

Electron microscopic identification of RNA- and DNA-containing structures in the preparations of isolated macronuclei of ciliates *Bursaria truncatella*

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Summary

RNA- and DNA-containing structures in the ciliate *Bursaria truncatella* were studied on ultrathin sections and spread preparations of individual macronuclei isolated at middle interphase. To label DNA-containing structures we used anti-DNA antibodies and protein A-colloidal gold complexes. To label RNP structures, complexes of gold with RNase A were used. Chromatin structures in the form of accumulations of nucleosome fibrils, partially decompacted or compact chromatin bodies were effectively labeled with anti-DNA antibodies. The accumulations of nucleosome fibrils were shown to be heterogeneous. Some of them corresponded to partially or completely decompacted inactive chromatin bodies, while others – to active chromatin. The latter contained granules of various sizes (20–50 nm) labeled with RNase-gold. The data obtained also showed that homogeneous accumulations of 20–30 nm granules, which often intermingled with accumulations of nucleosome fibrils, corresponded to nucleolar granular component. Such accumulations were in close contact with nucleosome fibrils, which points out the close connection between nucleolar structures and chromatin in macronuclei of *B. truncatella*.

Key words: chromatin, ciliates, immunoelectron microscopy, macronucleus, RNP-structures, spread preparations

Introduction

Ciliates are unique unicellular organisms with nuclear dualism. Each ciliate cell contains two morphologically and functionally different types of nuclei: one or several germinal micronuclei, which are usually diploid and functionally inert, and usually one somatic macronucleus, which is DNA-rich and

transcriptionally active during vegetative growth. Unlike the nuclei of higher eukaryotes, macronuclei are highly polyploid, with specific organization of genome in a form of a set of relatively small DNA molecules (for reviews see: Raikov, 1995; Jahn and Klobutcher, 2002). All ciliate species can be divided into two large groups: (i) the species with “gene-sized” macronuclear DNA molecules (usually 0.5 –

20 kb, although some authors observed DNAs up to 63 kb in size), and (ii) the species with macronuclear DNAs of subchromosomal size (from several tens to several hundred kb in size) (Raikov, 1995). The absence of typical Metazoan chromosomes can determine a peculiar organization of chromatin and nucleolar domains in macronuclei that differ from organization observed in nuclei of higher eukaryotes. It was shown that in “gene-sized” species *Stylonychia lemnae* rRNA is processed in a bipartite and inverse manner compared to a typical metazoan nucleolar organization (Postberg et al., 2006). The nucleoli of another ciliate, *Didinium nasutum*, also display the bipartite structure, with an inverted disposition of its main components (Leonova et al., 2006, 2012, 2013).

Electron microscopic studies of isolated ciliates' macronuclei, prepared according to Miller's technique (Miller et al., 1970), gave important information about macronuclear chromatin organization. In “subchromosomal” ciliate species, macronuclear DNAs are typically organized into compact chromatin bodies (CB) 0.1 to 0.2 nm in size. It was shown for *Bursaria truncatella*, *Paramecium caudatum*, *Spirostomum ambiguum* and *Didinium nasutum* that upon prolonged incubation of isolated macronuclear chromatin in low salt buffer chromatin bodies gradually decondensed, and a “halo” of chromatin nucleosome fibrils appeared around them (Tikhonenko et al., 1984; Borkhsenius et al., 1988; Karajan et al., 1995; Leonova et al., 2004; Popenko et al., 2015). Such structural organization of chromatin bodies in macronuclei of “subchromosomal” ciliate species is similar to that of chromomeres of higher eukaryotic chromosomes (Cook, 1995). In the above mentioned studies isolated macronuclei were lysed in the low ionic strength solution and centrifuged onto the electronmicroscopic grid through the sucrose-paraformaldehyde layer. Thus, not only chromatin structures, but also nucleolar and other RNP structures, which are abundant in macronuclei, are present in such specimens. It is often impossible to differentiate unambiguously chromatin, RNP and other nuclear macromolecules on isolated electron microscopic preparations solely by their morphology.

The aim of this work was to identify RNA- and DNA-containing structures in the preparations of isolated macronuclei of ciliates *B. truncatella* using immune-gold and enzyme-gold techniques that had been effectively used for labeling on the

ultrathin sections. The isolated macronuclei from *B. truncatella* cells that completed their growth and differentiation were used.

Material and methods

The ciliates *B. truncatella* were collected in the pond of the Botanical Garden of the Russian Academy of Sciences (Moscow), cultivated at constant temperature (10 °C) in boiled tap water and fed three times a week with *Paramecium caudatum* cultivated separately.

COLLOIDAL GOLD COMPLEXES AND ANTIBODIES

Lupus mouse IgG antibodies 2C10 and H241 that bind native DNA (Jang et al., 1996) were kindly granted for investigations by Prof. B.D. Stollar. The colloidal gold with a mean particle size of 15-16 nm was prepared as described by Frens (1973). Complexes of protein A (Serva/Heidelberg/Germany) and RNase A (Serva/Heidelberg/Germany) with colloidal gold were prepared as described by Roth (1983) and Bendayan (1981), respectively. Determination of an optimal amount of protein necessary for the gold sol stabilization and purification of the complexes by centrifugation was carried out according to Roth (1983).

ULTRATHIN SECTION PREPARATION

The ciliates *B. truncatella* were fixed in 1% glutaraldehyde solution in 0.1M phosphate buffer, pH 7.2, for 1 h at room temperature. Then the cells were either additionally fixed in 1% OsO₄ in 0.1M phosphate buffer, pH 7.2, dehydrated in series of ethanol dilutions and embedded in Epon-Araldite by standard procedure, or immediately dehydrated and embedded in Lowikryl K4M resin (Sigma-Aldrich/USA) according to manufacturer's recommendations. The 50 to 80 nm thick sections were cut with an “Ultratome III” (LKB/Uppsala/Sweden), stained with aqueous uranyl acetate and lead citrate solutions and investigated in electron microscope JEM-100CX (JEOL/Tokyo/Japan) at 80 kV.

ULTRATHIN SECTION GOLD LABELING

For DNA labeling the Lowikryl K4M sections before staining were incubated with antibodies

2C10 or H241 against native DNA (Jang et al., 1996) in phosphate buffered saline (PBS) solution with 1% BSA and 0.2% Triton X100 for 1h at room temperature, and afterwards in PBS solution with protein A – colloidal gold, 1% BSA and 0.2% Triton X100. The procedure of RNA labeling on Lowicryl K4M sections was carried as described elsewhere (Popenko and Cherny, 1991; Popenko et al., 1991).

ISOLATED CHROMATIN PREPARATION AND GOLD LABELING

Macronuclei from mature cells that completed their growth and differentiation (6 to 10 h after vegetative cell division) were isolated manually in a solution containing 0.5 % Nonidet NP-40, 1 mM $MgCl_2$, 2 mM phosphate buffer (pH 7.8), then lysed in 0.1 mM borate buffer, pH 8.7 and incubated in the same buffer for 0-90 min. The nuclear material was layered on the 4% paraformaldehyde – 0.2M sucrose solution (pH 8.7, borate buffer) and centrifuged for 10 min at 5500 rpm/min onto the electronmicroscopic grid covered with freshly glow-discharged parlodion-carbon supporting film (Miller et al., 1970). The specimens were washed in 0.2% Kodak Photo-flo solution (pH 8.7, borate buffer), air dried and rotary shadowed with Pt-Pd alloy at 6° angle.

For labeling DNA-containing structures the electron microscopic grids after washing in 0.2% Kodak Photo-flo solution were briefly rinsed in distilled water and transferred on the 50 μ l drops of PBS solution containing 1% bovine serum albumin type V (Sigma/USA), 0.1% NP-40 and antibody 2C10 or H241. After incubation for 20 min at room temperature the grids were twice briefly washed in PBS and placed on the drops of PBS solution containing 0.1% NP-40, 0.02% polyethyleneglycol 25000, and protein A-gold complex for 20 min at room temperature.

To label RNA-containing nuclear structures, the grids after washing in 0.2% Kodak Photo-flo solution were briefly rinsed in distilled water and transferred on the 50 μ l drops of a solution containing 20mM NaCl, 10 mM phosphate buffer pH 7.2, 0.02% polyethyleneglycol 25000 and RNase-gold complex for 5 min at room temperature. The concentration of RNase-gold was adjusted spectrophotometrically (optical absorbance at 525 nm $A_{525}=0.05-0.5$).

After gold labeling the grids were washed in 0.2% Kodak Photo-flo solution (pH 8.7, borate buffer), briefly rinsed in distilled water, air dried and either stained in 1% aqueous uranyl acetate solution or rotary shadowed with Pt-Pd alloy.

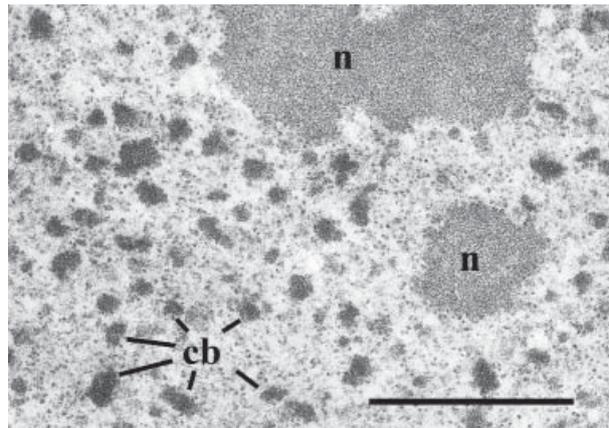


Fig. 1. An ultrathin section of *Bursaria truncatella* macronucleus of a mature cell completed its growth and differentiation. Abbreviations: cb – chromatin bodies, n – nucleoli. Scale bar: 1 μ m.

Results

Figure 1 shows a fragment of an ultrathin section of a macronucleus of mature *B. truncatella* cell which completed its growth and differentiation. Inactive chromatin in the macronucleus is arranged in compact electron-dense chromatin bodies (CB). Numerous nucleoli are scattered over the macronucleus. They have well distinguished granular and fibrillar components. Many small (20-30 nm) granules and granular bodies separated from the nucleolar granular zone are present in the karyoplasm near the nucleoli; 20-30 nm granules are well distinguished within them (Fig. 1).

A similar section treated with RNase-gold complex is shown in Figure 2. Among all the macronuclear components, the nucleoli are most intensely labeled with the RNase-Au. Gold particles are distributed rather evenly over the whole plane of nucleoli sections. The small granular bodies near nucleoli are also labeled with RNase-gold complexes (Fig. 2). Gold particles are absent from CB and are located mainly in the nucleoli and macronuclear karyoplasm, this concurs well with available cytochemical and biochemical data on the RNA distribution in eukaryotic nuclei. At the same time, many gold particles are located at the CB borders (Fig. 2).

Figure 3 shows *B. truncatella* sections treated with anti-dsDNA antibodies and protein A-gold complex. The gold particles are mainly located over chromatin bodies and in the karyoplasm near them. Since the patterns of gold particles

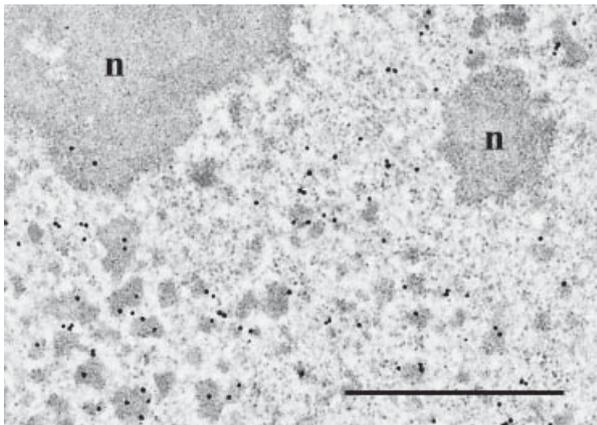


Fig. 2. An ultrathin section of *B. truncatella* macronucleus treated with RNase-colloidal gold complex. Abbreviations: n – nucleoli. Scale bar: 1 μ m.

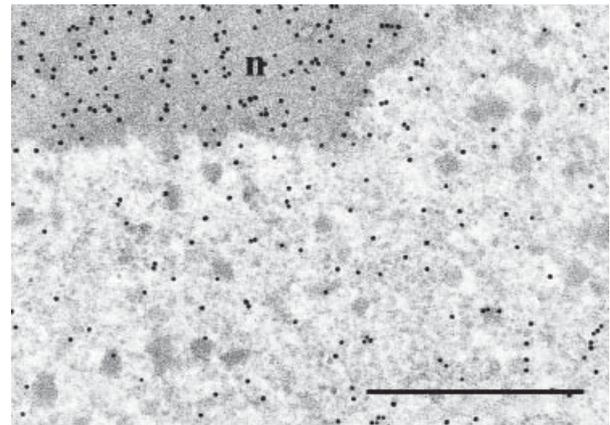


Fig. 3. An ultrathin section of *B. truncatella* macronucleus treated with antibody 2C10 against native DNA and protein A-colloidal gold complex. Abbreviations: n – nucleoli. Scale bar: 1 μ m.

distribution observed with 2C10 and H241 were virtually the same, the micrographs obtained using 2C10 antibody are only shown. A few gold particles are located over nucleoli. The label is virtually absent in the regions near nucleoli where the small granular bodies are seen.

Various isolated nuclear structures observed in the specimens prepared by Miller's technique (Fig. 4, A-F). The accumulations of compact CB are typical for the specimens incubated 0 to 5 min in 0.1 mM borate buffer. However, sometimes in the same specimens accumulations of partially decompact CB were observed (Fig. 4, A). It can be explained by different chromatin activity in various regions of *B. truncatella* macronucleus. As the incubation time in the low salt buffer increased, the CB gradually decompact (Fig. 4, B). After 90 min, almost all chromatin was presented by accumulations of nucleosome fibrils (Fig. 4, C, D). All these structures were labeled well with anti-DNA antibody and protein A-gold complex (Figs 5; 6, A-D). DNA was effectively labeled both in compact and completely decompact CB (Fig. 6, D, E, F). In some preparations nuclear pore complexes can be observed closely adjacent or connected with macronuclear chromatin. They were not labeled with 2C10-protein A-gold (Figs 5, B; 6, A).

Accumulations of nucleosome fibrils were not homogeneous. Granules of various sizes (20 to 50 nm) were present in them. In some cases nucleosome fibrils were intermingled with accumulations of homogeneous in size 20-35 nm granules, resembling the granules and granular bodies near nucleoli in

the ultrathin sections (Fig. 1). Such structures were observed earlier in the macronuclear chromatin of the ciliate *D. nasutum* (Karajan et al., 1995). The 20-35 nm granules were not labeled with anti-DNA antibodies (Figs 5, A; 6, A, B). Such granules were observed also at the periphery of rounded structures, often in contact with nucleosome fibrils. After treatment with anti-DNA antibody and protein A-gold complex the gold particles were only located in the central part of rounded structures (Fig. 6, C, G, H). These data allowed us to identify these structures as nucleoli, and 20-35 nm granules – as pre-ribosomal particles.

When RNase-gold was used for labeling at low concentrations (OD525 = 0.02), the few gold particles were observed mainly in the accumulations of nucleosome fibrils with 20 to 50 nm granules (Fig. 7, A). Compact CB and nuclear pores were devoid of label (Fig. 7, B, C). However, the specificity of RNase-gold complex for labeling RNA-containing structures in isolated nuclear material was worse than on ultrathin sections. It was obviously seen at higher concentrations when the gold label was observed over both DNP and RNP structures (Fig. 7, D). In the rounded nucleolar structures gold particles were located both in the central and peripheral regions (Fig. 7, E, F).

Figure 8 shows an accumulation of coiled granular fibrils 20-30 nm thick. In the central part of such accumulations electron dense centers 100-200 nm in diameter are often seen (Fig. 8, A). The coiled granular fibrils are organized in chrysanthemum-like structures, which can be observed in more

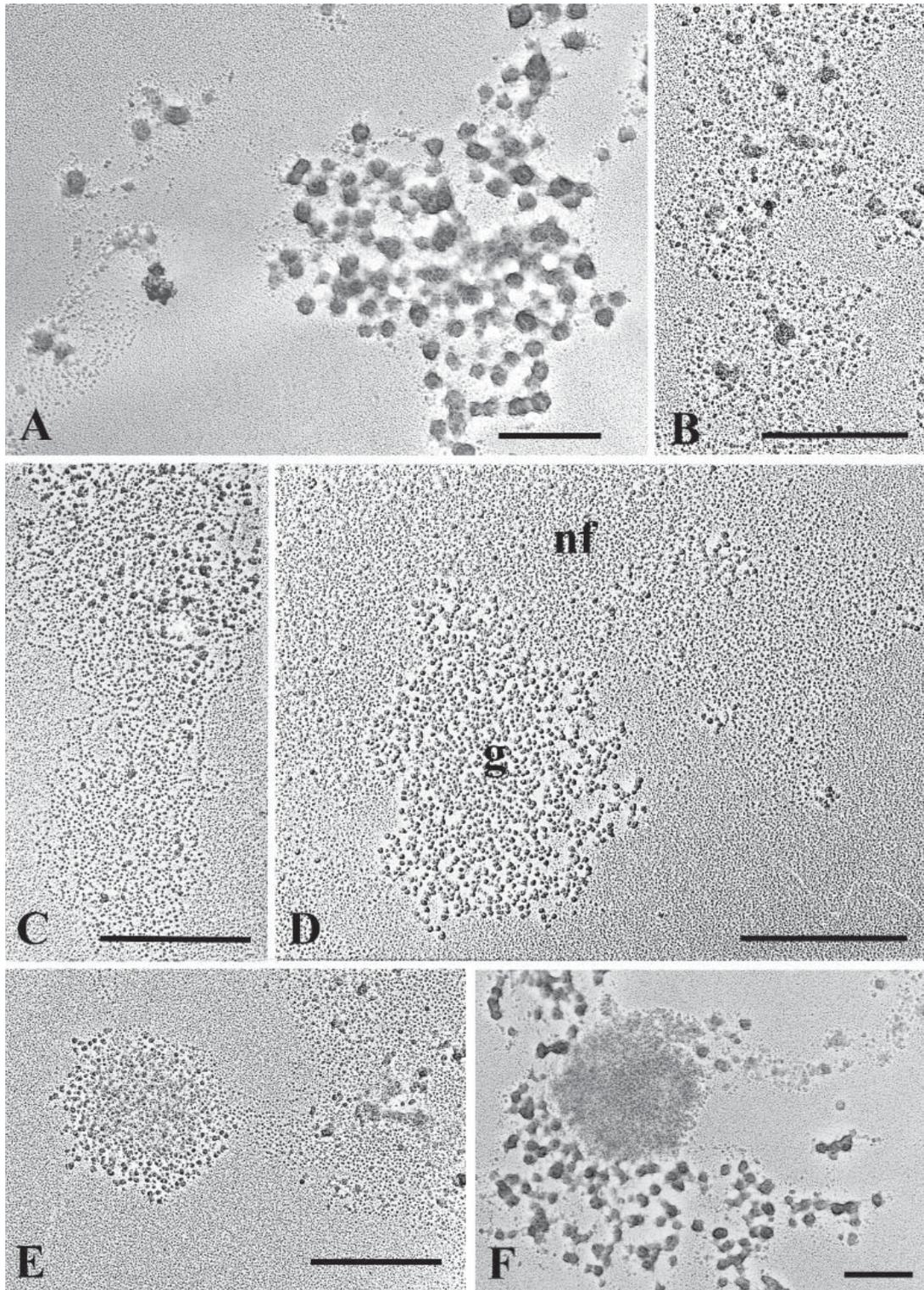


Fig. 4. Nuclear structures from macronuclei of mature *B. truncatella* cells completed growth and differentiation. Incubation time in low salt buffer: 5 min (A), 10 min (F), 30 min (B, E), 60 min (C), 90 min (D). A – Compact and partially decompacted chromatin bodies; B – accumulation of partially decompacted chromatin bodies; C – accumulation of nucleosome fibrils with 20-50 nm granules; D – accumulation of 20-35 granules (g) in close contact with accumulation of nucleosome fibrils (nf); E, F – rounded granular structures connected with partially decompacted and compact chromatin bodies. Scale bars: 1 μ m.

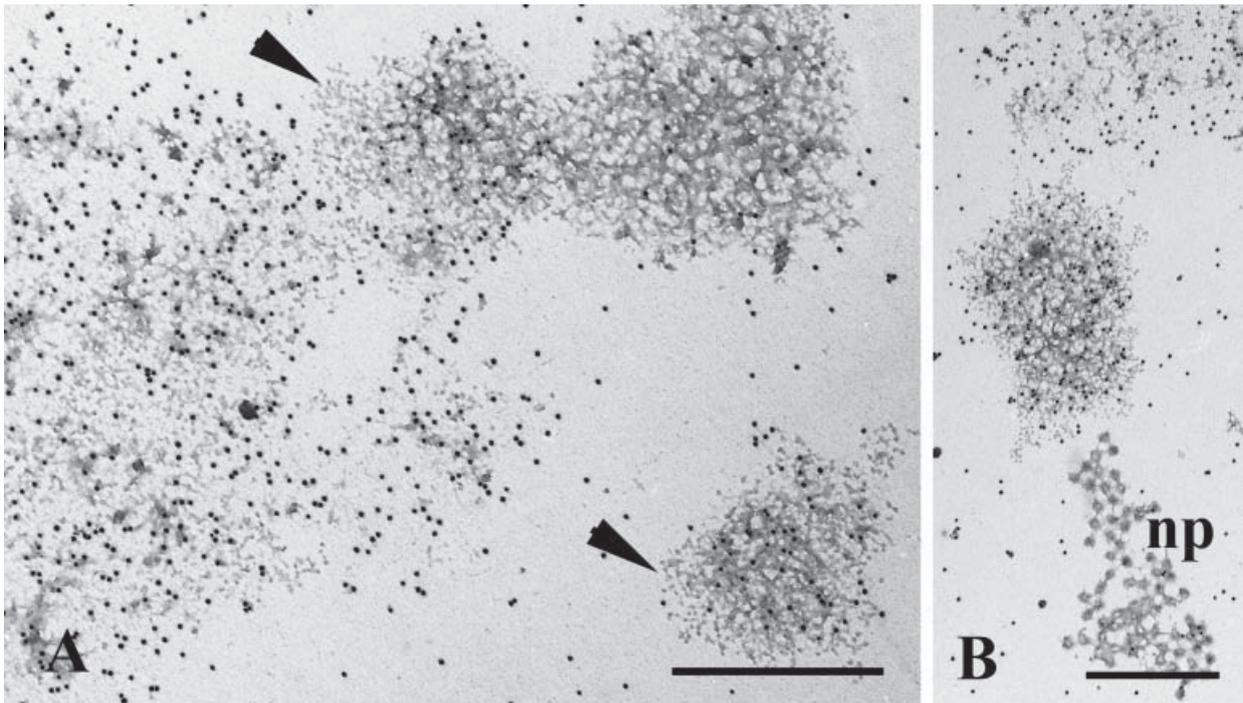


Fig. 5. Preparations of *B. truncatella* macronuclear structures treated with 2C10 antibody and protein A-gold complex. Uranyl acetate staining. *Arrowheads* indicate non-labeled granular structures. *Abbreviations:* np – nuclear pores. Scale bars: 1 μ m.

decompacted preparations (Fig. 8, B) or at the periphery of such accumulations (Fig. 8, A). Our data show that the coiled granular fibrils are not labeled either with RNase-gold or with anti-DNA antibodies (Fig. 8, C, D).

Discussion

In this work we used lupus mouse IG autoantibodies 2C10 and H241 that bind native DNA. These antibodies exhibit different specificity. Antibody H241 binds poly (dG-dC) but not poly (dA-dT), whereas 2C10 binds poly (dA-dT) (Stollar, 1990). The data obtained showed that these antibodies can be effectively used for exact identification of DNA-containing structures both on ultrathin sections and preparations of isolated chromatin. The use of colloidal gold particles \sim 15 nm in size allowed us to clearly visualize gold label both on uranyl acetate stained and metal shadowed preparations.

To label RNP-structures, we used complexes of colloidal gold with RNase A. This approach showed good results in determining RNP structures on ultrathin sections, in agreement with earlier data

(Bendayan, 1981, Popenko and Cherny, 1991). To avoid a degradation of RNP structures, we had to reduce the duration of RNase-gold treatment and increase concentration of the complex. At very low concentration of RNase-gold, compact CB, nuclear pores and remnants of cytoplasmic organelles, presented in the specimens, were not labeled. However, an increase of RNase-gold concentration led to a high background and nonspecific binding of RNase-gold complex both to DNA- and RNA-containing structures.

The approaches described enabled us to differentiate the material in the preparations of isolated *B. truncatella* macronuclei as follows.

(I) Accumulations of compact chromatin bodies 60–200 nm in size. They were intensely labeled with anti-DNA antibodies and protein A-gold complex.

(II) Accumulations of partially decompacted chromatin bodies (typical for the preparations dispersed to a small degree, 10–60 min in 2mM borate buffer) and nucleosome fibrils, observed after prolonged incubation (60–90 min), were intensely labeled with anti-DNA antibodies and protein A-gold complex. These accumulations were the result of decompaction of inactive chromatin bodies

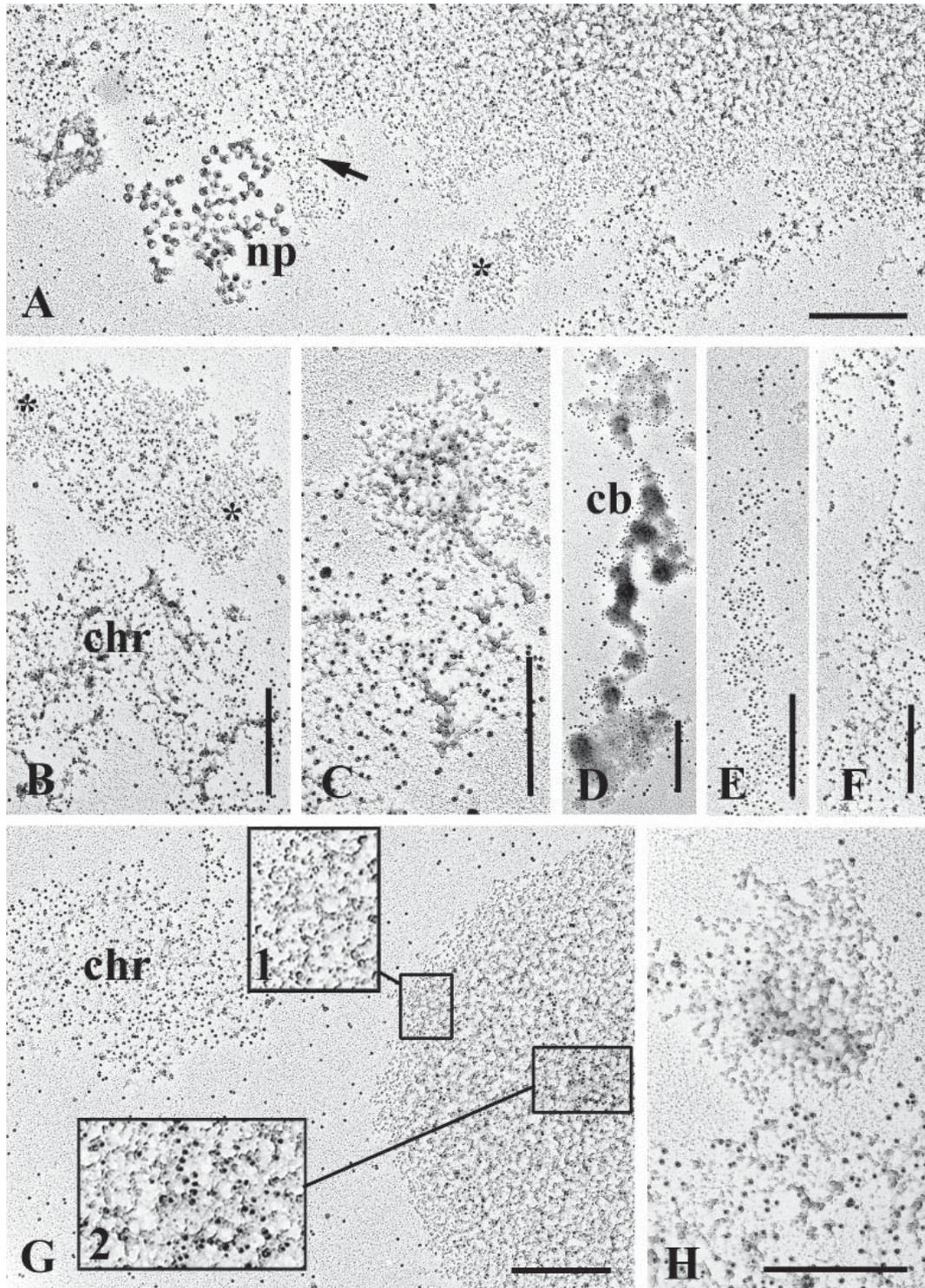


Fig. 6. Metal shadowed *B. truncatella* macronuclear preparations treated with 2C10 (A, C-H) or H241 (B) antibody and protein A-gold complex. A – General view, *arrow* indicates chromatin structures, *asterisk* – non-labeled granular accumulations; B – non-labeled accumulations of 20-35 nm granules (*asterisks*) intermingled with accumulation of nucleosome fibrils; C, H – accumulations of 20-35 nm granules with gold label in the central part; D, E, F – gold label over compact chromatin bodies, partially decompacted bodies and individual nucleosome fibril; G – rounded granular structure with the label in the central part (inlet 2), gold label is absent over granular periphery (inlet 1). *Abbreviations:* cb – chromatin bodies, np – nuclear pores, chr – decompacted chromatin. Scale bars: 1 μ m.

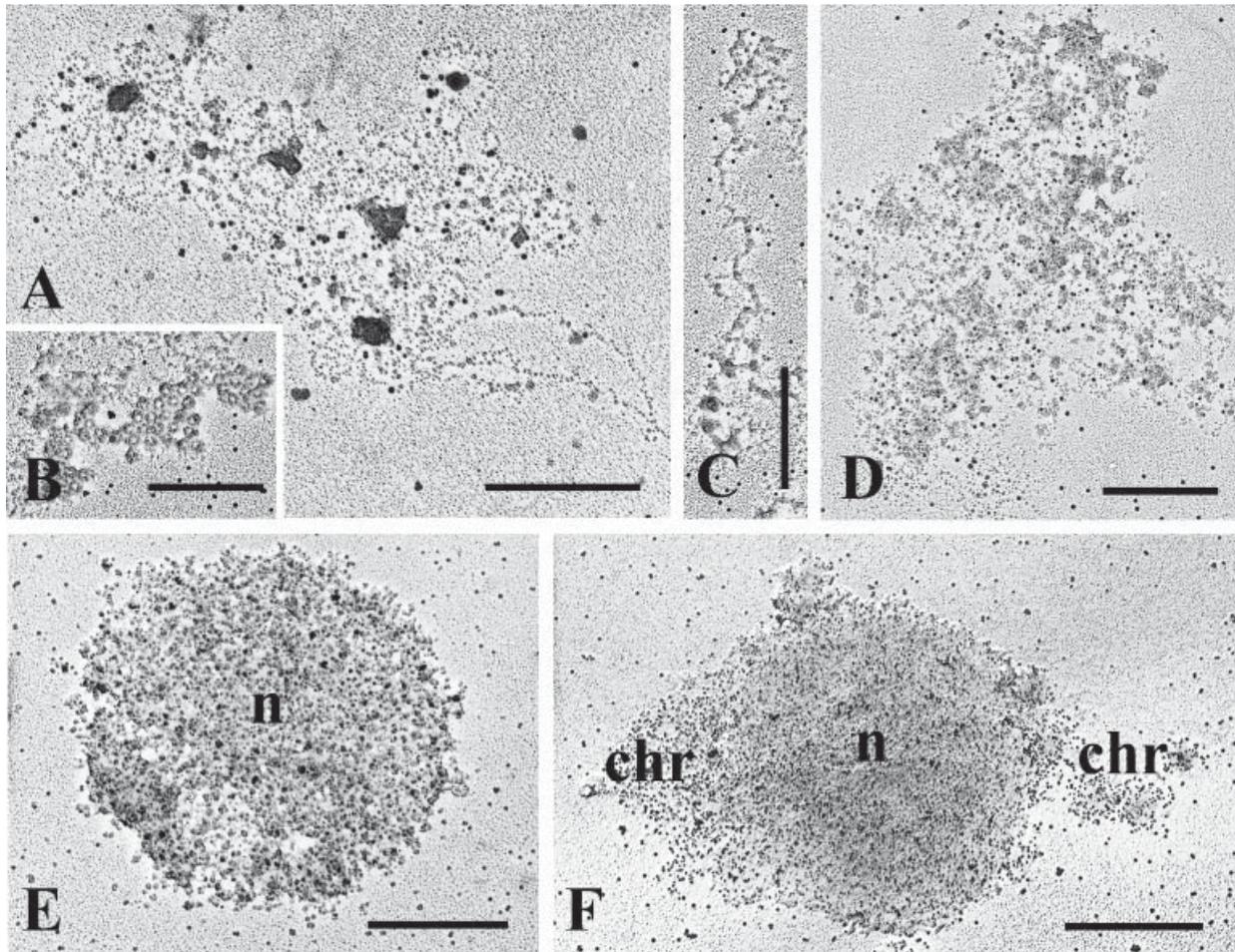


Fig. 7. *B. truncatella* macronuclear preparations treated with RNase-gold complex. Metal shadowing. A – Accumulation of partially decompacted chromatin bodies with individual 20 to 50 nm granules; B, C – non-labeled nuclear pores and a chromatin fibril; D – partially decompacted chromatin bodies labeled with RNase-gold at high concentration; E, F – rounded granular bodies (nucleoli) with adjacent chromatin structures. *Abbreviations:* chr – decompacted chromatin, n – nucleoli. Scale bars: 1 μ m.

under low ionic strength conditions.

(III) Accumulations of nucleosome fibrils, decompacted chromatin bodies and granules of various size (20-50 nm). The level of DNA labeling in these aggregates was lower than in the cases I-II, but some granules were labelled by RNase-Au. Such accumulations likely correspond to the active chromatin, containing RNP granules.

(IV) More or less rounded structures consisted of granules. They may be located alone or connected with accumulation of nucleosome fibrils. At the periphery of them, the homogeneous in size granules (20-40 nm) were observed. Only in the central part of such structures small compact pieces intensely labelled by anti-DNA antibodies and protein A-gold complex, can be observed. Such structures seem to

correspond to nucleoli with fibrillar centers inside.

(V) Accumulations of homogeneous granules 20-35 nm in size located alone or intermingled with accumulations of nucleosome fibrils or partially decompacted CB. They were not labeled with anti-DNA antibodies and likely correspond to nucleolar granular component. It can be often seen that such granular accumulations are in close contact with the nucleosome fibrils, which indicates the close connection between nucleolar structures and chromatin in macronuclei of *B. truncatella*.

Some structures looking like accumulations of coiled granular fibrils 20-30 nm thick (Fig. 8) were not labeled, neither with RNase-gold nor with anti-DNA antibodies. Such accumulations were observed earlier in isolated *B. truncatella* chromatin

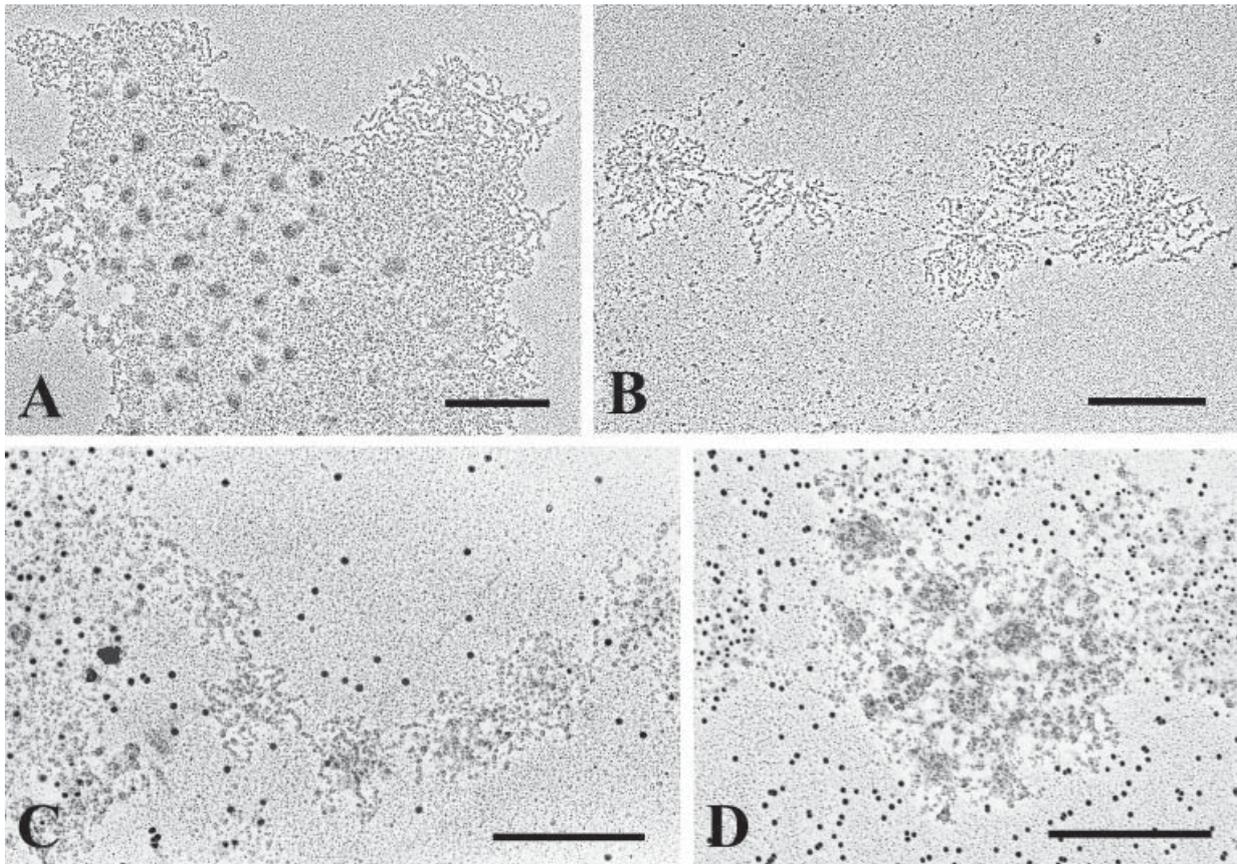


Fig. 8. Accumulations of coiled granular fibrils 20–25 nm thick not labeled either with RNase-gold or with anti-DNA antibodies. **A** – Compact accumulation; **B** – chrysanthemum-like structures; **C** – treatment with 2C10 antibody and protein A-gold complex; **D** – treatment with RNase-gold complex. Scale bars: 1 μm .

preparations at various stages of vegetative cell division (Popenko et al., 1988). Their nature remains unknown. It is possible to suggest that these accumulations are structural elements consisting, for example, of proteoglycans to maintain the volume of such water-bearing organisms as *B. truncatella*. Alternatively, they may represent accumulations of RNP structures necessary for further stages of *B. truncatella* cell cycle, where RNA is shielded by a protein layer and is not labeled with RNase-gold complex.

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