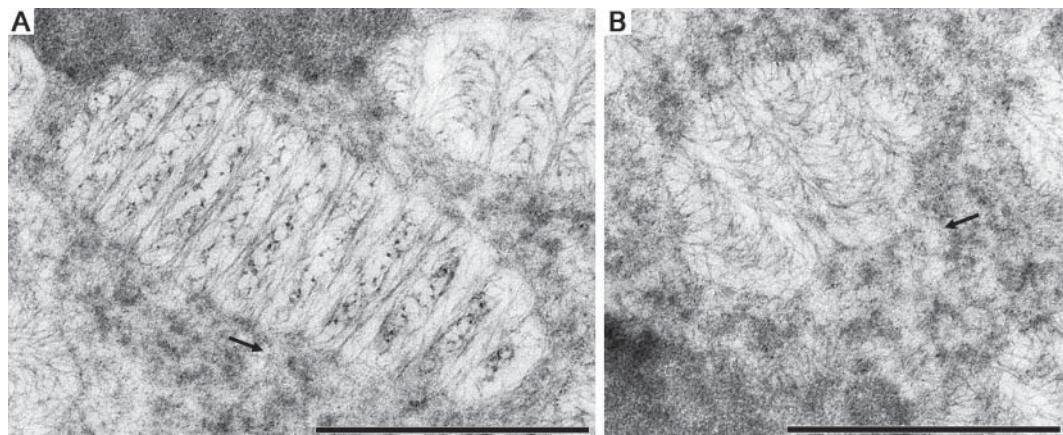


Fig. 4. Visualization of DNA fibers (arrows) that extend beyond the *P. minimum* type I chromosomes boundaries. A – longitudinal section; B – transverse section. Scale bars: 1 μm.

with electron-dense disks, such heterogeneity of chromatin is not visible in the ultrathin sections, but it reveals in the intermediately thick sections observed as stereo pairs.

The rosette-like complexes arise as the special sub-structures of chromatin as appeared from the studies of various uni- and multicellular eukaryotes. For instance, these complexes were detected in decondensed *in vitro* chromatin of ciliates (Tikhon-

enko et al., 1984), mammals (Zatsepina et al., 1983), and plants (Gornung et al., 1986). The similar structures were found in sections of partially deproteinized chromosomes of HeLa cells (Sheval and Polyakov, 2006). The rosette-like complexes were especially distinct in spread preparations of the deproteinized chromatin where each rosette appeared as a central granule with DNA loops stretching from it (Sonnenbichler, 1969a; Sonnen-

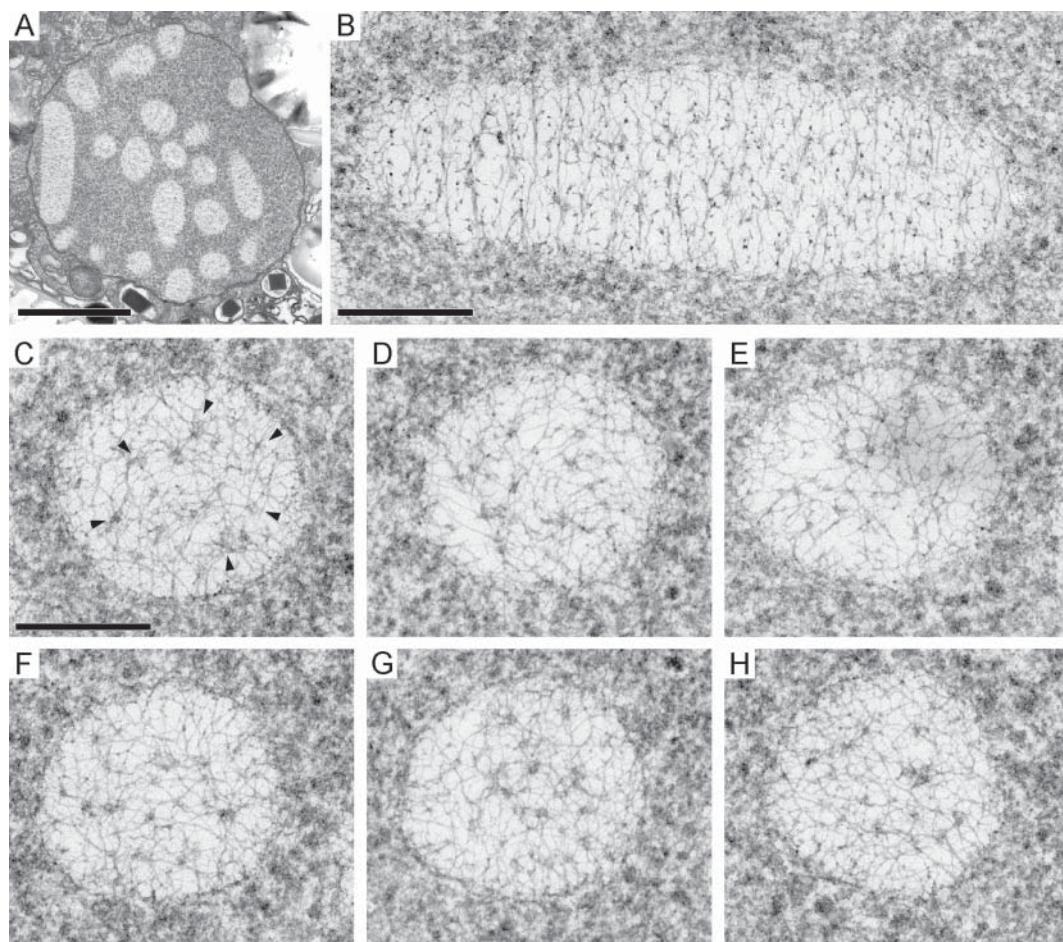


Fig. 5. Ultrastructural organization of the type II cell chromosomes of *P. minimum*. A – an overview of the interphase type II cell nucleus. There are no visible “disks,” which were the characteristic of chromosomes of type I (Fig. 1–4); B – longitudinal section; C–H – transverse serial sections. Rosette-like chromatin complexes are marked with *arrowheads*. Scale bars: 2 μ m (A) and 0.5 μ m (B, C).

bichler, 1969b; Comings and Okada, 1976; Okada and Comings, 1979; Prusov et al., 1983; Ascoli et al., 1988). These complexes are considered in most papers as the special condensed chromatin structural units – chromomeres – that become visible only after application of decondensation procedures and partial deproteinization of chromosomal material (see review: Kuznetsova and Sheval, 2016). However, they are indistinguishable in the condensed chromosomes without special treatments.

The data obtained in this study indicate the presence of discrete structural units within the interphase chromosomes of *P. minimum*. We infer that these chromatin structures have been preserved in the dinoflagellate chromosomes, even though these protists had lost histones during their evolution and the lower levels of their chromatin folding changed

dramatically in comparison with the majority of the eukaryotes. Nevertheless, this hypothesis requires experimental confirmation. Further studies of chromatin topology in the chromosomes of various dinoflagellate species are needed to elucidate the morphological diversity and possible evolution of this unique genetic apparatus.

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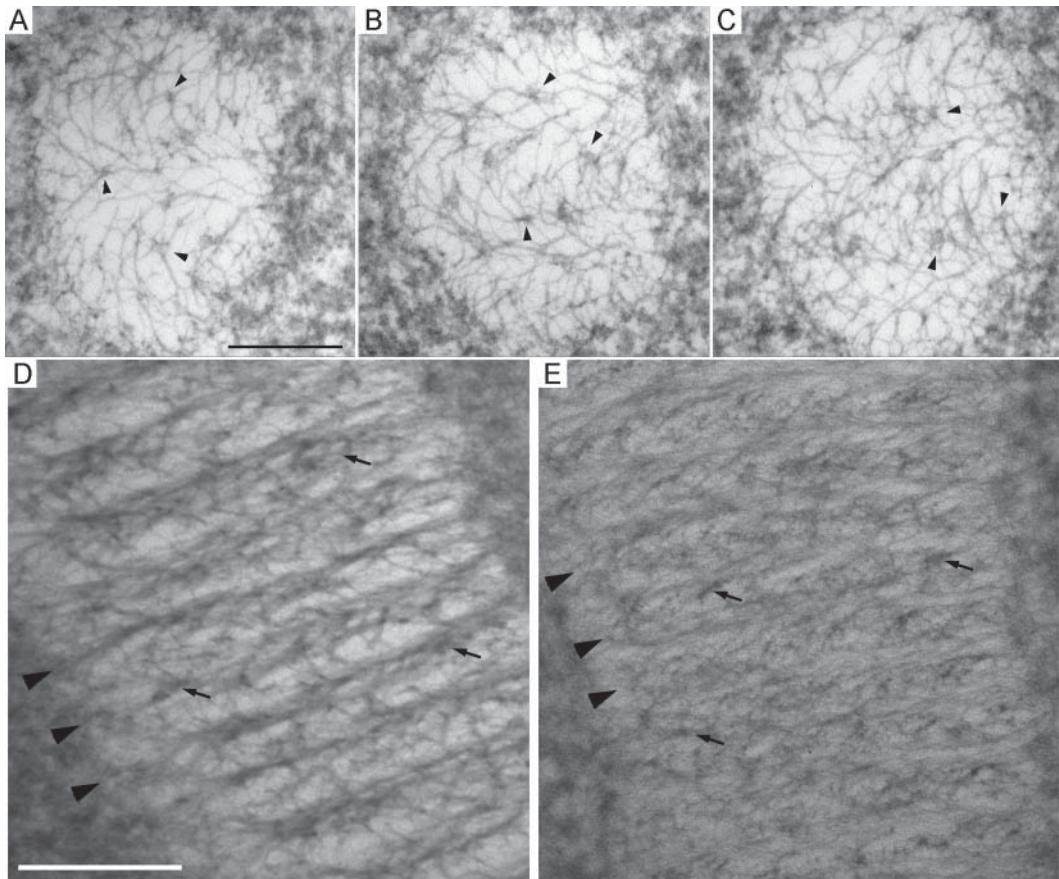


Fig. 6. Mixed features in the organization of chromosomes of *P. minimum*. A-C – transverse serial sections. The chromatin fibers demonstrate preferentially arch-like topology (from right to left border of the chromosome) with rosette-like complexes (arrowheads); D – stereo pair (red-cyan anaglyph) of chromosomes classified here as type I. There are discrete chromatin tangles, or rosette-like complexes (arrows) within the dense disks (large arrowheads); E – a stereo pair of chromosomes classified here as type II (i.e., without discernable disks). Disks (large arrowheads), as well as rosette-like complexes (arrows), are detected inside chromosomes. Scale bars: 0.2 μm.

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