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Localization of fibrillarin in the nucleoli of ciliate species with subchromosomal organization of macronuclear genome

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Summary

Fibrillarin is one of the highly evolutionary conserved nucleolar proteins. It plays an important role in the early processing and modifcation of pre-rRNA, ribosome assembly, maintenance of nuclear shape and cellular growth, and is essential for the early embryonic development. During the interphase, fibrillarin is found in nucleoli of higher eukaryotes, mainly in fibrillar centers and dense fibrillar components as well as in the Cajal bodies. At present, little is known about distribution of nucleolar proteins in ciliates. In this work, *Bursaria truncatella, Paramecium multimicronucleatum* and *Didinium nasutum* were studied. These ciliates belong to the species with subchromosomal organization of macronuclear genome. The aim of this work was to study localization of fibrillarin in their nucleoli using confocal microscopy and specific antibodies. It was shown that in the nucleoli of *B. truncatella* and P. *multimicronucleatum* fibrillarin mainly occurs in the central region of the nucleoli. In *D. nasutum*, on the contrary, nucleoli fibrillarin was located at the periphery of the nucleolar domain. These results are in good agreement with the electron microscopic data on morphology of the nucleoli that show an unusual, "inverted" location of the fibrillar component and the granular component in the nucleoli of *D. nasutum*.

Key words: ciliates, confocal microscopy, electron microscopy, fibrillarin, macronuclei, nucleoli

Introduction

The nucleolus is an essential structural component of the nucleus. In the nuclei of higher eukaryotes nucleoli assemble around the nucleolar organizer regions, where ribosomal DNA (rDNA) genes cluster in tandemly repeated rDNA arrays. The main role of the nucleolus is ribosome biogenesis. In the nucleolar domain ribosomal RNAs are synthesized, processed and assembled with ribosomal proteins. In eukaryotic cells, up to 80% of RNA synthesis is devoted to rRNA transcription, which is indispensable to the preservation of ribosome production and thus protein synthesis capacity (Nemeth and Langst, 2011).

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In the nucleolus 28S, 18S and 5.8S ribosomal RNAs (rRNAs) are transcribed as a single 47S ribosomal RNA (rRNA) precursor transcript, which is subsequently cleaved to form the mature 28S, 18S and 5.8S rRNAs, post-transcriptionally modified through interaction with small nucleolar ribonucleoproteins and additional protein-processing factors and, finally, assembled with the many ribosomal proteins before interaction with the export machinery and transport to the cytoplasm (Hadjiolov, 1985). Although the nucleolus is primarily associated with ribosome biosynthesis, recent data evidence that it has additional functions such as cell cycle progression, gene silencing, biogenesis of multiple ribonucleoprotein particles (Hernandez-Verdun et al., 2002), control of cell survival and cell proliferation (Carmo-Fonseca et al., 2000).

Three main structural subregions can be electronmicroscopically defined by their texture and contrast in nucleoli of higher eukaryotes (reviewed in Gébrane-Younès et al., 2005). Fibrillar centers (FC) are rounded clear fibrillar areas 0.1–1.0 μm in size. They are partly or completely surrounded by the dense fibrillar component (DFC) of compact texture. The dense fibrillar component is highly contrasted and is formed by closely packed fibrils. It was established that the sites of active rDNA transcription are localized at the interface between the FCs and the DFC. The non-transcribed part of the rDNAs as well as the polymerase I complexes and the transcription machinery such as the upstream binding factor (UBF) and topoisomerase I are localized in the fibrillar centers (Goessens, 1984). The fibrillar centers and dense fibrillar component are embedded in the granular component which mainly consists of granules 15–20 nm in diameter. It was shown that early processing of the pre-rRNAs occurs in the dense fibrillar component and late processing in the granular component (Goessens, 1984).

However, recent studies showed that such "classical" tripartite nucleolar organization is an evolutionary acquisition of higher eukaryotes. For example, the nucleoli in yeasts have only two morphologically distinct nucleolar components. The emergence of tri-compartmentalized nucleoli coincides with the transition between anamniotic and amniotic vertebrates (Thiry et al, 2011).

Recent advances in a large scale purification of nucleoli and protein analysis through mass spectrometry has provided a lot of information about the nucleolar proteome (Andersen et al., 2002). More than several hundred plant and human proteins that stably co-purify with isolated nucleoli were characterized. Interestingly, a comparison of nucleolar proteome data from humans and budding yeast showed that ~90% of human nucleolar proteins have clear yeast homologues and that the nucleolar proteome is largely conserved through evolution (Andersen et al., 2005).

One of the highly evolutionary conserved nucleolar proteins is fibrillarin. Fibrillarin homologues are present in the cells of various species from Archaea to human (Henriquez et al., 1990; Amiri, 1994; Amin et al., 2007). Fibrillarin plays an important role in early processing and modification of pre-rRNA, ribosome assembly, and is also essential for early embryonic development (Fomproix et al., 1989; Tollervey et al., 1993; Newton et al., 2003). Moreover, fibrillarin plays a critical role in the maintenance of nuclear shape and cellular growth (Amin et al., 2007). During interphase, fibrillarin is prominently found in nucleoli mainly in fibrillar centers and dense fibrillar components as well as in the Cajal bodies (Azum-Gelade et al., 1994; Amin et al., 2007).

At present, little is known about distribution of nucleolar proteins in ciliates. In these protists, nucleoli are located in the macronuclei – transcriptionally active highly polyploid somatic nuclei. Ciliate nucleoli are very diverse in morphology (Raikov, 1982), which can be partly explained by peculiarities of macronuclear genome organization. Contrary to genomes of higher eukaryotes, macronuclear genome is represented by a set of relatively short DNA molecules. All ciliate species can be divided into two groups: the ciliates with macronuclear DNA molecules of subchromosomal size (from several tens up to several hundred kbp), and those with gene-sized (mainly 0.5–25 kb) macronuclear DNAs (Raikov, 1995). Each of these DNA molecules is terminated by telomeres at both ends, lacks a centromere and is usually considered as a nano-, or mini-chromosome (Zagulski et al., 2004; Pimenov et al., 2006; Jung et al., 2011).

Postberg et al. (2006) showed that spherical nuclear structures, which fulfil nucleolar function in the gene-sized ciliate *Stylonychia lemnae*, do not represent true nucleoli in a classical use of the term, because processing of rRNA occur in a bipartite and inverse manner compared to typical metazoan nucleolar organization. Postberg et al. (2006) assumed that the absence of typical chromosomes could

determine an alternative spatial organization of *S. lemnae* nucleolar bodies.

In this work, *Bursaria truncatella, Paramecium multimicronucleatum* and *Didinium nasutum* were studied. These ciliates belong to the species with subchromosomal organization of macronuclear genome. The aim of this work was to study localization of fibrillarin in their nucleoli using confocal microscopy. The results obtained were compared with electron microscopic data on morphology of nucleoli in these species.

Material and methods

Cultures

Bursaria truncatella were sampled in a pond in the Tsytsin Main Moscow Botanical Garden of the Russian Academy of Sciences (Moscow, Russia) and cultivated at 10–12 °C in boiled tap water. The laboratory strain of *Didinium nasutum* (Sciento, UK) used in this work was cultivated at room temperature in boiled tap water. Both *B. truncatella* and *D. nasutum* were fed with *Paramecium caudatum* cultivated separately. *P. multimicronucleatum* and *P. caudatum* cultures were received from the Centre of Core Facilities "Culture Collection of Microorganisms" (St. Petersburg State University) and cultivated in lettuce medium inoculated with *Enterobacter aerogenes*.

Immunolabeling

Ciliate cultures in logarithmic growth phase were used. The interphase macronuclei were isolated in the solution containing 0.5% Nonidet P-40 (Serva, Germany) in 2 mM KH_2PO_4 buffer, pH 7.5, briefly rinsed in PBS (140 mM NaCl, 7.5 mM K_2HPO_4, and 2.5 mM NaH_2PO_4, pH 7.3) and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Fixed macronuclei were mounted on cover glasses, washed in PBS (3 times × 5 min), incubated overnight at 4°C in anti-fibrillarin antibody (Abcam, ab5821) diluted 1:200 with PBS containing 2% BSA (bovine serum albumin), washed in PBS (3 times × 5 min), incubated overnight in Alexa Fluor 555 donkey anti-rabbit IgG (Abcam, ab150062) diluted 500 times with PBS and washed 2 times in PBS (5 min). The specimens were embedded in Slowfade medium (Molecular probes Inc., USA) containing DAPI (1.5 mkg/ml) for DNA counterstaining, and sealed with nail polish.

Confocal imaging

Eight-bit digital images were acquired using an inverted fluorescence confocal microscope Leica DMI 6000 CS equipped with a Leica TCS SP5 laser scan unit (Leica Microsystems) and operated with the Leica application software. All confocal laser scanning microscopy images were obtained using a HCX PL APO 63X/1.40-060 OIL CS oil immersion objective. The DAPI signal was acquired by excitation at 405 nm and detection in the 450–510 nm range. Alexa 555 fluorescence was acquired by excitation at 543 nm and detection in the 550-650 nm range.

Electron microscopy

Ciliates were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) for 1 h at room temperature. The material was dehydrated in a graded series of alcohol and embedded in Epon-Araldite. Serial sections were obtained using a LKB III ultratome (LKB, Sweden) and stained with uranyl acetate and lead citrate according to the standard procedure. The specimens were viewed through a JEM-100CX electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

Results

Figures 1, A-C show confocal microscopy micrographs of a *B. truncatella* macronucleus. In Fig. 1, A, where the total macronuclear DNA was stained with DAPI, nucleolar domains looked like unstained or weakly stained areas. Detection of fibrillarin with specific antibodies showed that fibrillarin was located in the central part of nucleolar domains (Figs 1, B, C). The distribution of fibrillarin label intensity was not uniform. From one to several bright areas surrounded by less intensely stained material were seen in the central part of each nucleolar domain. Taking into account the fact that fibrillarin mainly occurs in the dense fibrillar component, it was possible to assume that both solitary nucleoli and groups of several nucleoli were present in the macronucleus. This was well confirmed by electron microscopic data (Figs 1, D, E). On the ultrathin sections *B. truncatella* nucleoli had a complicated shape. Dense groups formed by several nucleoli of different size and shape were often seen (Fig. 1, D). Nucleoli had well-defined fibrillar component in the center and a thin layer

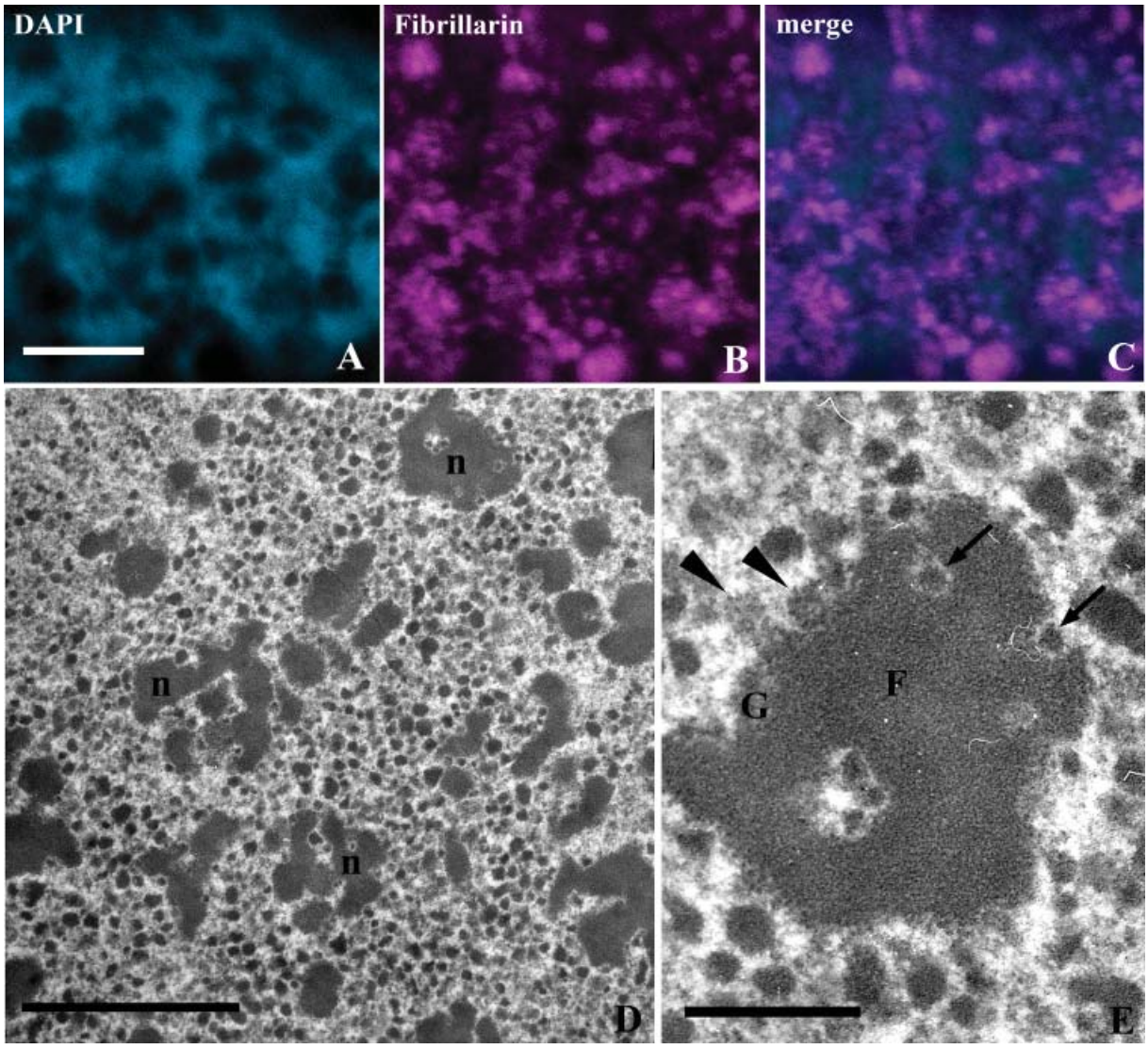

Fig. 1. Confocal microscopy (A-C) and electron microscopy (D, E) of the nucleoli in the interphase macronuclei of *Bursaria truncatella*. A – Total macronuclear DNA staining with DAPI; B – immunolabeling of fibrillarin with specific antibodies; C – merge of A and B images; D, E – ultrathin sections of *B. truncatella* macronuclei at different magnifications. *Abbreviations*: n – nucleoli, G – granular component, F – fibrillar component, *arrows* – intranucleolar chromatin bodies, *arrowheads* – small granular bodies about 120 nm in size formed by 20 nm RNP granules. Scale bars: A, B, C - 1 μm, D – 2 μm, E – 0.5 μm.

of the granular component at the periphery. Many 20 nm RNP granules and small granular bodies disconnected from the nucleolus granular region were seen in the macronucleus karyoplasms near the nucleoli. These granular bodies were about 120 nm in size and were formed of 20 nm RNP granules (Fig. 1, E).

With reasonable probability, the low thickness of the granular component is due to the high rate of pre-ribosomal particle transfer to the cytoplasm necessary to ensure a high level of protein synthesis in such large unicellular organism as *B. truncatella*.

Similar pattern of fibrillarin distribution was also observed in the nucleoli of *P. multimicronucleatum* (Figs 2, A-C). In interphase *P. multimicronucleatum* macronuclei, the nucleoli were of rounded shape with a fibrillar component in the center and fibro-granular and granular material at the periphery. Single nucleoli can be located close to each other forming large aggregates (Figs 2, D, E). A difference

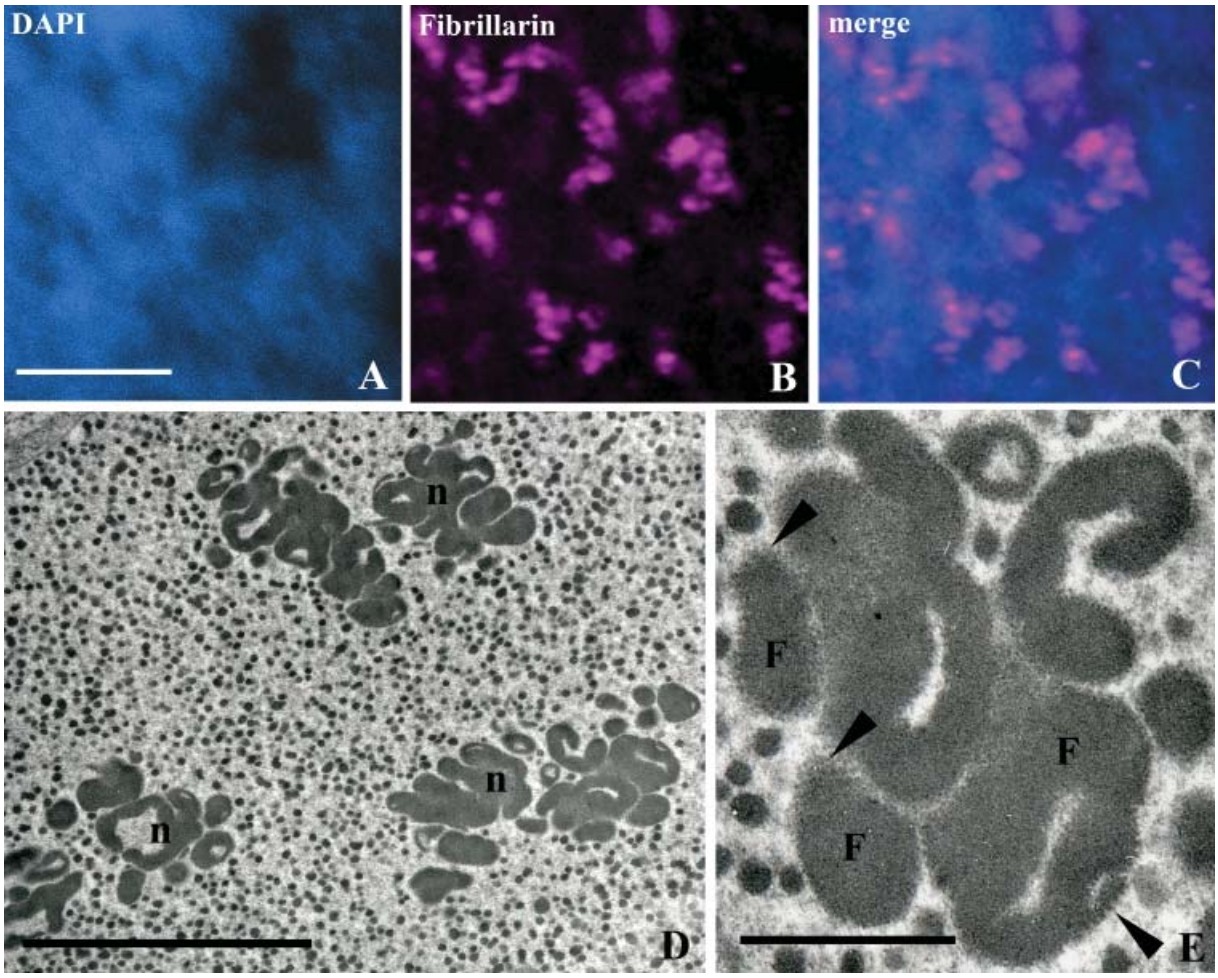

Fig. 2. Confocal microscopy (A-C) and electron microscopy (D, E) of the nucleoli in the interphase macronuclei of *Paramecium multimicronucleatum*. A – Total macronuclear DNA staining with DAPI; B – immunolabeling of fibrillarin with specific antibodies; C – merge of A and B images; D, E – ultrathin sections of *P. multimicronucleatum* macronuclei at different magnifications. *Abbreviations*: n – nucleoli, F – fibrillar component, *arrowheads* –granular component, *arrows* – intranucleolar chromatin bodies. Scale bars: A, B, C – 1 μm, D – 5 μm, E – 1 μm.

between organization of the granular component in *P. multimicronucleatum* and *B. truncatella* is that in *P. multimicronucleatum* the fibro-granular component sometimes exfoliates from the nucleoli, forming extended lamellas about 75 nm thick and up to 1500 nm and more in length. RNP granules in these lamellas are arranged in an ordered form (Fig. 2, E).

A completely different pattern of fibrillarin distribution was observed in *D. nasutum*. One can see in Fig. 3 (A–C) that fibrillarin is located at the periphery of nucleolar domains. It correlates well with the electron microscopy data showing that the fibrillar component in the trabecular form lies at the periphery, while the inner part of the nucleoli is filled with granular material (Figs 3, D, E). It is well seen in Fig. 3, C, that fibrillarin is unevenly distributed. A lot of brightly colored spots are visible in the region of fibrillar component at the periphery of the nucleoli.

Discussion

The data obtained show that spatial organization of nucleoli in ciliate species with subchromosomal organization of macronuclear genome is different. Fibrillarin is the major nucleolar methyltransferase. During interphase fibrillarin is mainly found in the dense fibrillar component of the nucleoli of higher

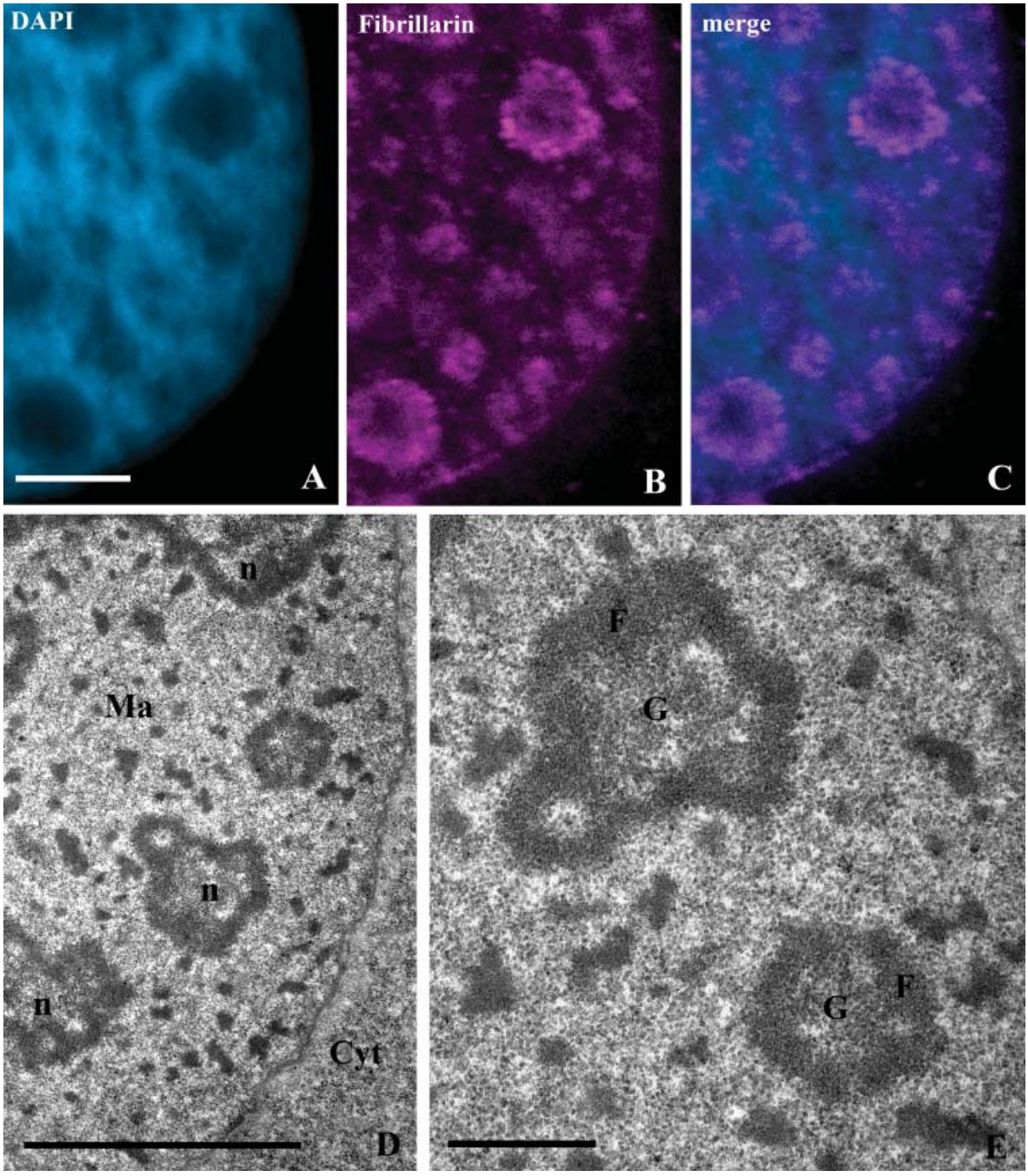

Fig. 3. Confocal microscopy (A-C) and electron microscopy (D, E) of the nucleoli in the interphase macronuclei of *Didinium nasutum*. A – Total macronuclear DNA staining with DAPI; B – immunolabeling of fibrillarin with specific antibodies; C – merge of A and B images; D, E – ultrathin sections of *D. nasutu*m macronuclei at different magnifications. *Abbreviations*: Ma – macronucleus, Cyt – cytoplasm, n – nucleoli, G – granular component, F – fibrillar component. Scale bars: A, B, C - 1 μm, D – 2 μm, E – 0.5 μm.

eukaryotes, where it participates in methylation and processing of pre-rRNA. In the nucleoli of *B. truncatella* and *P. multimicronucleatum* these processes occur in the central region. In *D. nasutum*, on the contrary, fibrillarin is located at the periphery of the nucleolar domain.

Using three-dimensional electron microscopy reconstructions, it has been shown that *D. nasutum* nucleoli, looking on the single sections like individual separate structures, are parts of the large complicated branchy nucleolar networks (Leonova et al., 2006, 2012, 2013). 3D models built on the basis of serial ultrathin sections show that in these nucleolar domains the fibrillar component is located at the periphery, while the granular part is in the central part of the nucleolus (Leonova et al., 2006). Taken together with confocal data on fibrillarin localization, it evidences that processing of rRNAs in *D. nasutum* nucleoli occurs from the periphery of the nucleolus towards its center.

The nucleoli with such "inverted" direction of vectorial processing of rRNA transcripts were observed by Postberg et al. (2006) in the *S. lemnae* nucleolar bodies. The authors assumed that the absence of typical chromosomes could determine such alternative spatial organization as observed in *S. lemnae* nucleoli (Postberg et al., 2006). However, our data indicate that the small size and the absence of typical chromosomes cannot be the only factors determining the inverted spatial organization of some ciliate nucleoli. Indeed, all three ciliate species studied here refer to the species with macronuclear DNA of subchromosomal size (Popenko et al., 1998, 2015; Rautian and Potekhin, 2002). However, only in one of them we observed the unusual location of the fibrillar component and fibrillarin at the periphery of the nucleoli. Besides, the nucleolar domains with the "inverted" direction of rRNA processing were found both in the species with gene-sized and subchromosomal macronuclear genomes.

Fibrillar centers in the nucleoli of higher eukaryotes look like clear rounded areas surrounded by dense fibrillar component. We did not observe any of such "classical" fibrillar centers in the nucleoli in the ciliates studied. It coincides with the idea that the "tripartite" structure of the nucleolus is an evolutionary acquired feature of higher eukaryotes. The transition between "bipartite" and "tripartite" nucleoli correlates with a striking expansion in the size of intergenic rDNA spacers that separates the pol I transcription units in rDNA arrays and this has been suggested to underlie the specialization of a single fibrillar component in two distinct compartments: the fibrillar center and the dense fibrillar component (Thiry and Lafontaine, 2005; Hernandez-Verdun et al., 2010; Thiry et al., 2011).

In the nucleoli of *B. truncatella* and especially of *D. nasutum*, which are of large size, a heterogeneous distribution of fibrillarin is well seen. The presence of well-defined stained spots allows us to suggest that synthesis of rRNA occurs in spatially separated loci. At present, an issue of which structures play the role of fibrillar centers in ciliate nucleoli cannot be considered as fully resolved. However, on the ultrathin sections one can see the chromatin bodies located in close proximity or inside the nucleolar material. With high probability such chromatin bodies, completely or partially surrounded by the fibrillar component, can play a role of nucleolar organizing regions in ciliate nucleoli (references in Raikov, 1995; Sabaneyeva et al.,1984; Sabaneyeva, 1997; Leonova et al., 2012).

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