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Research paper

Testis-specific products of the *Drosophila melanogaster sbr* gene, encoding nuclear export factor 1, are necessary for male fertility

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ABSTRACT

The evolutionarily conserved nuclear export factor 1 (NXF1) provides mRNA export from the nucleus to the cytoplasm. We described several testis-specific transcripts of the *Drosophila melanogaster nxf1* gene designated "*sbr*" in this species via different PCR approaches and CAGE-seq analysis. Characteristically, most of them have truncated 3′UTRs compared with those in other organs. In addition to regular transcripts, there are shorter transcripts that begin in intron 3 of the *sbr* gene. These short, 5′-truncated testis-specific transcripts vary in terms of transcription start site and their ability to exclude or retain the last 237 nucleotides of intron 3 in their 5′UTR. Using an anti-SBR antibody against the C-terminal portion of this protein, we detected the major SBR protein (74 kDa) in all analyzed organs of the fly as well as a new smaller protein (60 kDa) found only in the testes. This protein corresponds to the detected *sbr* transcripts that start in intron 3, based on its molecular mass. We investigated the sbr^{12} allele of the *sbr* gene, which is lethal in homozygous females and causes dominant sterility in heterozygous males. Sequencing of the sbr^{12} gene allele revealed a 30-bp deletion in exon 9 without a frame shift. Western blot analysis with an SBR-specific antibody revealed two bands of the expected size in the testes of heterozygous males. Thus, a mutant protein along with the normal protein presents in the testes of lethal allele-bearing flies and the described shorter testis-specific variant of SBR may account for male sterility.

1. Introduction

The variety of products derived from a single gene is one facet of gene function specialization. Transcript diversity is a characteristic feature of the *nxf* (nuclear export factor) gene family. The product of *nxf1*, the main gene in this family, is responsible for the transport of most mRNAs from the nucleus to the cytoplasm in different eukaryotic organisms (Herold et al., 2000, 2001; Sasaki et al., 2005). This universal function is necessary for all cells with active transcription. The NXF1 protein belongs to a "transport receptor" family. As a receptor, NXF1 nonspecifically interacts with cellular mRNAs through adaptor proteins, and NXF1 also functions as a transporter via interaction with nucleoporins to ensure RNP (ribonucleoprotein) complex transfer through the nuclear pores (Katahira et al., 1999; Bachi et al., 2000; Fribourg et al., 2001; Lévesque et al., 2001; Schmitt and Gerace, 2001; Thakurta et al., 2004; Viphakone et al., 2012). The *Drosophila melanogaster small bristles* (*sbr*) gene is an ortholog of the *nxf1* genes in other organisms and thus, based on its function, has been designated "*Dm nxf1*" (Wilkie et al., 2001; Herold et al., 2001; Tretyakova et al., 2001). It is X-linked, and the majority of the known alleles are lethal in a homozygous or hemizygous state in females or males, respectively (FlyBase http://flybase.org). Mutations described at the *sbr* locus exhibit a broad spectrum of pleiotropic effects, including dominant male sterility (Dybas et al., 1983; Geer et al., 1983; Golubkova et al., 2015). Given that not all *sbr* alleles cause male sterility, it can be assumed that there are some tissue-specific functions of the *sbr* gene in spermatogenesis, and these functions are impaired in certain cases (e.g., sbr^{17} and sbr^{12} mutant males). Previously, in addition to the canonical 3.5-kb *sbr*-encoded transcript found in all *D. melanogaster* organs, we detected shorter testis-specific transcripts (1.9 kb and 2.8 kb) (Ivankova et al., 2010). It is noteworthy that short testis-specific *nxf1* transcripts have not been described for mammalian *nxf1* genes. However, in *Homo sapiens* and *Mus musculus* there are the paralogous genes *nxf2* and *nxf3*, which are characterized by exceptional

Abbreviations: NXF, nuclear export factor; UTR, untranslates region; Dm, *Drosophila melanogaster*; PCR, polymerase chain reaction; APA, alternative polyadenylation; DSE, downstream sequence element; CDD, Conserved Domain Database; USE, upstream sequence element; DPE, downstream promoter element; CAGE, cap analysis of gene expression.

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or predominant expression in the testes (Herold et al., 2000; Herold et al., 2001; Yang et al., 2001; Sasaki et al., 2005). In *D. melanogaster*, the *nxf* family includes four genes (Herold et al., 2001) and their role in spermatogenesis is poorly understood.

Herein, we determined in detail the organization and origin of testis-specific *sbr* transcripts. We provide evidence that most *sbr* transcripts have truncated 3′UTRs in the testes, and the start points for the testis-specific 1.9-kb transcripts are localized in intron 3. Western blot analysis exploring antibodies to the C-terminal fragment of the SBR protein identified universal (74 kDa) and testis-specific, short (60 kDa) forms of SBR in *D. melanogaster*. Furthermore, the deletion of 10 aa in the testis-specific SBR protein leads to dominant male sterility, implying the significance of this protein in spermatogenesis.

2. Material and methods

2.1. D. melanogaster strains

- *Oregon-R*
- sbr^{12} *(l(1)24/45)/FM6v, y* sc^{8} *dm B*
- *Df(1)v-L4,* ras^{2} m^{D}*/FM6 l, y sc8 dm B/Dp(1;Y)* y^{+} v^{+}
- sbr^{10} *(l(1)ts403); bw; st*

For DNA isolation and sequencing of the mutant sbr^{12} allele, we used heterozygous adult females obtained by crossing sbr^{12}*/FM6v, y* sc^{8} *dm B* females with sbr^{10} *(l(1)ts403); bw; st* males. DNA isolation and sequencing were performed as described by Golubkova et al. (2009).

2.2. 5′ and 3′ RACE-PCR for the determination of the transcript ends

Total RNA was isolated from testes and heads of wild type *D. melanogaster* (*Oregon-R*) using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was generated with a Mint RACE cDNA amplification kit (Evrogen, Russia), which permits the flanking of the 5′ and 3′ ends of the transcripts with special adaptors. For RACE-PCR we used gene primers specific for *sbr*:

	5′ RACE		3′ RACE	
Round I	ex7R1	CGAAGGCATTGATATGGAGAG	ex4F	ACTCTTGAGTGCTCTATTGGCAG
Round II	ex7R2	CACAGATAGGATGCCTTCGTT	ex9F	GGTGGTGCCACAGAATAATG
Round III	ex6-7R	CTCTCCGTCCAACTTAACCA		

Amplification parameters were selected according to the manufacturer's recommendations. Major homogenous PCR products (after round III in 5′ RACE and round II in 3′ RACE) that were not present in the control reaction with gene-specific primers alone were isolated from an agarose gel and cloned into the TA vector pAL-TA (Evrogen, Russia). *E. coli* cells were transformed with the vector; plasmids from several positive clones were isolated and sequenced using the standard M13 primer.

Additionally, we performed reverse transcription PCR (RT-PCR) using the same cDNA described above originating from the testes as well as cDNA simultaneously synthesized from RNA templates from the heads of the same males. There were three RT-PCR reactions in total. The reverse primer specific to exon 5 (5′GGGAACGGCAGGGATTGTTC) was used in all 3 RT-PCR reactions. The forward primers for PCR product detection were: (1 and 2) specific to intron 3 (5′TATTATTGCTTACATCTACAGACGT and 5′GATAATTTCGGTCGCTGCTATCT) and (3) specific to exon 4 (5′CAGCATGGAGGCGTTTAAGGG).

2.3. Anti-SBR antibody production

For antibody production, we used the SBR protein fragment corresponding to the majority of exon 9. A DNA fragment of 417 bp (73–489 bp of *sbr* gene exon 9) was cut out from the genome subclone 14.5 BB (DS03615) (Tretyakova et al., 2001) at NcoI and HinfI restriction sites. This DNA fragment was cloned into the expression vector pET23d with a C-terminal 6^{x}His-tag. After *E. coli* transformation, the C-terminal fragment of SBR protein was isolated from the lysed *E. coli* on Ni-Sepharose columns.

A 14-kDa purified fragment of the SBR protein was used to immunize mice. Isolation and purification of polyclonal mouse antibodies were performed by O.S. Morenkov (The Institute of Cellular Biophysics, RAS, Pushchino). The obtained antibodies were used for Western blot analysis of the proteins from different organs of adult *D. melanogaster*.

2.4. Western blot analysis

Tissue samples (ovaries, testes and heads) were dissected in PBS solution from adult flies. SDS-PAGE was performed in 15% reducing gels. Our primary anti-SBR antibodies were diluted 1:500, and the secondary anti-mouse antibodies conjugated with horseradish peroxidase (GE Healthcare UK Limited, UK), were diluted 1:4000. Detection was performed with a Western blotting detection reagent kit (GE Healthcare UK Limited, UK) and Kodak photographic film (Eastman Kodak Company, USA).

2.5. Analysis of CAGE-seq data

CAGE (Cap Analysis of Gene Expression) (Kodzius et al., 2006) combined with next-generation sequencing technologies allows for TSS (transcription start site) mapping at the genome-wide level. In contrast to RNA-seq, CAGE-seq allows for the possibility of correctly mapping alternative promoters, even if both major and alternative transcripts overlap for the most part and have different expression levels.

Raw CAGE reads were downloaded from the NCBI SRA (Sequence Read Archive) database (http://www.ncbi.nlm.nih.gov/sra), and the sample IDs were SRR488272 and SRR488285. CAGE reads are single-end and 36 nt in length and contain a 9-nt CAGE tag that must be cut.

Analysis of CAGE-seq data was performed in several steps. In the first step, the reads that did not contain the tags were removed. Then, the CAGE tags were removed from the remaining reads. In the second step, the reads, deprived of the CAGE tags, were aligned to the *D. melanogaster* genome (release version 6.03, FlyBase http://flybase.org) using Bowtie2 (Langmead et al., 2009; 2012). In the third step, the reads that aligned with a MAPQ (mapping quality) of less than 10 (MAPQ = −10 log10p, where 'p' is the probability of misalignment) were removed from the analyzed group of reads. If there were 3 or more reads with the same start in a cluster, such clusters of reads were used for further analysis. Finally, TPM (tags per million reads) values were calculated for each mapping position remaining after filtration of each sample. A final picture of the transcript start points was drawn with the Matplotlib package (Hunter, 2007) for Python.

3. Results

3.1. The sbr gene allele with a male sterility phenotype comprises a 30-bp deletion that does not shift the ORF

The sbr^{12} allele is one of the 9 recessive lethal alleles among 23 known alleles of the *sbr* gene (FlyBase http://flybase.org). Viable males, heterozygous at the sbr^{12} allele, can be obtained by using the duplication *Dp(1;Y)* y^{+} v^{+} (FlyBase ID: FBab0003206). Besides the alleles y^{+} and v^{+} this duplication includes also the sbr^{+} and B^{+} alleles. Heterozygous males sbr^{12}*/Dp(1;Y)* y^{+} v^{+} exhibit a dominant male sterility phenotype. This phenotype is allele-specific because the other

Fig. 1. (A) Intron–exon structure of the *Drosophila melanogaster sbr* gene. Exons that were sequenced in the sbr^{12} allele are indicated. The 30-nt deletion in exon 9 of the sbr^{12} allele with the 5-nt direct terminal repeats (open boxes), that do not allow to indicate the breakpoints of the deletion exactly, is shown. Shown above are the possible variants of the deleted sequence. (B) The structure of *sbr* transcripts with shortened 3′UTR that are identified in the testes: 1 — fully spliced; 2 — starting from alternative promoter in intron 3; 3 — starting from alternative promoter in intron 3, with alternative splicing of the last 237 nt of intron 3.

lethal *sbr* alleles, such as sbr^{0}, sbr^{5}, or sbr^{6} do not cause male sterility in the presence of the duplication $Dp(1;Y)\ y^{+}\ v^{+}$.

To determine what changes in the *sbr* gene sequence distinguish the sbr^{12} allele from the wild type, we amplified and sequenced all coding sequences of the sbr^{12} allele that comprise the following three regions of the gene: exons 1–3, 4–5 and 6–10 (Fig. 1A). Introns 3, 5, the 5′UTR and the 3′UTR were not included in the analysis.

Given that sbr^{12} is a lethal allele, sequencing was performed using DNA from sbr^{12}/sbr^{10} heterozygous females. It was possible to control heterozygosity via known SNPs for the sbr^{10} allele (Korey et al., 2001; Golubkova et al., 2009). As a control, we used genomic DNA isolated from homozygous sbr^{10} females.

In the sbr^{12} allele, we discovered 3 neutral substitutions: C531T in exon 1, G670T in exon 2 and T12491C in exon 7; we also identified 5 substitutions in the introns: C870G in intron 2, A12264C, G12288T, T12292A, C12387T in intron 6; and finally we found the deletion 2del12294 in intron 6. Importantly, the sbr^{12} allele contains a 30-bp deletion in exon 9. It is noteworthy that there are direct 5-nucleotide terminal repeats in the deletion breakpoint region (Fig. 1A). The presence of short direct repeats at the breakpoint suggests a recombination mechanism underlying its origin (Shaffer and Lupski, 2000). This finding is of particular interest given that sbr^{12} mutation was discovered among MR (male recombination)-induced sex-linked recessive lethal mutations (Eeken et al., 1985).

The deletion of 30 bp does not shift the reading frame in the mutant gene sbr^{12}. Thus, in the resulting mutant SBR^{12} protein, 10 amino acids from 526 to 535 (TIFITNATHE) are not present. The first 6 of these amino acids belong to the NTF2-like domain and the rest are part of the linker between the NTF2-like and UBA-like domains (domain layout from the Conserved Domain Database (CDD), NCBI http://www.ncbi.nlm.nih.gov/cdd) (Fig. 2). In spite of the fact that $sbr^{12}/Dp(1;Y)\ y^{+}\ v^{+}$ males possess the normal sbr^{+} allele, presence of the mutant sbr^{12} allele results in a dominant negative effect on male fertility. This suggests that the mutant SBR^{12} protein with deletion prevents normal spermiogenesis and motile sperm formation (Golubkova et al., 2015).

Previously, we have shown that in *D. melanogaster* testes, specific 1.9-kb and 2.8-kb *sbr* transcripts are expressed (Ivankova et al., 2010).

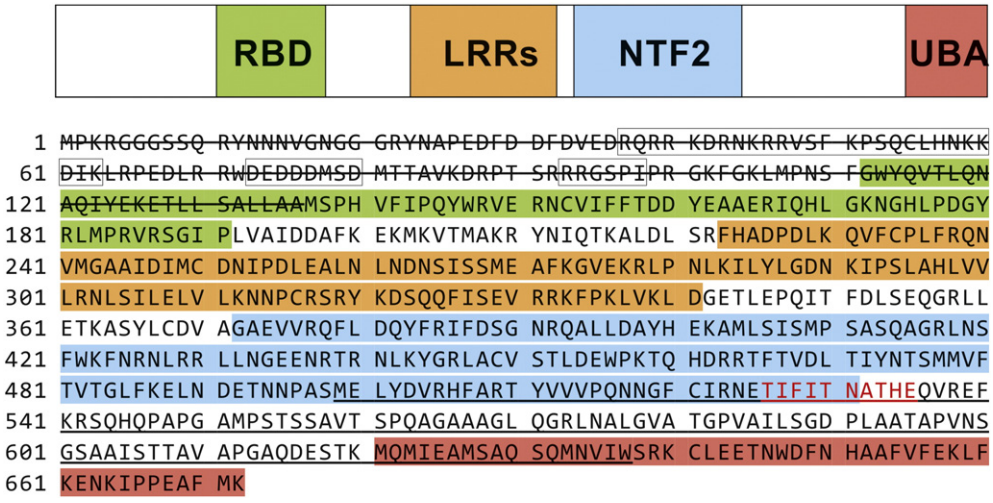

Fig. 2. Domain structure of the SBR protein (according to the Conserved Domain Database (CDD), NCBI http://www.ncbi.nlm.nih.gov/cdd). The sbr^{12} 10-aa deletion (TIFITNATHE) is shown in red. Domains are highlighted with different colors: RBD — RNA-binding domain; LRR — leucine-rich repeats; NTF2-like — nuclear transport factor 2-like; UBA-like — ubiquitin-associated-like. Note: Strikeout amino acids are apparently absent in the short testis-specific SBR protein — "SBR-t" (536 aa). The nuclear localization signal (NLS) is in the box (as it was predicted in Zhang et al., 2011). Underlining indicates the SBR protein fragment used as an antigen in anti-SBR antibody production.

Molecular features of the testis-expressed transcripts have not been described yet.

3.2. Testis-specific transcripts of the sbr gene start from an alternative promoter and are truncated at the 3′ end

To further characterize the *sbr*-originated testis-specific transcripts, we performed RACE-PCR analysis. First of all, we have shown that the short 1.9-kb testis-specific transcript of *Drosophila* males starts in the largest *sbr* intron 3 according to 5′ RACE-PCR. We experimentally demonstrated the presence of short *sbr* transcripts in the *D. melanogaster* testes belonging to two major types (Fig. 3). The transcripts of the first type start in intron 3. The transcripts of the second type also start in intron 3, upstream from the transcripts of the first type (Figs. 1B, 4), but differ from the first type transcripts in that they do not contain the last 237 nt of intron 3, apparently due to alternative splicing.

Identification of the transcription start sites (TSSs) by the CAGE-seq method revealed among the testis-expressed *sbr* gene transcripts starting both in exon 1 and intron 3. In contrast, all head-expressed *sbr* transcripts started only in exon 1 (Fig. 5). In intron 3, TSSs are mapped within 300–212 nt before the exon 4 start, with the major peak at the 244 nt position (9613 nt position in the gene) that does not contradict the RACE-PCR results (Fig. 4).

For the next step, to confirm that in intron 3 there are alternative promoters that are utilized in the testes and that regulate the synthesis of the two transcript types discussed above, we verified the 5′ RACE-PCR results by RT-PCR. We used several primer sets with different forward primers and a common reverse primer (Fig. 6). The reverse primer is located in the adjacent exon, which enables us to verify the normal splicing of intron 4. The PCR product that starts in exon 4 (forward primer #3) could be found both in the heads and testes. The primer sets with the forward primers from intron 3 (##1, 2) only gave a PCR product with template cDNA from the testes. If primer #1 (upstream the 237-nt region, which is removed as an intron in the second-type transcripts) was used, only one PCR product was detected. Its size and sequence confirmed the excision of the 237 nt. When primer #2 (within the 237-nt region) was used, the PCR product corresponded to the first-type transcripts in which the 237-nt region was retained.

It is important to note that the translation frame of the 5′-truncated transcripts was not shifted compared to the main protein, if the translation of the short testis-specific transcripts starts from the AUG codon in exon 4 (Fig. 4). As a result, the 5′-truncated testis-specific SBR protein should lack 136 N-terminal amino acids compared to the full-length protein, including the first 25 aa of the RNA-binding domain. Meanwhile, the beginning of the SBR protein includes nuclear localization signal (NLS) whose location is conservative in evolution (Zhang et al., 2011) (Fig. 2). As a result, the short testis-specific protein apparently cannot fulfill the universal function of nuclear mRNA export, demonstrating specialization features.

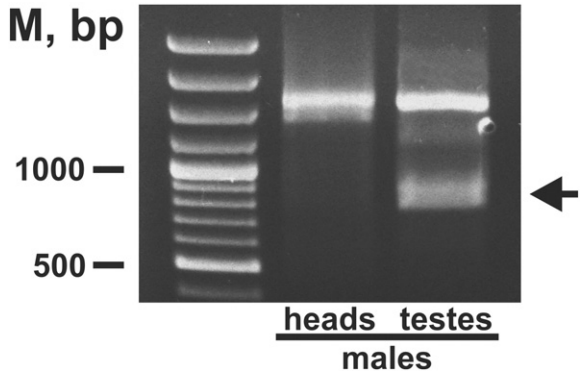

Fig. 3. The results of 5′ RACE-PCR, round III, with samples from *Drosophila* males, performed with the same reverse primer in exons 6 and 7 (ex6-7R). The amplification product in the testes (indicated by an arrow) is shorter than products in the heads that indicate alternative promoter usage in the testes.

3′ RACE-PCR revealed that most of the testis-expressed *sbr* transcripts have truncated 3′UTRs. Such transcripts that varied in size contain only 44–124 nt out of 757 nt typical for the full-length canonical 3′UTR (Figs. 7, 8). Therefore, in the testes, even the fully spliced (normal) transcripts are shorter (2.8 kb) compared with those in other organs (3.5 kb), as we have shown previously (Ivankova et al., 2010). Truncation of 3′ end is common for different mRNAs species in the testes. It has been shown that testis-specific factors are responsible for 3′ end formation in mammals (Dass et al., 2007; Di Giammartino et al., 2011).

3.3. The testis-specific short form of the SBR protein in D. melanogaster

Short testis-specific transcripts of the *sbr* gene, which start in intron 3, provide 5′-truncated mRNA templates for the synthesis of a short SBR protein that is truncated at the N-terminus. This protein should lack the part encoded by exons 1–3 of the full-length SBR protein (Fig. 2).

To detect this protein, we produced antibodies against the C-terminal fragment of the SBR protein (Fig. 2, underlined) and used them for Western blot analysis. The known major form of the SBR protein with a molecular mass of 74 kDa was found in all examined tissue samples from *D. melanogaster* (Fig. 9). Moreover, in addition to this canonical protein (74 kDa), a short 60-kDa form of SBR was found only in the testes of males with different genotypes. Its molecular mass perfectly corresponded to that of the putative protein encoded by short testis-specific transcripts. Moreover, in the heterozygous $sbr^{12}/Dp(1;Y)\ y^+\ v^+$ mutant males that have the sbr^{12} deletion and the sbr^+ allele, the SBR protein (at least its short testis-specific form) was detected as expected in two forms: normal and truncated as a result of deletion (Fig. 9, line 4). Therefore, the anti-SBR antibody developed recognized all forms of the SBR protein, and the deletion of 10 aa in the region used as the antigen did not affect the antibody binding.

4. Discussion

4.1. Alternative polyadenylation of sbr transcripts in the testes

For mRNAs of ubiquitously expressed genes, alternative polyadenylation (APA) is more wide-spread than for tissue-specific genes (Lianoglou et al., 2013). In *Drosophila*, the *sbr* gene, whose main function is nonspecific mRNA export from the nucleus to the cytoplasm, apparently follows this rule. Multiple 3′UTR isoforms are a common characteristic of most house-keeping genes. Various 3′UTRs are capable of gene expression regulation at the post-transcriptional level (Lianoglou et al., 2013). There may be several alternative polyadenylation sites within the 3′UTR of an mRNA that may strongly influence its fate. Target sequences for miRNAs and other regulatory elements that interact with RNA-binding proteins are common in the 3′UTR (Jing et al., 2005; Bartel, 2009; Fabian et al., 2010; Miura et al., 2013; Hollerer et al., 2014). If such sequences are not retained in the mature mRNA as a result of APA, mRNA stability, cytoplasmic localization and/or translation efficiency may be strongly affected (Lewis et al., 1995; Edwalds-Gilbert et al., 1997; Zhao et al., 1999; Moore, 2005; Yan and Marr, 2005; Ji et al., 2011; Di Giammartino et al., 2011; Matoulkova et al., 2012; Hafez et al., 2013; Lianoglou et al., 2013; Tian and Manley, 2013).

In the 3′UTR of *sbr* mRNA, there are numerous major and minor hexanucleotide PASs (polyadenylation sites) that represent potential polyadenylation sites as well as USE (upstream sequence element) motifs before them. Fig. 8 depicts the possible post-transcriptional regulation of this gene via alternative polyadenylation that corresponds to 3′ RACE results (Fig. 7) and shows specific poly(A) sites that may be used in the testes, heads and ovaries. Our experimental data demonstrates truncations in the 3′ end transcripts of *sbr* in the testes comparing with the transcripts from the heads and ovaries. Sequence motifs

```
     TATA                                      *      *      *             *DPE↓      *    ▼              <--
9545 TATATAATGTATATAGTAGAAAGTGATTTAAGTTCAGAGAAGATTTCAGTAAGCTAACTGAATAAAGTCAAATATTATTGCTTACATCTACAGACGTgta
     --237-bp intron start---------------------------------------uORF------------------------uORF---------
9645 cgtgattcttcctatttttagacatttagagctatctctccgttttacacactccattggatgatctatcgcagaaccaacacgaaatatggagcactgc
     --------uORF----uORF--------uORF-------------------------------------------------------------------uORF
9745 tggcaaaacatgagttcatgagttggccgatgagataatttcggtcgctgctatctgtgcaccatataagtttatataataatcgacagacgacgtctaa
     --------------237-bp intron end-->                                                       start-codon
9845 tggtttcccttttttccgcatttttcttttttgcagTTACAAAACGCCCAGATATACGAAAAGGAAACACTCTTGAGTGCTCTATTGGCAGCGATGTCGCCA
```

Fig. 4. Sequence of the *sbr* fragment: from the end of intron 3 to the beginning of exon 4 (in bold). The arrowhead shows start point detected by 5′ RACE-PCR and sequencing of first-type transcripts, without alternative splicing. Asterisks — start points of the second-type transcripts with the excluded last 237-bp of intron 3. Major transcription start site identified by CAGE-seq assembly is marked by the arrow and the last 237 bp of intron 3 are indicated. Regulatory promoter elements are indicated as TATA —TATA-box promoter; DPE — downstream promoter element. The 237-nt intron and upstream ORF start codons (uORFs) in the 5′UTR are marked as possible control translation efficiency elements.

potentially important for APA, such as poly(U)-tract, USE, PAS, and DSE (downstream sequence element) motifs, are found in certain places in the 3′UTR of the *sbr* mRNA (Fig. 8).

It is noteworthy that *sbr* transcripts detected in the testes, both full-length and shorter transcripts, had truncated 3′ termini. At the same time, polyadenylation, which is typical for testes RNA, was not found in the canonical full-size *sbr* transcripts isolated from the heads or ovaries of the flies (Fig. 7). In the case of *sbr* gene expression, tissue specificity is not limited by the formation of an alternative 3′UTR. Another cause of *sbr* transcript variability is alternative transcription start sites. Alternative promoters are a source of short testis-specific transcripts corresponding to short testis-specific SBR proteins.

4.2. Short transcripts starting within intron 3 give rise to the testis-specific SBR protein

Testis-specific promoters in intron 3 of the *sbr* gene do not have one fixed transcription start site (TSS) (Figs. 4, 5). Such promoters are designated as “broad”, “weak” or “wide” (Atkinson and Halfon, 2014). They are common both in mammals (Carninci et al., 2006; Juven-Gershon et al., 2008; Juven-Gershon and Kadonaga, 2010) and in *Drosophila* (Rach et al., 2009; Hoskins et al., 2011).

In mammalian genomes, there are different types of promoters: TATA and CpG-rich (Carninci et al., 2006). In the *Drosophila* genome, there are promoters containing the TATA-box — TATAWAAR (~30% of promoters), as well as the Initiator (Inr) element TCAKTY (~65% of promoters, functioning either together with the TATA-box or independently) and Downstream Promoter Elements (DPEs) RGWYV and others (Ohler et al., 2002; Gershenzon et al., 2006). The majority of these elements are present in region containing intron 3 of the *sbr* gene (Fig. 4). Further studies are needed to answer questions regarding the specific significance of these elements for transcription efficiency in the testes.

Both the 3′UTR and 5′UTR are important for the fate of a transcript. Either an upstream ORF (uORF) (Medenbach et al., 2011) or an intron (Bicknell et al., 2012) in the 5′UTR are the factors that control translation efficiency in mammals. Exclusion or retention of the 237-bp intron may, therefore, regulate the translation of testis-specific transcripts.

The existence of several TSSs does not change the protein-coding portion of the shortened *sbr* transcripts. All of the described *sbr* transcripts are in principle capable of encoding the same protein — the testis-specific shortened SBR isoform, designated “SBR-t”. In comparison with full-size SBR protein, SBR-t lacks the N-terminal portion with a nuclear localization signal (NLS) (Fig. 2). The absence of the NLS may restrict transport of the SBR-t into the nucleus. SBR-t also lacks a portion of the RNA-binding domain. As a result, the shorter, testis-specific SBR-t protein cannot fulfill the main canonical function of the full-size SBR protein and, thus, may possess certain other functions outside the nucleus, in the cytoplasm. Cytoplasmic localization is a characteristic of the mammalian NXF family proteins, expressed in the testes (Herold et al., 2000; Sasaki et al., 2005; Tan et al., 2005). We also showed

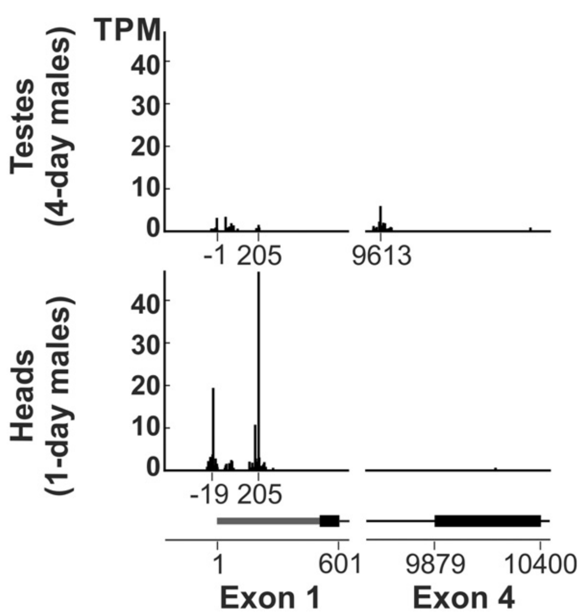

Fig. 5. The transcript start points of the *sbr* gene in the testes and heads of *D. melanogaster* males, according to the analysis of the CAGE-seq data. Total *sbr* expression in the *Drosophila* heads is higher than in the testes, as it is usually observed. The start points for testis-specific transcripts are localized both in exon 1 and intron 3 of the *sbr* gene; for “heads” samples it is located only in exon 1. TPM (tags per million reads) values were calculated for each mapping position in exon 1 and intron 3 of the *sbr* gene. The localization of the major peaks and *sbr* gene (gene ID: 43944) nucleotide numbering is indicated.

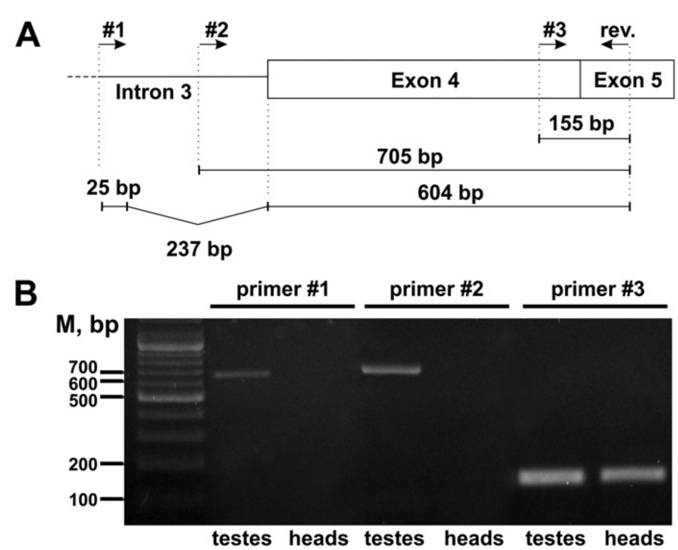

Fig. 6. Scheme (A) and results (B) of RT-PCR with cDNA from the testes and heads of adult *D. melanogaster* males. There is one common reverse primer in exon 5 (rev.) and three forward primers in intron 3 (##1, 2) and in exon 4 (#3). In cases where the forward primer is mapped in intron 3, the RT-PCR product is found in the testes only. The length of the RT-PCR product from primer #1 verifies the 237-bp intron excision from the transcripts of the second type that begin in intron 3 and do not contain the last 237 bp of the intron due to alternative splicing. The bigger size of the RT-PCR product from primer #2 is the result of retention of the 237-bp intron.

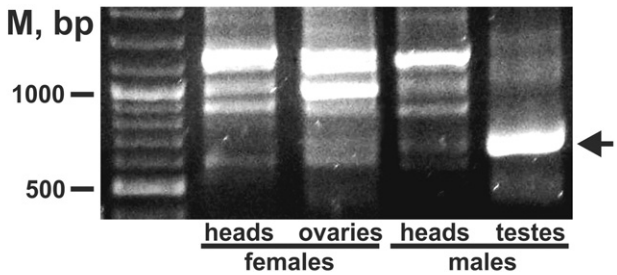

Fig. 7. The results of 3′ RACE-PCR, round II, with different organ samples, performed with the same forward primer in exon 9 (ex9F). The major PCR amplification product in the testes (indicated by an arrow) is shorter than products in the heads or ovaries.

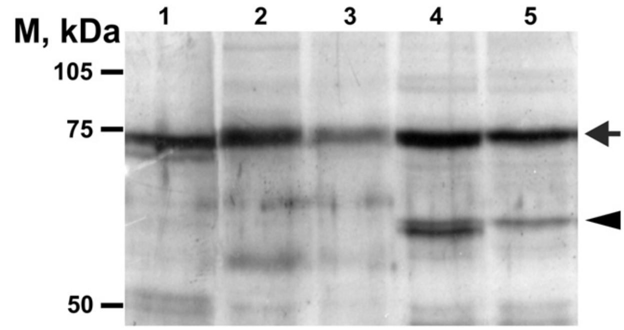

Fig. 9. Western blot analysis of the proteins isolated from the organs of adult *D. melanogaster* carrying different *sbr* alleles. The arrow indicates the universal canonical SBR protein, and the arrowhead indicates the short testis-specific protein SBR-t. Double band is evident in the testes of heterozygous *sbr*^{12}-bearing males (lane 4). 1 – ovaries of *Oregon-R* females 2 – heads of *sbr*^{12}/*Dp(1;Y) y*$^{+}$ *v*$^{+}$ males 3 – heads of *FM6v, y sc8 dm B*/*Dp(1;Y) y*$^{+}$ *v*$^{+}$ males (without *sbr*^{12} deletion)4 – testes of *sbr*^{12}/*Dp(1;Y) y*$^{+}$ *v*$^{+}$ males 5 – testes of *FM6v, y sc8 dm B*/*Dp(1;Y) y*$^{+}$ *v*$^{+}$ males (without *sbr*^{12} deletion)

the localization of SBR protein in the cytoplasm in some stages of spermatogenesis (Atsapkina et al., 2010; Golubkova et al., 2015). Unfortunately, the antibody described in this paper does not allow distinguishing SBR and SBR-t proteins.

4.3. Sterile *sbr*^{12}-heterozygous males of *D. melanogaster* do not form motile sperm

NXF proteins function as large macromolecule complexes based on their structure. Thus, the LRR domain is responsible for protein–protein interactions (Liker et al., 2000), and the NTF2-like domain determines binding with its permanent partner protein p15, often termed "NXT1" (Bachi et al., 2000). The deletion present in the *sbr*^{12} allele affects the NTF2-like domain (Fig. 2) and causes disturbances in spermatogenesis. Heterozygous *sbr*^{12} males are sterile and do not have motile spermatozoa in the testes (Golubkova et al., 2015). These defects are allele-specific and dominant; even normal expression of the SBR protein (one dose) in the same cell with the mutant protein does not result in the formation of functional sperm. Thus, a question arises as to why the NTF2-like domain is so important for the completion of spermatogenesis. To begin with, it was noted that if there is a defect in NXT1 (the permanent partner of NXF1), many different testis-specific mRNAs no longer accumulate (Caporilli et al., 2013). Then, it was recently found that the surface of the NTF2-like domain is also important for interaction of the export receptor with mRNA (Katahira et al., 2015). Therefore, *sbr*^{12} deletion may affect NXF1-NXT1 dimer formation and structure, changing its affinity to specific target mRNAs.

Furthermore, the NTF2-like and UBA-like domains of the NXF1 proteins are responsible for interactions with nuclear pore complexes (NPCs) (Braun et al., 2001). Previously, using antibodies specific to the C-terminal fragment of the SBR protein, we showed that SBR is localized on the convex side of the crescent-shaped sperm nucleus during the individualization of spermatids (Atsapkina et al., 2010). NPCs focus on the same side of the nuclear envelope during the individualization of spermatids (Fuller, 1993; Fabian and Brill, 2012). The nuclear envelope is the most convenient compartment for the localization of temporally untranslated mRNAs whose products are necessary for spermiogenesis.

Thus, we can hypothesize that specialized testis-specific function of the *sbr* gene is important for the mRNA biogenesis that is necessary for the formation of motile spermatozoa. Further investigation is needed to dissect the molecular functions of this testis-specific protein and address questions concerning its mRNA targets.

5. Conclusions

Apart from its universal function, i.e., mRNA export from the nucleus to cytoplasm, the *sbr* (*Dm nxf1*) gene in *D. melanogaster* has testis-specific functions. Thus, we demonstrated that there are testis-specific alternative transcripts of this gene and a shortened SBR protein (60 kDa) that is found only in the testes. The described testis-specific transcripts result from alternative splicing and differ in their transcription start sites. We sequenced the *sbr*^{12} allele of the *Dm nxf1* gene and found a 30-nt deletion in this allele that does not shift the ORF. Given that the *sbr*^{12} allele exhibits dominant male sterility, it was concluded that this deletion, which results in the absence of 10 aa, is responsible for this phenotype. It was also shown that the first 6 aa of the deletion belong to the NTF2-like domain, which likely leads to the functional disability of mutant proteins to provide sperm motility.

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                         minPAS                                          *               poly(U)-tract
13561 CCGCCUGAGGCUUUUAUGAAGUAAAUCGCAUAGGAGUUUCCGUAGGACAGAGCCGCGUGCCACAUCCACAUAAUCGAAUGCUGUUUUUUUUUUUUUGGUU
       USE majPAS        ***   *                     *                          DSE
13661 UUGUAAUUAAAUUUUAAAAUUAUUAGAGAAACCUCUAUAUAAUAAUAAUAAUUAAUAUUAUUAAGCUGCGAAGUUGUGUGCACAUUCGGGCAGUAGCAAU

13761 UAUUAUCCCAGCACUGCGGGCAAUGUGCAUCAACGAUCACAGUUCUUCGAUAGAUUAGUUUAGCUCUCUUUAAGUUCCGUCCGCAGAUCCGCUGGUCUAC

13861 AUUGAGGCCAGGACGAGUCUGCGAACGGUAGUCCCUUUAGAGUUAAAGUUGUUUUAGAUUCCUAAGCCAAACACCUUCAAACACACACAACACGACAACA
        majPAS
13961 CUAUUAAACGUAAUGCAACAAUGUUGUCGAAUCGAAAGAAACCCAAUUUUUAUUUUAAUAUAUACGACGAUACCGAAACCAAGGCGAUGGUCGAAAAGCA
                         USE                                       USE            majPAS
14061 UGGAUCGCGCCAAUAAUUCUAUAUUCCCCGCUUUCCCAGAUCCUCGACUCGCCUUACAUUUUGUAUACAAAUACCAUAGAAUAAAAAGAAACAUUUUGAC
         USE        USE
14161 CACUGUAAAAAUUUUGUAACGCUCGGAAACCAAAAUUACCUUUAUUUCUUAAUAGAAAAAAAUUAUUCGAUUUACAAUACACGCUUAGCCGUAAAUUCAA
                 USE      minPAS                       USE         majPAS
14261 UUGAAUUUAAGUGUAAGAUAUAUAAAAAUUAUAUACAUUAAGACAAAAGUGUAGAUUCAUAAAUAAAUUUUUCAGAAUAGAAU
```

Fig. 8. Sequence of the *sbr* 3′UTR. The end of the coding region is shown in bold. Asterisks mark the ends of transcripts detected by 3′ RACE-PCR. Important polyadenylation elements are depicted as poly(U)-tract, USE — upstream sequence element, majPAS — major polyadenylation site, minPAS — minor polyadenylation site, DSE — downstream sequence element.

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