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Peculiarities of Spermatogenesis in *Drosophila melanogaster*: Role of Main Transport Receptor of mRNA (Dm NXF1)

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Abstract—Spermatogenesis in both the model object *Drosophila melanogaster* and mammals, including humans, is characterized by the specificity of the regulation of gene expression at both the transcriptional and translational levels, which is manifested in the existence of testis-specific transport mRNA receptors, NXF (nuclear export factor). By using antibodies to the C-terminal part of the Dm NXF1 protein (SBR), a considerable amount of this protein is found to be present at all stages of spermatogenesis. At early stages of spermatogenesis, we have shown the cytoplasmic localization of the Dm NXF1 protein. This protein is located in the nucleus or in the nuclear envelope at the stage of rounded spermatids. During spermatid elongation, the Dm NXF1 protein is located polarly and disposed only along one side of the extended spermatid nucleus, while, at the stage of spermatid individualization, it is translocated into the spermatozoan tail in the form of large cytoplasmic granules.

Key words: transport mRNA, spermatogenesis, Drosophila, NXF

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Abbreviations used: mRNA, matrix or informational ribonucleic acid; RNP, ribonucleoprotein complex; NXF (nuclear export factor), factor of nuclear export of mRNA.

INTRODUCTION

The processes that occur during spermatogenesis are characterized by evolutionary conservatism and are similar in different animals (Fuller, 1993; Guraya, 1987). During spermatogenesis, male germ cells are submitted to differentiation and fast polarization, which requires the transport and certain localization of proteins, mRNA, and organoids. For instance, it is known that, in mouse male germ cells, protein TB-RBP (testis brain RNA-binding protein), or translin, transports certain mRNAs in the composition of ribonucleoprotein complexes (Han et al., 1995; Morales et al., 2002). In particular, this involves mRNA of genes of X chromosomes, which are expressed exclusively in male postmeiotic germ cells and are characterized by delayed translation (Morales et al., 2002). Haploid spermatids are connected by intercellular bridges and form syncytium, which provides equal conditions for cells containing different chromosome sets. It can be said that genetically haploid chromatids are phenotypically diploid (Braun et al., 1989; Fuller, 1993; Morales et al., 1998). Another classic example is the mRNA of protamines, which are synthesized at the stage of round spermatids, transported, and stored in the cytoplasm in the composition of mRNP for several days before the beginning of translation at the stage of elongated spermatids (Balhorn et al., 1984; Giorgini et al., 2001; Kleene et al., 1984).

One of the peculiarities of spermatogenesis is that genes exist that are expressed in no tissues except the testis. The genes specific of spermatogenesis involve the male fertility factors coupled to Y chromosome and some genes located in the X chromosome and autosomes (Wakimoto et al., 2004). Furthermore, many genes are shown to contain testis-specific paralogs (Mikhaylova et al., 2008) and, in some cases (HIPK3, etc.), the testis-specific products are formed as a result of alternative splicing (Elliot and Grellscheid, 2006).

The *small bristle (sbr)* gene belongs to the conservative family of *nxf* genes (nuclear export factor); therefore, in *D. melanogaster*, it has another name, i.e., *Dm nxf1* (Herold et al., 2000, 2001; Tretiyakova et al., 2001; Wilkie et al., 2001). Orthologs of the *Dm nxf1* gene in nematodes (*Ce nxf1*), mice (*Mm nxf1*), and humans (*Hs nxf1*) encode major transport receptors of mRNA and are expressed in all tissues (Tan et al., 2000; Herold et al., 2001; Sasaki et al., 2005). For mammals, it is known that genes-paralogs *nxf1* have the tissue-specific character of expression, which is believed to be able to be connected with specialization of their function with respect to peculiar mRNAs

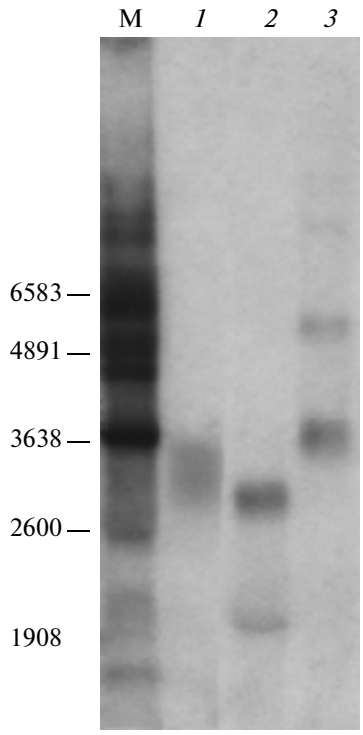

Fig. 1. Transcripts of *Dm nxf1* (*sbr*) gene of *Drosophila melanogaster* detected in different imago organs and in larval nerve ganglia by method of Northern blot hybridization with AJ probe. M is molecular weight marker with indication of size in nucleotides; *1.* RNA from the adult female ovaries; *2.* RNA from the adult male testes; *3.* RNA from nerve ganglia of larvae of the third age.

(Herold et al., 2001; Jun et al., 2001; Yang et al., 2001; Sasaki et al., 2005). The existence of paralogic genes of the mammalian nxf family whose expression is specific to the testis and brain and, based on the characteristic cytoplasmic localization of products of these genes, we have developed the concept that NXF factors are connected to temporarily nontranslated mRNAs (Herold et al., 2000, 2001; Jun et al., 2001; Yang et al., 2001; Sasaki et al., 2005; Tan et al., 2005; Tretiyakova et al., 2005; Lai et al., 2006; Takano et al., 2007; Katahira et al., 2008).

MATERIALS AND METHODS

Northern Blot Hybridization

The electrophoresis of total RNA was performed in 1.5–2% formaldehyde-agarose gel (4–6 μg RNA per band). RNA fractioned in gel was transferred onto a Hybond-N+ nylon membrane (Amersham, England) under vacuum conditions using a VacuGene XL blotter (Amersham, England) at 70 mbar. RNA was additionally fixed by UV cross-linking irradiation at 312 nm for 1 min. cDNA of the *sbr* gene (GenBank/EBI/Accession no. AJ318090.I.-AJ) was used as a probe. The cDNA fragment corresponding to the sequence of 137–672 amino acids in SBR protein was inserted into the pGEX5X-3 vector for restriction sites EcoRI and NotI and served as a matrix for obtaining labeled samples. To label probes, a Random Primed DNA Labeling Kit (Amersham, England) and [α-^{32}P]ATP were used. Hybridization was performed in a hybridizational stove (Rock 'N' Roll^{TM}, Boekel) at 68°C for 8–12 h in solution of the following composition: 2×–0.1× SSC, 0.1–1% SDS. To remove the unspecifically bound label, after hybridization the membrane was washed under rigid conditions: 0.1 × SSC, 0.1% SDS at 68°C three times for 30 min. Then the membrane was exposed by using the X-ray film (Kodak BIO MAX, England) and an enhancing screen at –70°C for 24–48 h.

As molecular weight standards, RNA-markers G3191 (Sigma, United States) were used.

Imunocytological Analysis of Localization of Dm NXF1 Protein

Testes were isolated from a 3–5-day-old male *Drosophila melanogaster* of standard laboratory strain of wild type Oregon-R. The testes were prepared in saline (testis buffer was 183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, pH 6.8) by carefully removing seminal vesicles, additional glands, the urethra, and other components of the male urogenital system. The tissue was fixed in 4% paraformaldehyde for 7 min and washed in PBST solution (0.1% Tween-20 in PBS (the phosphate buffered saline (Ashburner, 1989)). 3% bovine serum albumin (BSA) in PBST was used as the blocking reagent. Incubation with primary antibodies (1 : 400 in PBST+BSA) obtained in mice was performed overnight at 4°C. Washing to remove primary antibodies was conducted using PBST solution. Incubation with secondary antibodies (to mouse immunoglobulins) was performed in the PBST+BSA solution for 1 h at 37°C. As secondary antibodies, the antibodies from Invitrogen (United States) labeled with fluorochromes Alexa Fluor 488 and Alexa Fluor 546 were used. After washing away secondary antibodies in PBST, testes were incubated with DAPI (4',6-diamidino-2-phenylindole) (1 μg/ml in PBST) for 10 min, washed once in PBS, and mounted under coverslip in the medium Vectashield (United States). The preparations were analyzed under a Leica TCS SP5 confocal microscope at the Center for the Study of the Ultrastructure and Molecular Composition of Biological Objects KHROMAS at the Biological Faculty of St. Petersburg State University.

RESULTS AND DISCUSSION

We have shown the presence of the testis-specific transcript (Fig. 1) and the nervous tissue-specific transcript (Ivankova et al., 2010). Despite the evolutionary conservatism of the *nxf1* genes that have orthologs in different organisms, including humans, the testis-spe-

cific transcripts of *Mm nxf1* and *Hs nxf1* genes in mammals have not been described. It is possible that, in mammals, a function important for spermatogenesis was acquired by gene paralogs (*Mm nxf2* and *Hs nxf2*, *Mm nxf3* and *Hs nxf3*), which have the testis-specific character of expression (Herold et al., 2000, 2001;; Yang et al., 2001; Sasaki et al., 2005; Wang, Pan, 2007). The existence in *D. melanogaster* of the testis-specific product of the *Dm nxf1* gene can reflect the initial stage of specialization of transport receptors of the NXF family.

In the course of studying the participation of the Dm NXF1 (SBR) protein in Drosophila spermatogenesis, we performed an immunocytological analysis of the localization of the Dm NXF1 (SBR) protein at various stages of spermatogenesis. First of all, we have shown that the protein localization changes depending on the stage of spermatogenesis. At early stages of spermatogenesis, in mitotically dividing cells, the Dm NXF1 protein is present as individual granules located in the region of the nucleus. Significant amounts of Dm NXF1 protein can be seen at the stage of primary spermatocytes; it is present as granules both in the nucleus and in the cytoplasm (Fig. 2a). Primary spermatocytes appear as a result of four consecutive mitotic divisions, which lead to the formation of 16-cell cysts, in which all cells develop synchronously and are connected by cytoplasmic bridges, which form the syncytium (Fuller, 1993). The syncytial development is the characteristic peculiarity of formation of male germ cells in different animals (Burgos and Fawcett, 1955; Fawcett, 1961; King and Akai, 1971; Rasmussen, 1973). The maintenance of intercellular connections within the cyst is important for the synchronization of processes that occur during the differentiation of spermatozoa (Fawcett, 1961) and the provision of equal conditions for haploid sex cells containing different sex chromosomes (Braun et al., 1989; Caldwell and Handel, 1991). These connections are preserved until the moment of the individualization of spermatids (Fuller, 1993).

The distinguished peculiarity of the spermatogenesis of many animals is that the transcription of genes occurs, not only in the interphase diploid, but also in the meiotic and haploid cells (Kleene, 2001, 2003), with the synthesis of different mRNAs being translated not at once after transcription. Transcripts can be preserved for several days in a state inaccessible for translation, which occurs during spermiogenesis (Kleene, 1996, 2003; Steger, 1999; Kotaja et al., 2006). Spermiogenesis is the process of conversion of spermatids into mature spermatozoa, which is characterized by formation of the complex morphological structures that provide spermatozoon with functions of the unicellular organism capable for perception of signals from environment and the goal-oriented movement by competing with other spermatozoa in the active search for oocyte. During spermiogenesis, the translation of mRNAs that were previously in the composition of RNP complexes that block translation is activated (Kleene, 2001, 2003; Iguchi et al., 2006).

The Dm NXF1 protein is present mainly in the nucleus at the stage of primary spermatocytes when they are ready to start meiosis (Fig. 2a). The nucleus contains the area in which the Dm NXF1 protein is absent. This area seems to correspond to nucleolus. At this stage and in the period preceding the formation of a 16-cell cyst composed of diploid primary spermatocytes, on the surface, it is possible to see 1 or two bright granules (Fig. 2a, arrow) that contain the Dm NXF1 factor. The synchronous behavior of these granules and their location on the surface of the nucleus allows one to suggest that they might be connected with centrosome.

The centrosome plays a peculiar role in spermatogenesis and determines the polarity of stem cells by providing contact with the niche and asymmetrical division of stem cells (Yamashita et al., 2007). Spermatocytes of Drosophila contain two pairs of orthogonal centrioles ten times larger than those of other cells (Gonzalez et al., 1998). In spermatogenesis, centrioles are necessary not only for cell division, but also for formation of the spermatozoon flagella (Rieder et al., 2001; Basto et al., 2006). The flagella, which are derivatives of centrioles, usually have two additional central microtubules, radial spokes, and dinein arms providing mobility of the flagellum (Dawe et al., 2007). The disturbance of the formation of an axoneme (flagellum) leads to mature spermatozoa that are deprived of mobility (Bisgrove and Yost, 2006). During fertilization, the centrosome, which enters the oocyte together with spermium, participates in the formation of astral microtubules, which are responsible for the transport of pronuclei to one another (Rieder et al., 2001).

In addition to spermium, mRNAs, which are able to affect the processes connected with the fusion of pronuclei and subsequent embryonic mitoses, also enter the oocyte (Ostermeier et al., 2004). Most likely, the posttranscriptional fate of spermatogenic RNAs depends on the complexes in which they pass their biogenesis. Most likely, the nuclear export and subsequent biogenesis of RNAs synthesized in spermiogenesis need peculiar RNA-binding proteins, including transport receptors. We have shown that the heat shock on sex cells of the males at the larval stage leads to the appearance of offspring with a disturbed number and set of not only paternal, but also maternal chromosomes (K'ergaard and Mamon, 2007).

The obtained data have allowed us to suggest the existence of factors that enter the oocyte together with spermium and able to affect the process of divergence of both the paternal and maternal chromosomes after fertilization. The process of the formation of these factors coincides with the premeiotic period of spermatogenesis and seems to be achieved with the participation of the product of the *Dm nxf1* gene (K'ergaard and Mamon, 2007). The connection of different mRNAs

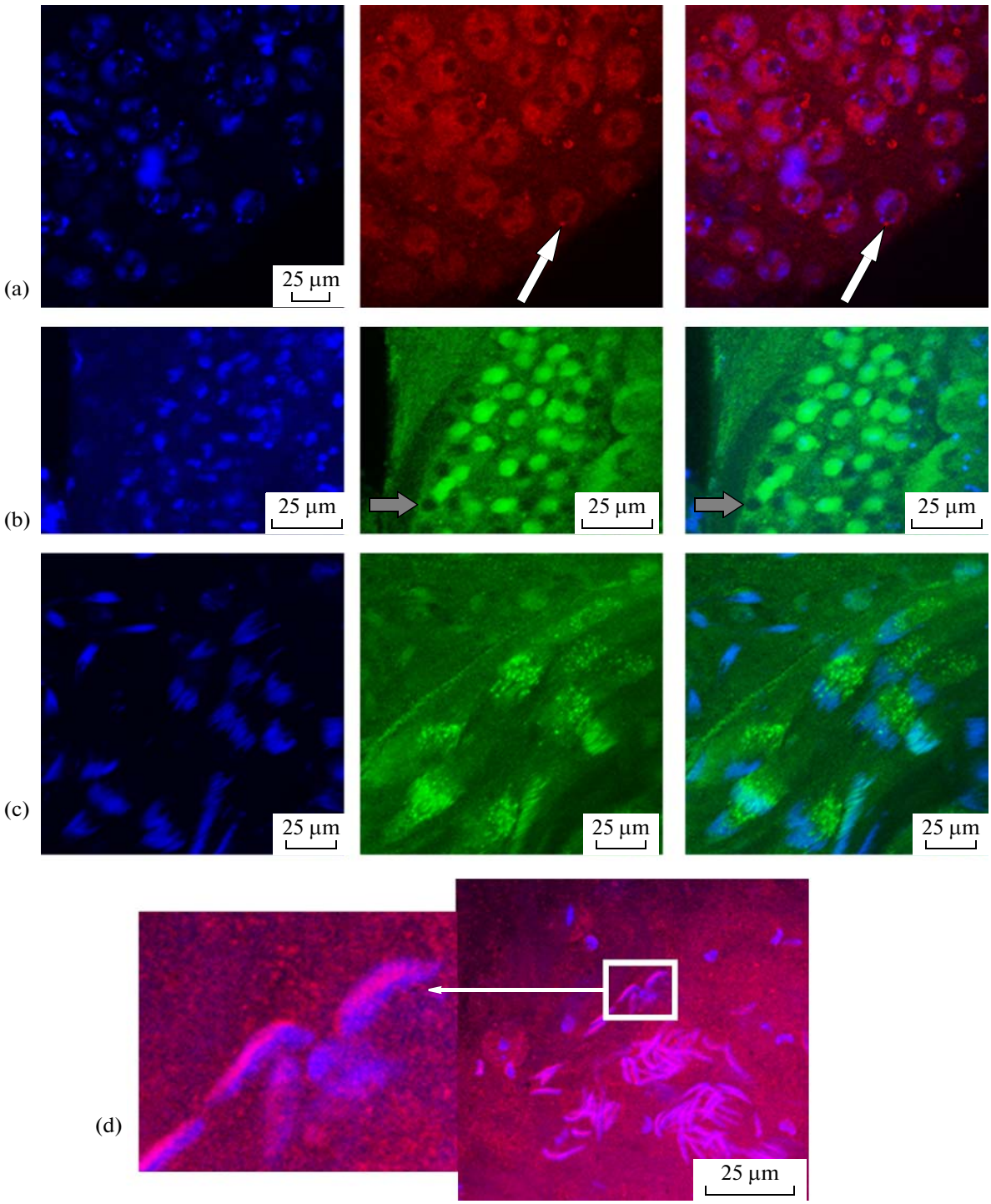

Fig. 2. Localization of the DmNxf1(SBR) protein with use of antibody at various stages of spermatogenesis of *Drosophila melanogaster*. (a) localization of protein at the stage of primary spermatocytes (scale 10 μm); SBR protein is detected in both nucleus and cytoplasm of spermatocyte in form of granules; chromosomes are decondensed and are represented by three discrete regions in each nucleus; arrow indicates granule located on surface of nuclear envelope. (b) spermatids at onion stage (scale 25 μm). Protein concentration in nuclear envelope increases at onion stage, unstained structures (arrow) represent Nebenkern. (c) the final stage of spermatid elongation before individualization (scale 25 μm); the SBR protein leaves condensed nuclei and is translocated in large granules into formed spermatozoa tails. (d) spermatids at canoe stage occur in first stage of elongation (scale 25 μm); at this stage, the SBR protein moves onto one side of the formed spermium head; presented is the testis site containing spermatids at the canoe stage and a magnified image of several elongated heads of the future spermatozoa (carried out); red indicates secondary antibody labeled with Alexa Fluor 546, blue indicates DAPI. (a) 1. staining with DAPI, 2. secondary antibody labeled with Alexa Fluor 546, 3. merged picture; (b, c) 1. staining with DAPI, 2. secondary antibody labeled with Alexa Fluor 488, 3. merged picture.

with centrosomes, which leads to asymmetry in the distribution of cytoplasmic determinants between daughter cells (Lambert, Nagy, 2002), makes the hypothesis that the Dm NXF1 factor can participate in the biogenesis of centrosome and the factors connected with it as an RNA-binding protein rather attractive (Mamon, 2008).

At the onion stage, which corresponds to the stage of rounded spermatids, the Dm NXF1 protein is also located in both the nucleus and cytoplasm (Fig. 2b). At this stage, 64 haploid cells that form the cyst enter spermiogenesis, i.e., the process of formation of mature spermatozoa (Fuller, 1993). In the cytoplasm near the nucleus, as a rule, one large granule that includes the Dm NXF1 protein is seen, whereas, in the mitochondrial structure called a "Nebenkern" (Fig. 2b, arrow), the Dm NXF1 protein is not detected.

At the stage of spermatid elongation, chromatin is condensed and the nucleus shape changes. It should be noted that, at this stage, the Dm NXF1 protein is located on one side of the nucleus (Fig. 2d). This may be because, in the course of elongation, the readjustment of the nuclear membrane is accompanied by a shift in the nuclear pore complexes to one side of the nucleus (Fuller, 1993).

In the course of individualization at the final stage of spermatogenesis, the Dm NXF1 protein leaves nucleus and in the composition of multiple granules moves to the distal end of the spermatozoan tail (Fig. 2c). The Dm NXF1 factor at the end of spermatogenesis may be submitted to proteasomal degradation, which is promoted by the presence of a UBA-like domain (ubiquitin-associated domain). Proteasomes are used to eliminate the spent proteins. The testicle-specific proteasome form, Prosα6T, in *D. melanogaster* is found in the granules that accompany the complex of individualization (Zhong and Belote, 2007). This complex, the main components of which are actin cones, moves to the distal end of the spermatozoan tail by forming a new membrane, thereby individualizing each mature sex cell (Noguchi et al., 2008). The disposition of granules containing proteasome Prosα6T is dynamic with respect to the actin cones. When the individualization complex only begins to gather around the elongating and condensing nucleus, granules are seen in front of the actin cones. When the individualization complex leaves the nucleus, the actin cones and granules cross and the granules move behind the actin cones; mutants for Prosα6T manifest as male sterility (Zhong and Belote, 2007).

The localization and concentration of the Dm NXF1 factor change depending on the stage of spermatogenesis. Dynamical changes in the localization of the SBR protein at early stages of spermatogenesis allow it to be suggested that this protein not only controls the export of mRNA from the nucleus, but also performs cytoplasmic functions.

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