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Nascent Polypeptide-Associated Complex as Tissue-Specific Cofactor during Germinal Cell Differentiation in *Drosophila* Testes

G. L. Kogan, N. V. Akulenko, Yu. A. Abramov, O. A. Sokolova, E. A. Fefelova, and V. A. Gvozdev*

Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, 123182 Russia

**e-mail: gvozdev@img.ras.ru*

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Abstract—During the process of spermatogenesis, the proliferation of spermatogonia (stem cell descendants) is replaced by their differentiation in growing spermatocytes responsible for the preparation to meiosis, which is accompanied by a cardinal change in transcriptional programs. We have demonstrated that, in drosophila, this process is accompanied by a splash of the expression of β-subunit of nascent polypeptide-associated complex (NAC) associated by ribosomes. Nascent polypeptide-associated complex is known as a chaperone involved in co-translational protein folding. This is the first case of the detection of tissue-specific co-translational NAC cofactor in multicellular eukaryotes. It is proposed that spermatocyte specific NAC is involved in the modulation of the expression of the proteins that provide the functioning of subsequent stages of spermatogenesis.

Keywords: βNAC, spermatogenesis, spermatocytes, tissue-specific ribosomes

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INTRODUCTION

Nascent polypeptide-associated complex (NAC) is a conservative protein that can be detected in all eukaryotes (from yeasts to humans) and consists of α- and β-subunits that are dimerized by the interaction of NAC domains [1–3]. Mutations and deletions of the ubiquitously expressed β*NAC* gene in mice, drosophila, and nematode lead to the death of embryos at early stage of the development [4–6]. It is assumed that ubiquitously expressed NAC in eukaryotes is a co-chaperone that provides the folding of newly synthesized peptides and co-translational protein transport in mitochondria and lysosomes [7–9]. NAC is involved in the cell response to stress, as well as interferes with the processes of apoptosis [6, 10–14]. The studies on yeasts and nematode demonstrated that NAC provides a translocation of newly synthesized proteins into the membrane and endoplasmic reticulum lumen and suppresses nonspecific contacts between ribosomes and translocons, which prevents the erroneous transport of mitochondrial proteins into the endoplasmic reticulum [8, 15]. Presumably, NAC provides the delivery of methionine-aminopeptidase to newly synthesized polypeptides [15, 16]. However, the molecular function of NAC protein as a factor associated with the ribosome remains insufficiently studied.

Previously, we found a family of highly homologous genes in the *Drosophila melanogaster* genome that are specifically expressed in testes [17] and encode paralogs of ubiquitously expressed NAC β-subunit encoded by a unique *bicaudal* gene [5]. The detection of tissue-specific genes in drosophila that encode the tissue-specific NAC β-subunits indicated the involvement of this protein variant in the ensuring of specialized cellular function. Bioinformatic analysis of genomes of different drosophila species demonstrated that the copies of tissue-specific β*NACtes* genes, which are expressed in testes, multiplied in the genome of the precursor of sister species (i.e., species closely related to the *Drosophila melanogaster*) and are absent in more phylogenetically distant species [18]. The analysis of the divergence of paralogs of the β*NACtes* genes indicated the involvement of directional selection in their evolution; however, their adaptive function remains unexplained [19].

In the present work, we demonstrated that a sharp increase in the expression of βNACtes is associated with the beginning of differentiation of mitotically dividing germinal cells in growing spermatocytes, in which, as is known, a new program of gene transcription in the process of spermatogenesis is performed [20–22]. This program provides biogenesis of new types of mRNA, the translation of which can also occur in later stages of spermatogenesis, when the appropriate proteins are involved in the processes of meiosis of mature spermatocytes and in following stage of spermiogenesis. It seems to us more interesting an to assume of the important role of tissue-specific NAC protein as a modulator of the translational control of mRNA types in spermatocytes [22–24]. The conception about the role of NAC protein in the

choice of mRNA for the translation is within the modern discussion of the heterogeneity of ribosome populations and nonrandomness of the selection by ribosomes of certain mRNA for the translation. The specifics of this choice can be determined both by tissue-specific ribosome proteins and by the ribosome protein factors with which it is associated [25, 26].

EXPERIMENTAL

Production of antibodies to βNACtes protein. Full-sized fragments of open reading frame of two paralogues of the β*NACtes* gene (*CG32601* and *CG18313*) were obtained by the RT-PCR method using the total RNA of testes and the primer pairs as follows:

CG32601: CTTACCAAACATATGGATTTCAAGAAGCTG,
AAATGAGAATTCCTAATCTTCGTCCTCTGACACC;
CG18313: TCTTAACAAACATATGGATTTCAACAAGCG,
ATATGAGAATTCCTAATCTTCGTCCTCGGAGACC.

These fragments were cloned in the pET28b vector (Novagen). Plasmids containing the β*NACtes* gene region were sequenced and expressed in the *Escherichia coli* BL21 (Rozetta) strain. Recombinant proteins containing six histidine residues at the N-terminus were purified by Ni-affine chromatography in native conditions according to the manufacturer's protocol (GE Healthcare). Rats were immunized by purified recombinant βNACtes proteins (10 mg per rat). Antibodies to two βNACtes paralogs detected (Western-blot analysis, dilution 1 : 1000) similar mobility of βNACtes proteins and specificity in immunostaining testes (1 : 100 dilution). The βNACtes mobility corresponded to molecular weight 37–38 kDa different from the calculated one (28.5 kDa).

Western-blot analysis. Testes (30–40 pairs) were extracted from males, the age of which did not exceed 2 days, homogenized in the buffer containing 8 M urea, 10 mM Tris-HCl (pH 8.0), 100 mM NaH_2PO_4, centrifuged for 15 min at 13000 rev/min. Supernatant was separated by electrophoresis in polyacrylamide gel under denaturing conditions (SDS-PAGE) according to a standard protocol, the proteins were transferred into the PVDF membrane (Immobilon-P, Millipore). Before the staining with antibodies, the membrane was incubated for 1 h in the blocking buffer (0.2% Tropix I-Block, Applied Biosystems), then 1 h with antibodies in the blocking buffer, and 1 h with secondary antibodies conjugated with alkaline phosphatase, washed by phosphate buffered saline (PBS) containing 0.1% Tween-20 , and developed by means of the chemiluminescent detection system (BioRad Chemi Doc MP Imaging System). Monoclonal rat antibodies to HSP90 (ADI-SPA-835 ENZO Life Sciences) in a dilution of 1 : 5000, monoclonal mouse antibodies to β-tubulin (Monoclonal anti-β-Tubulin, Sigma) in a dilution of 1 : 1000, and monoclonal mouse antibodies to β-actin (ab8224, Abcam) in a dilution of 1 : 2500 were used.

Immunostaining. The fixation and immunostaining of testes of 1–2-day males were conducted according to the method [27]. Polyclonal rat antibodies to βNACtes (1 : 100), polyclonal rabbit antibodies to lamin (1 : 1000 obtained from P.A. Fisher), secondary antibodies to rat and rabbit antigens conjugated with fluorescent dyes (Alexa 488, Alexa 546, Invitrogen) were used.

Detection of NACtes protein in ribosomes and polysomes. Frozen testes (500 pairs) were homogenized in a Dounce homogenizer in 500 μL buffer for isolating polysomes (PB, 25 mM Hepes pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, RiboLock RNase inhibitor (Thermo Scientific)) in a concentration 40 units/mL, 0.015% digitonin, 1% NP-40, 0.5% sodium deoxycholate, and 100 μg/mL cycloheximide. The Protease Inhibitor Cocktail Tablets were used according to the company's recommendations. The fragments of the cells and mitochondria were removed by centrifugation at 12000 *g* and 4°C for 20 min. Post-mitochondrial supernatant (500 μL, 10 units of the solution optical density at 260 nm) was applied on a linear sucrose density gradient (11 mL, 10–50% (w/v)) prepared in PB a day before (5.5 mL 10% sucrose was applied in a centrifuge tube, 5.5 mL 50% sucrose were added, covered with parafilm, and placed horizontally for 2 h, after which they were carefully moved to a vertical position and left for the night at 4°C), centrifuged in the Beckman SW40 Ti rotor at 38000 rev/min and 4°C for 3.5 h. Twenty-five fractions (500 μL each) were selected for analysis. The proteins of each fraction were precipitated by 10% trichloroacetic acid and left for the night in ice. Precipitates were separated by centrifuging at 12000 *g* and 4°C for 30 min, washed twice by cooled acetone, dissolved in 30 μL buffer for the preparation of the sample for Western-blot analysis.

Ribosome isolation and immunoprecipitation. To obtain ribosomes, 1000 pairs of frozen testes of the Batumi line flies were homogenized in PB buffer, post-mitochondrial supernatant was centrifuged in the Beckman SW40 Ti rotor at 39000 rev/min for 4 h at 4°C. The precipitate was washed by PB buffer and dissolved in buffer for immunoprecipitation (IB, 50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitor cocktail (Roche), as described above, 40 units/mL RiboLock RNase inhibitor and 0.5% CHAPS). Magnetic particles of Dynabeads Protein G (Invitrogen by Thermo Fisher Scientific), which was associated with antibodies to

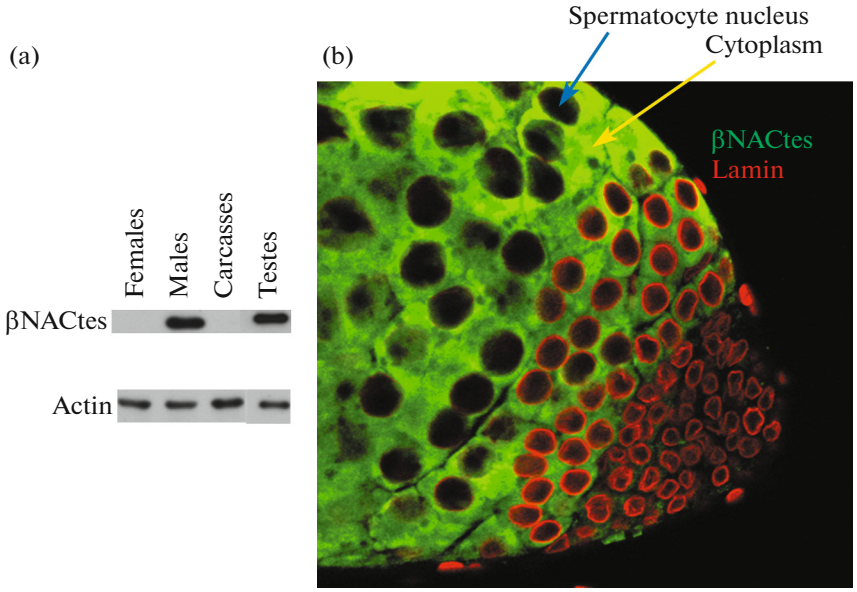

Fig. 1. Specificity of βNACtes protein expression in germinal cells of testes. (a) Western-blot analysis of extracts of Batumi flies; (b) βNACtes protein is localized in spermatocyte cytoplasm; βNACtes, green; lamin (nuclear envelop), red.

βNACtes, were used for immunoprecipitation, which was conducted according to the company's recommendations. A total of 300 μL of ribosome extract containing 2.0 μg protein/μL were added to 50 μL magnetic particles conjugated with antibodies to βNACtes. The suspension was incubated and rotated on a vortex for 1 h at 20°C, washed five to six times by PBS containing 0.1% Tween-20. A volume of 500 μL of the Trizol reagent (Life Technologies) was added to magnetic particles and thoroughly mixed, after which a 1 : 10 volume of chloroform was added and the suspension was left for 10 min in ice, then centrifuged at 10000 rev/min for 5 min at 4°C. A total of 1 μg glycogen (Thermo Scientific) was added to the supernatant, after which RNA was precipitated by an equal volume of isopropanol for a night at –20°C, washed by 70% ethanol, and dissolved in water treated with diethylpyrocarbonate. DNA was removed by DNase I (Ambion) for 1 h at 37°C.

Reverse transcription with subsequent polymerase chain reaction. Total RNA from testes, testis ribosomes, and the ribosome fraction obtained as a result of precipitation by antibodies to βNACtes was isolated using Trizol reagent (Life Technologies) according to the company's recommendations. Reverse transcription of RNA was conducted using oligo-dT primers (Sileks, Russia) and SuperScriptII reverse transcriptase (Invitrogen) according to the company's method. The amount of transcripts was estimated using real-time PCR with Hot Start DNA polymerase (SibEnzyme, Russia) and SYBR Green1 dye on the DT-96 instrument (DNA Technology, Russia). The results were normalized on the amount of the *RPL32* (*Rp49*) gene mRNA. The following primer pairs were used:

βNACtes–AATCGTGTTCGTCCTCGCAA,
CCGATGGCACCTTCTCATTATCCG;
Vasa–TTGAGCAAGTTGTTGGAGGAT,
TTTGATGTCTGAACGAGGTG.

RESULTS AND DISCUSSION

Detection of βNACtes Subunit in Testis Spermatocytes

Using antibodies to the NACtes β-subunit (that we obtained), we determined the type of cells containing βNACtes. Nucleotide sequences of paralogs of the β*NACtes* genes differ slightly. The two most different paralogs (*CG32601* and *CG18313*), the identity of nucleotide sequences of which exceeded 90%, were cloned to obtain antibodies. As expected, antibodies to two recombinant proteins that correspond to two different paralogs yield identical results in Western-blot analysis and immunostaining. Previously, we demonstrated the transcriptional activity of each β*NACtes* copy using RT-PCR; therefore, it was accepted that the immune analysis registers a total activity of all five amplified paralogs in the *D. melanogaster* genome (Fig. 1). Western-blot analysis detects βNACtes protein in extracts of testes and whole males, but not in extracts of females and males with remote testes (carcasses) (Fig. 1a, see colored insert).

Immunostaining demonstrated that the significant expression of the NACtes protein is only registered at the stage of differentiation of growing spermatocytes (Fig. 1b). Insignificant staining is observed near the tip of the testis, where mitotic divisions of spermatogonia (spermatocyte precursors) occur. A sharp boundary between the weak staining of spermatocyte

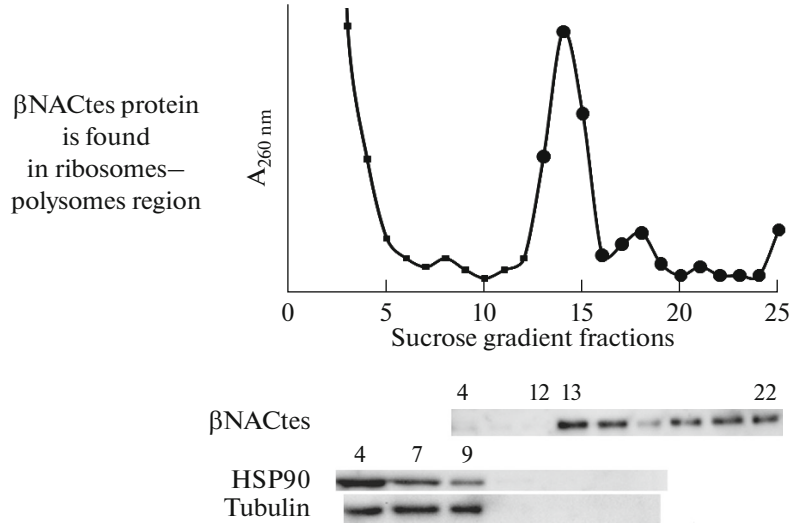

Fig. 2. NACtes protein is associated with ribosomes and polysomes. Fractioning of post-mitochondrial extract of testes in sucrose gradient. Absence of βNACtes protein in cytosol (fractions 4–9) enriched by tubulin and HSP90 heat shock protein was demonstrated. Results of protein detection in fractions by means of Western-blot analysis are presented. Fractions containing (●) and not containing βNACtes (■).

precursor cells (in which, according to [28], the process of spermatocyte differentiation only begins) and the intensive staining of differentiated spermatocytes can be seen. In mitotically dividing germinal undifferentiated cells, it is already possible to detect transcripts of the β*NACtes* genes by RT-PCR method in individuals homozygous for the *bam* mutation, which prevents the differentiation of spermatocytes and results in the accumulation of undifferentiated germinal cells. A semiquantitative analysis by the RT-PCR method demonstrated that the content of βNACtes mRNA differ insignificantly from the amount of ribosomal RpL49 protein mRNA and exceeds the content of Vasa mRNA (known marker of germinal eukaryotic cells). The beginning of spermatocyte differentiation apparently launches the translation of βNACtes subunit. Somatic cells adjacent to spermatocytes are not stained by antibodies to βNACtes. It is possible to conclude that tissue-specific NACtes protein is only produced in germinal cells. We did not observed the βNACtes staining at later post-meiotic stages of spermatogenesis. Thus, a strict confinement of NACtes to the stage of growth and maturation of spermatocyte, in which a new transcriptional program providing meiosis and subsequent spermatogenesis stages is sharply activated, was detected.

NACtes Protein Is Associated with Ribosomes

The βNACtes protein is accumulated in the cytoplasm of spermatocytes and is not detected in the nucleus (Fig. 1b). Since we only monitor the NACtes protein based on the presence of the β-subunit, it was interesting to determine whether this protein is associated with ribosomes as well as the usually studied ubiquitously expressed NAC protein [13]. Separation in the sucrose gradient of the testes extract, from which mitochondria were removed, demonstrated that NACtes is almost completely absent in cytosol and is associated with the 80S peak of ribosomes and polysomes (Fig. 2). This result clearly indicates the existence of yet unidentified ribosomal function of NACtes.

It is possible that the NACtes marks ribosomes are intended for translating certain mRNA produced in spermatocytes. This assumption is based on ideas about the existence of heterogeneous ribosome population, in which ribosomes may differ in the composition of ribosomal proteins or in associated factors providing a specific control of certain mRNA translation [26]. It was previously demonstrated that minor weakly expressed variant of the NAC protein β-subunit (found in the *Saccharomyces cerevisiae* yeast cells) is associated (unlike the main NAC) with ribosomes that are involved in the translation of predominantly ribosomal proteins and mitochondrial proteins encoded by nuclear genome [7, 29]. Our preliminary experiments do not exclude the involvement of ribosomes that contain NACtes in selective association with specific mRNA of spermatocytes. We compared the presence of mRNA encoding the βNACtes and Vasa marker protein of germinal cells in RNA preparations from total ribosome fraction and ribosomes containing the βNACtes separated from the total fraction by immunoprecipitation using the antibodies to the βNACtes. The relative amount of the Vasa mRNA (encoding RNA helicase of germinal tissues) in the immunoprecipitate exceeded its content in RNA isolated from the total ribosome fraction by three times (Fig. 3). On the contrary, the content of βNACtes mRNA in immuno-

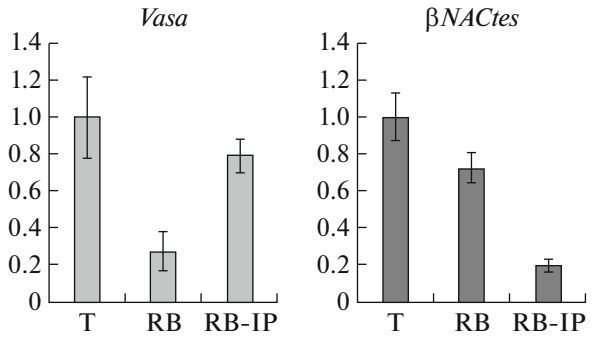

Fig. 3. Relative content of *Vasa* and β*NACtes* transcripts in RNA preparations obtained from testes (T, accepted for 100%), testicle ribosomes (RB), and ribosomes immunoprecipitable with antibodies to βNACtes (RB-IP). Results of RT-PCR are normalized to the content of Rp49 transcripts.

precipitated ribosomes was almost four times lower than in the total ribosome preparation (Fig. 3).

This result demonstrates the preferential association of the Vasa mRNA with ribosomes containing the NACtes protein, while the βNACtes mRNA (actively translated in spermatocytes) is apparently predominantly associated with ribosomes that do not contain NACtes. This preliminary result, which indicates the existence of the selective association of ribosomes that contain NACtes with specific mRNA should be confirmed by the high-throughput sequencing of mRNA samples to identify specific mRNA. This approach can answer the question of whether NACtes functions are associated with mRNA translation in spermatocytes and/or NACtes is involved in the association of ribosomes with specific mRNA, the translation of which occurs at later stages of spermatogenesis. The assumption about the involvement of associated with ribosomes NAC protein in the choice of translated mRNA and in the generation of specialized ribosomes is only new relative to NACtes proteins. The conception about the functional ribosome heterogeneity determined by the presence of specialized ribosomal proteins or proteins associated with ribosomes is widely discussed [25, 26, 30], and the detection of functionally specialized ribosomes that control the translation of important regulatory proteins is an argument in its favor [31].

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