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Localization of RNA transcription sites in insect oocytes using microinjections of 5-bromouridine 5'-triphosphate

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Abstract: In the present study we used 5-bromouridine 5'-triphosphate (BrUTP) microinjections to localize the transcription sites in oocytes of insects with different types of the ovarium structure: panoistic, meroistic polytrophic, and meroistic telotrophic. We found that in an insect with panoistic ovaries (*Acheta domesticus*), oocyte nuclei maintain their transcription activity during the long period of oocyte growth. In insects with meroistic ovaries (*Tenebrio molitor* and *Panorpa communis*), early oocyte chromosomes were found to be transcriptionally active, and some transcription activity still persist while the karyosphere, a compact structure formed by all condensed oocyte chromosomes, begins to develop. At the latest stages of karyosphere development, no anti-Br-RNA signal was registered in the karyosphere.

Key words: Insects - Oocyte nucleus - Karyosphere - Karyosome - Transcription - BrUTP incorporation - Microinjections

Introduction

In oogenesis of many animals including insects with meroistic ovaries, the so-called karyosphere (=karyosome) is formed at different stages of a long period of diplotene [14]. In broad terms the karyosphere represents a compact "knot" of the condensed chromosomes joined together in a limited area of the large oocyte nucleus. Karyosphere formation is a wide-spread phenomenon being described in more than 120 animal species which belong to 4 phyla (see [14] for a review). At the same time, oocyte chromosomes in many other animals including insects with panoistic ovaries seem not to form the karyosphere [20,29,30].

It is generally assumed that oocyte chromosomes united into the karyosphere are transcriptionally silent [5,22,24,27]. At the same time, several authors have documented the incorporation of ^{3}H-uridine into the karyosphere of some insect species [9,10,26,34,35]. This indicates that oocyte genome is not fully inactivated in these species while the karyosphere is formed. However, some contradictions came from the ultrastructural studies on insect oocyte nuclei revealed, in many cases, a high condensation of chromatin at the latest stages of karyosphere development in some species [1,7,8,31]. Also, immunogold labeling electron microscopy revealed a prominent redistribution of RNA polymerase II and pre-mRNA splicing factors from the periphery of chromatin blocks to other nuclear domains at the end of karyosphere development [1,7,8,32]. These findings apparently suggest that the cessation of chromosome transcription activity must occur at this stage because many pre-mRNAs are spliced co-transcriptionally [2,4,12,25]. In the present paper we provide a direct confirmation of this supposition.

We used 5-bromouridine 5'-triphosphate (BrUTP) microinjections into the ooplasm of diplotene oocytes to localize the sites of transcription at different stages of nuclear morphogenesis in insects. We used oocytes of the mealworm, *Tenebrio molitor* (meroistic telotrophic ovaries), the scorpionfly, *Panorpa communis* (meroistic polytrophic ovaries), and the house cricket, *Acheta domesticus* (panoistic ovaries). In former two species the karyosphere is formed, and the stages of its morphodynamics were previously described and can be easily distinguished [1,8]. In cricket oocytes the karyosphere is not formed, and diplotene chromosomes have a lamp-brush structure [20,30].

We found that oocyte chromosomes in the cricket remain their transcription activity until the latest stage of oocyte growth while in insects with meroistic ovaries (*T. molitor* and *P. communis*) we were able to localize RNA transcription sites only at earlier stages of karyosphere development, and no detectable incorporation of BrUTP into the latest karyosphere was revealed.

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Materials and methods

Animals. Specimens of *Tenebrio molitor* L. (Coleoptera: Polyphaga, Tenebrionidae) and *Acheta domesticus* L. (Orthoptera: Gryllidae) were kept in laboratory. Specimens of *Panorpa communis* L. (Mecoptera: Panorpidae) were collected in June in the village of Toksovo (Leningrad Region, Russia).

Tissue preparation and light microscopy. Single ovarioles were isolated in Ringer's solution for insects (0.75% NaCl, 0.035% KCl, 0.021% $CaCl_2$) or in Grace's insect medium (Sigma). Ovarioles were slightly squashed on a coverslip by a microscope slide. In the case of large vitellogenic oocytes, oocyte nuclei were manually isolated before squashing. Non-fixed preparations were analyzed in a Leica DM IRB fluorescent microscope equipped with Nomarski differential interference contrast (DIC) optics. In this case, 0.5-1.0 μg/ml 4,6-diamidimo-2-phenylindole (DAPI) was added in a medium before squashing to reveal DNA. DIC and fluorescent images of the same nuclei were taken with a Leica DFC 320 digital camera; images were combined and adjusted with Adobe Photoshop. Other ovarioles were used for microinjection experiments (see below). After microinjection, the squashes were prepared, then frozen in liquid nitrogen, fixed in 2% paraformaldehyde in 96% ethanol for 45 min, rinsed in 70% ethanol and PBS, and stored in PBS before immunostaining [15,21].

Microinjections. Microinjections were performed using an Eppendorf 5242 microinjector and a Leica DM IRB microscope equipped with a Narishige micromanipulator. Ovarioles were held and the microinjections were carried out like shown in Fig. 1. Oocytes were injected into the cytoplasm with 100 mM BrUTP (Sigma) in 140 mM KCl and 2 mM PIPES, pH=7.4 [37,38]. After microinjection, ovarioles were kept in a moist chamber for 30-60 min, then squashed and fixed. As a control, non-injected oocytes were kept at the same conditions.

Immunostaining and confocal microscopy. The squashes were blocked in 10% fetal serum (Gibco) in a diluent containing 1% BSA, 0.5% Tween 20, and 0.1% sodium azide in PBS for 10 min, stained at 4°C overnight in a moist chamber with mouse monoclonal anti-bromodeoxyuridine (anti-BrdU) antibody (clone BU-33, Sigma; 1:500 in the diluent). Anti-BrdU antibodies are known to recognize bromouridine with high specificity and affinity [36]. After staining in primary antibody solution, the samples were rinsed in PBS and incubated with a FITC-conjugated goat anti-mouse secondary antibody diluted 1:200 for 1.5 h. After washing in PBS, the specimens were stained for 1 min with 1 μg/ml of the DNA-specific dye To-Pro-3 (Molecular Probes), rinsed again in PBS, mounted in Vectashield medium (Vector Laboratories, USA), and examined with a Leica TCS SL laser scanning confocal microscope equipped with Argon (488 nm) and Helium-Neon (633 nm) lasers permitting the imaging of FITC (green, emission 518 nm), and To-Pro-3 (red, emission 661 nm), respectively. Confocal images were taken with ×63 (NA 1.32) objective. Merged images were obtained using Leica Confocal Software. Contrast and relative intensities of the images were adjusted with Adobe Photoshop.

Results

The mealworm *Tenebrio molitor* is an insect with typical meroistic telotrophic ovaries [35]. In oocytes occupying the "neck" region of the ovariole, the chromatin is considerably decondensed [8]. This stage corresponds to the early previtellogenesis and the stage 3 according to Ullmann [35]. Only a few condensed

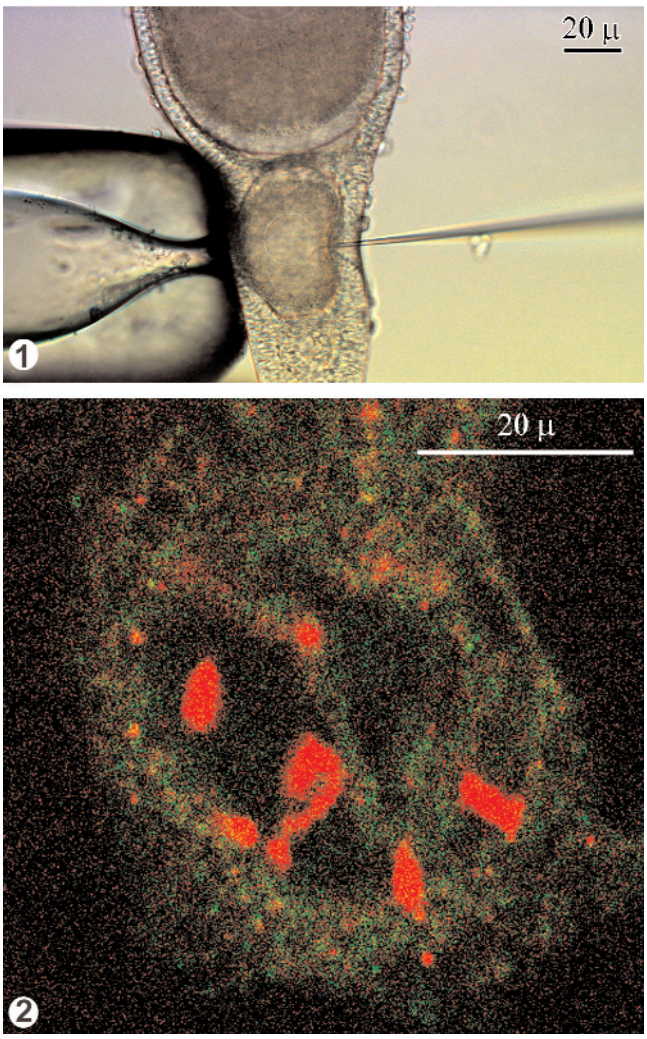
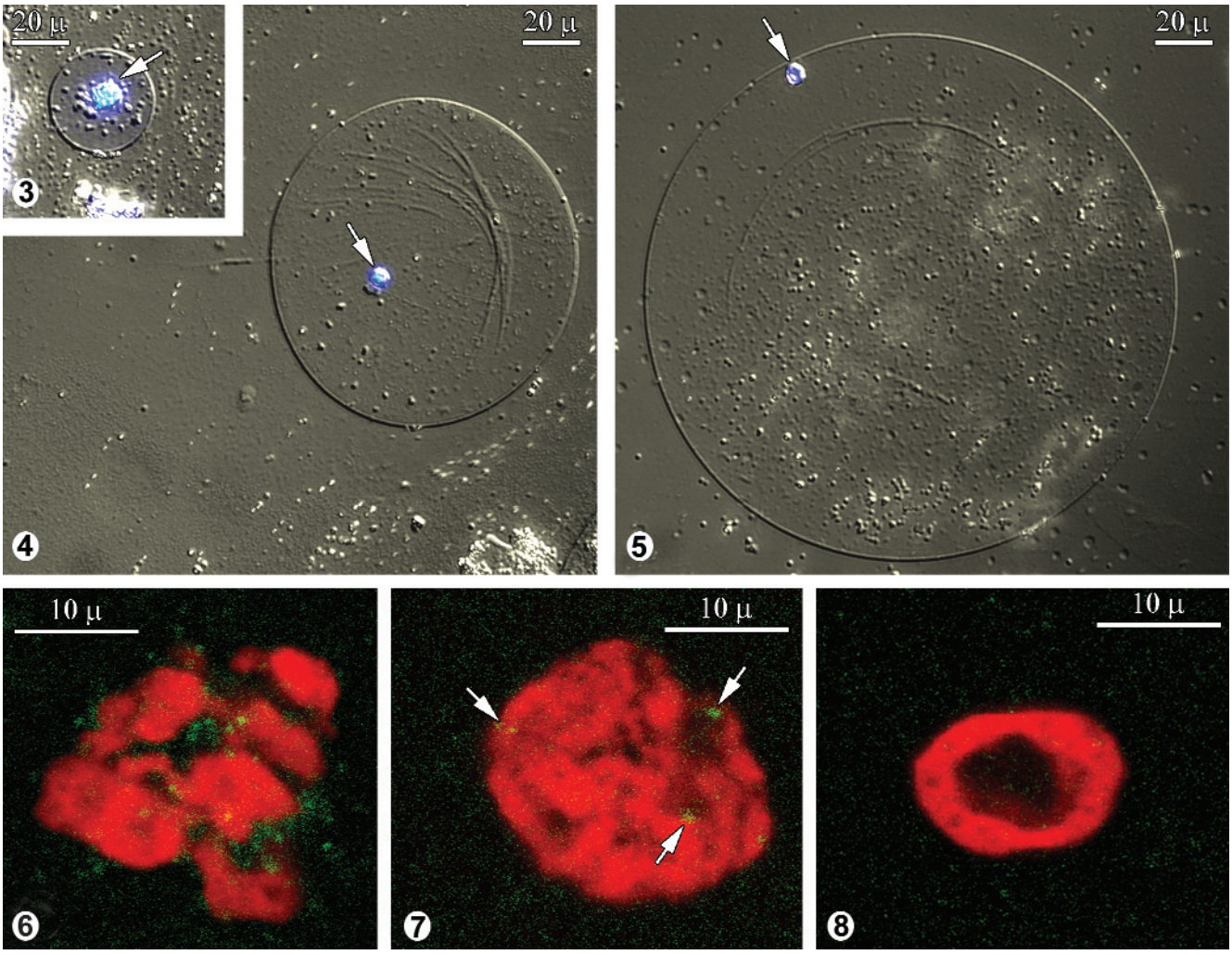


Fig. 1. A previtellogenic oocyte of *Tenebrio molitor* during microinjection experiment. **Fig. 2.** A nucleus of *T. molitor* early previtellogenic oocyte after BrUTP microinjection. Anti-Br-RNA signal (green) is diffusely distributed through the nucleus along with the decondensed chromatin (To-Pro-3 staining, red). Larger blocks of the condensed chromatin reflect the beginning of karyosphere formation at this stage.

chromatin blocks can be observed within the nucleus at this stage (Fig. 2). After BrUTP microinjection, the diffuse distribution pattern of anti-Br-RNA signal was revealed (Fig. 2).

The process of karyosphere formation in *T. molitor* begins at early diplotene when an oocyte enters the vitellarium [8,35]. This stage corresponds to the mid previtellogenesis (stage 4 [35]). The key stages of karyosphere development in *T. molitor* oocytes are shown in Figs. 3-5. The early karyosphere looks like a loose chromatin knot. While an oocyte grows, the chromatin gradually condenses, and the latest karyosphere in *T. molitor* oocytes is a true "sphere" because a prominent cavity arises inside. This stage is not clearly seen in squashes under a routine microscope but it can be easily identified using an analysis of serial con-



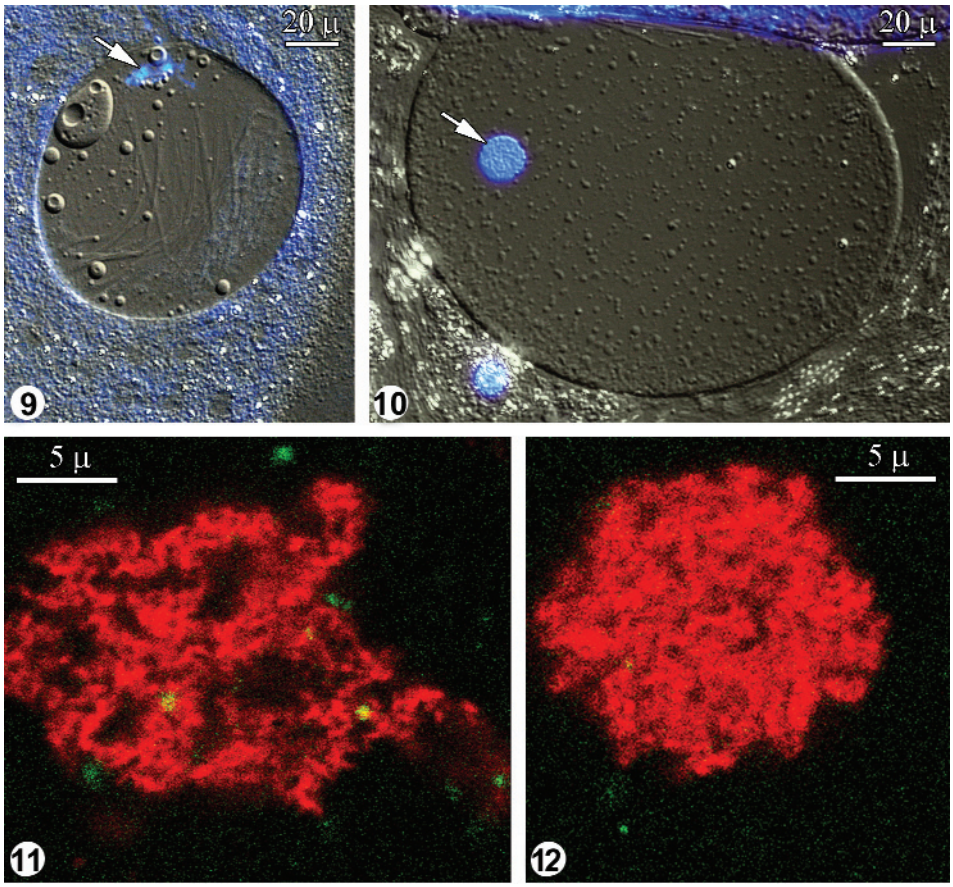
Figs. 3-5. Squashed non-fixed nuclei of *T. molitor* oocytes at the stages of mid previtellogenesis (Fig. 3), early and late vitellogenesis (Figs. 4, 5) viewed at the same magnification. Nomarski optics + DAPI staining. Arrows indicate the karyosphere. Note, that the karyosphere is the only DNA-containing oocyte nuclear structure. **Figs. 6-8.** *T. molitor* karyospheres of about the same stage nuclei as in Figs. 3-5 after BrUTP microinjections into the ooplasm. In the early karyosphere (Fig. 6), anti-Br-RNA signal (green) is clearly seen while at the intermediate stage of karyosphere development only a few fluorescent foci are observed (Fig. 7, arrows). The late karyosphere does not incorporate BrUTP (Fig. 8). Chromatin was stained with To-Pro-3 (red).

focal optical sections (or at the ultrastructural level; see [7,8]).

The results of our microinjection experiments with *T. molitor* oocytes at the consecutive stages of karyosphere development are shown in Figs. 6-8. The incorporation of BrUTP into the early karyosphere (late previtellogenesis; stages 4-5 [35]) was revealed in some places at the periphery of the condensed chromatin (Fig. 6). At the intermediate stage of karyosphere development (early vitellogenesis; stage 6 [35]), a number of fluorescent anti-BrUTP dots within the karyosphere decreases significantly (Fig. 7). In a random confocal optical section of this stage karyosphere, no signal is often registered, and a serial analysis of all sections was always applied. Finally, we did not reveal any prominent anti-Br-RNA signal when BrUTP was injected into the oocytes at the stage of the late karyosphere (mid vitellogenesis; stage 7 [35]) (Fig. 8).

The scorpionfly *Panorpa communis* is an insect with typical meroistic polytrophic ovaries [28]. Karyosphere formation in this species begins earlier than in *T. molitor*, in early previtellogenic oocytes [1]. In *P. communis* we were not able to inject very young oocytes due to their small size and significant flattening. Unlike *T. molitor*, the karyosphere in *P. communis* never acquires an internal cavity and does not look like a sphere [1]; see also Figs. 9-10.

The distribution pattern of anti-Br-RNA signal within the intermediate stage karyosphere in *P. communis* oocytes (late previtellogenesis) was similar to that in *T. molitor*, and several, not numerous, fluorescent dots were observed at the periphery of the con-



Figs. 9-10. Squashed non-fixed nuclei of *Panorpa communis* late previtellogenic (Fig. 9) and vitellogenic (Fig. 10) oocytes. Nomarski optics + DAPI staining. Arrows indicate the karyosphere. **Figs. 11-12.** *P. communis* karyospheres of about the same stage oocyte nuclei as in Figs. 9-10 after BrUTP microinjections into the ooplasm. Several anti-Br-RNA fluorescent dots (green) are observed in the early karyosphere (Fig. 11) while the late karyosphere (Fig. 12) does not incorporate BrUTP. Chromatin was stained with To-Pro-3 (red).

densed chromatin (Fig. 11). No visible reaction was observed at later stages of the karyosphere development in vitellogenic oocytes (Fig. 12).

The house cricket *Acheta domesticus* is an insect with typical panoistic ovaries [23]. As seen in Figs. 13-15, the karyosphere is not formed in *A. domesticus* oocyte nuclei.

In the nuclei of all injected oocytes at different stages of their growth, clear incorporation of BrUTP was revealed (Figs. 16-19) although a level of the fluorescence was less than it could be expected. Anti-Br-RNA signal was registered as bright dots located along the chromosomes. While an image of a single chromosome was zoomed, the characteristic distribution pattern of anti-Br-RNA signal was revealed. As shown in Figs. 20-21, BrUTP incorporates in discrete zones rather than along the whole chromosome. In control preparations no incorporation of BrUTP was observed (Fig. 22).

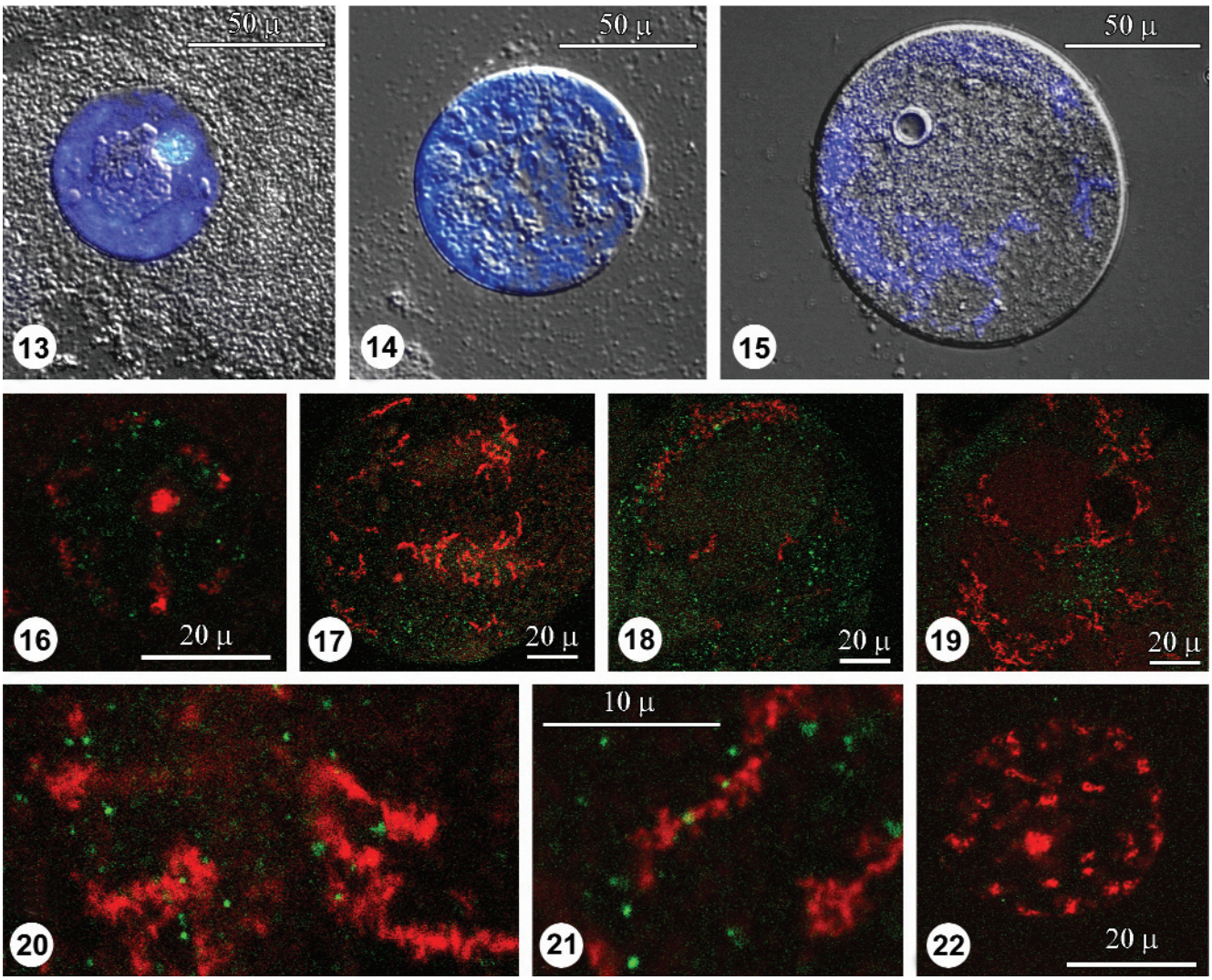
Discussion

During oogenesis, the oocyte must acquire large stores of macromolecules including different RNAs, and in broad terms there are two kinds of oocytes [22]. Insects with panoistic ovaries have oocytes of the first type, and they are characterized by the autosynthetic source of RNAs. Insects with meroistic ovaries have oocytes of the second type which depend on the nutritive cells providing an oocyte with large amounts of RNAs [3,33].

In the present work we provide direct evidence that in an insect with panoistic ovaries, *A. domesticus*, oocyte nucleus maintains its own transcriptional activity at the diplotene stage. Nevertheless, not all genes are apparently expressed at the defined time, since anti-Br-RNA signal exhibited a discrete pattern along the chromosomes. It is unknown which genes are expressed in *A. domesticus* oocytes at the defined stage of oogenesis.

A prominent feature of oogenesis in insects with meroistic ovaries is the formation of the karyosphere described in all species studied [14]. The mechanisms of karyosphere formation and factors involved in chromosome condensation during the karyosphere development are poorly known. Recently, an analysis of the mutations in *Drosophila* has shown that the mutation in the *nhk-1* gene encoding a kinase that phosphorylates histone H2A in meiosis leads to a rearrangement of oocyte chromatin, and the karyosphere is not formed in mutant oocytes [16].

In the present study we found that before the karyosphere formation, the distribution pattern of anti-Br-RNA signal is typical for a transcriptionally active cell [11]. This result is in a good accordance with our previous data obtained using immunogold labeling



Figs. 13-15. Squashed non-fixed diplotene nuclei of *Acheta domesticus* previtellogenic (Figs. 13-14) and vitellogenic (Fig. 15) oocytes. Nomarski optics + DAPI staining. Note, that the karyosphere is not formed in *A. domesticus* oocytes. Prominent DNA-positive body in Fig. 13 represents the large chromomere on chromosome 6, a result of rDNA amplification [19]. **Fig. 16.** Incorporation of BrUTP (green) in early diplotene oocyte nucleus of *A. domesticus*. Chromatin was stained with To-Pro-3 (red). **Figs. 17-19.** Fragments of larger *A. domesticus* oocyte nuclei, imaged at the same magnification. **Figs. 20-21.**. Zoomed images of single chromosomes showing the distribution pattern of anti-Br-RNA signal. **Fig. 22.** Control, non-injected oocyte nucleus at the same stage as in Fig. 16.

electron microscopy on the same stage oocytes. We reported that RNA polymerase II and some essential pre-mRNA processing factors are distributed at this stage within the whole nucleus in association with perichromatin fibrils [8] which are known to represent nascent pre-mRNA transcripts [11,13].

The formation of the karyosphere seems not to be a result of chromosome inactivation because the incorporation of BrUTP is still registered at earlier stages of karyosphere development. At the same time, the cessation of oocyte transcriptional activity does occur later as we showed in our BrUTP microinjection experiments. At the end of karyosphere morphogenesis, RNA polymerase II and pre-mRNA splicing factors were found earlier to be redistributed in the nucleus, from perichromatin regions to extrachromosomal nuclear bodies [1,7]. An accumulation of the components of gene expression disengaged from RNA transcription/processing cycles has been reported for oocyte nuclear bodies of different insects with meroistic ovaries [1,6,7,17,18].

Thus, our data confirm a suggestion (see Introduction) that silencing of oocyte genome is a characteristic feature of animals with the nutrimental (heterotrophic) type of oogenesis including insects with meroistic ovaries but at the beginning stages of karyosphere development, oocyte genome is not fully inactivated. Finally, in insects with panoistic ovaries, oocyte nuclei do maintain their transcription activity at the period of oocyte growth.

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