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Three levels of regulation lead to protamine and Mst77F expression in *Drosophila*

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Abstract

Differentiation from a haploid round spermatid to a highly streamlined, motile sperm requires temporal and spatial regulation of the expression of numerous proteins. One form of regulation is the storage of translationally repressed mRNAs. In *Drosophila* spermatocytes, the transcription of many of these translationally delayed mRNAs during spermiogenesis is in turn directly or indirectly regulated by testis-specific homologs of TATA-box-binding-protein-associated factors (tTAFs). Here we present evidence that expression of Mst77F, which is a specialized linker histone-like component of sperm chromatin, and of protamine B (ProtB), which contributes to formation of condensed sperm chromatin, is regulated at three levels. Transcription of *Mst77F* is guided by a short, promoter-proximal region, while expression of the Mst77F protein is regulated at two levels, early by translational repression via sequences mainly in the 5' part of the ORF and later by either protein stabilization or translational activation, dependent on sequences in the ORF. The *protB* gene is a direct target of tTAFs, with very short upstream regulatory regions of *protB* (–105 to +94 bp) sufficient for both cell-type-specific transcription and repression of translation in spermatocytes. In addition, efficient accumulation of the ProtB protein in late elongating spermatids depends on sequences in the ORF. We present evidence that spermatocytes provide the transacting mechanisms for translational repression of these mRNAs, while spermatids contain the machinery to activate or stabilize protamine accumulation for sperm chromatin components. Thus, the proper spatiotemporal expression pattern of major sperm chromatin components depends on cell-type-specific mechanisms of transcriptional and translational control.

Keywords

tTAF target genes; Protamine; Mst77F; Transcriptional regulation; Translational repression; Translational activation

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Introduction

The development of functional sperm requires stage- and cell-type-specific activation of numerous genes involved in the regulation of meiotic divisions, formation of the spermatid acrosome and flagellum, and extreme nuclear condensation. Developing male germ cells in both *Drosophila* and mammals store many translationally repressed mRNAs for later spermatid morphogenesis (Renkawitz-Pohl et al., 2005; Sassone-Corsi, 2002). In *Drosophila*, only a few genes are transcribed after the meiotic divisions (Barreau et al., 2008; Vibranovski et al., 2010), and therefore spermatid morphogenesis largely depends on stored, often translationally repressed mRNAs transcribed in spermatocytes (Fuller, 1993; Jayaramaiah Raja and Renkawitz-Pohl, 2005) though it cannot be excluded that stable proteins persist or mRNAs are translated before and after meiosis such as β 2-tubulin (Michiels et al., 1993). *In-situ* hybridization experiments on adult testes indicate that at least 2100 genes are specifically activated in spermatocytes, and the majority of these transcripts persists during spermiogenesis (Zhao et al., 2010).

A striking example of such stored transcripts arises from a common feature of mammalian and *Drosophila* spermatid differentiation—the replacement of histones by protamines, which results in highly compact chromatin. The protamine-like proteins Protamine A (Mst35Ba, ProtA) and Protamine B (Mst35Bb, ProtB) and the linker histone-like protein Mst77F are major chromatin components of mature sperm in *Drosophila*. The corresponding mRNAs are transcribed in young spermatocytes but are translated only several days later, during late spermatid elongation stages (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2007). Live imaging of testes and single cysts from flies carrying a *protB-eGFP* transgene revealed that accumulation of ProtB takes place 50–60 h after meiosis. Thus, *protB* mRNAs are stored in an untranslated state up to 6 days (Awe and Renkawitz-Pohl, 2010). Likewise, the sperm chromatin protein Mst77F is expressed in late spermatids from mRNAs transcribed in spermatocytes and stored. Mst77F genetically interacts with β 2-tubulin, which indicates a further function of Mst77F (Fuller et al., 1989; Jayaramaiah Raja and Renkawitz-Pohl, 2005; Tweedie et al., 2009). Indeed, the Mst77F protein colocalizes with microtubules during nuclear shaping, for which it is essential (Jayaramaiah Raja and Renkawitz-Pohl, 2005; Rathke et al., 2010).

In *Drosophila* and in mammals, “non-prototypical” homologs of subunits of core promoter recognition complexes have been shown to guide cell-type-specific transcription (Goodrich and Tjian, 2010). In *Drosophila* spermatocytes, testis-specific homologs of TATA-box-binding-protein-associated factors (tTAFs) regulate the expression of many spermatid differentiation genes. Often these genes are transcribed solely in spermatocytes. Five such tTAFs have been characterized and proposed to act in a complex: Cannonball (Can; dTAF5 homolog), No hitter (Nht; dTAF4 homolog), Meiotic arrest (Mia; dTAF6 homolog), Spermatocyte arrest (Sa; dTAF8 homolog), and Ryan express (Rye; dTAF12 homolog) (Hiller et al., 2004, 2001). Germ cells from male flies mutant for any of these tTAF genes do not enter meiotic division and spermiogenesis. Although the cells mature spermatocytes, they fail to express the high levels of many spermiogenesis-relevant genes of normal spermatocytes (Hempel et al., 2006; Hiller et al., 2004; White-Cooper et al., 1998), which suggests that tTAFs directly and/or indirectly control robust transcription of many genes

required for spermatid morphogenesis. Of these spermiogenesis-relevant, tTAF-dependent genes, so far only the promoters of *fzo*, *Mst87F*, and *dj* are known to be bound by tTAFs (Chen et al., 2005, 2011). The transcripts of each of these genes are spatiotemporally translated in a distinct, controlled manner. *fzo* encodes a GTPase relevant for mitochondrial morphogenesis (Hales and Fuller, 1997), *Mst87F* encodes an outer-dense fiber protein component of the sperm tail (Kuhn et al., 1988), and *dj* encodes a histone-like protein that is both a transient nuclear component and a persisting component of mitochondrial derivatives of the sperm tail (Santel et al., 1998). Whether other genes are under direct control of tTAFs remains to be investigated, especially as it has been proposed that the function of tTAFs partly counteracts Polycomb to allow transcription of spermiogenesis-relevant genes (Chen et al., 2005, 2011).

Here, we identified the sperm chromatin component ProtB as a direct transcriptional target of the tTAFs. We provide evidence that ProtB and *Mst77F* expression is regulated at three levels: transcription by tTAFs, translational repression, and selective activation of translation and/or protein accumulation in late spermatids dependent on the specific ORF.

Materials and methods

Fly strains and culture

Drosophila melanogaster strains were maintained on standard medium at 25 °C. *w¹* (Klemenz et al., 1987; Michiels et al., 1993) was used as *wild-type* unless otherwise specified. *can¹²* mutants (Hiller et al., 2001) were used for chromatin immunoprecipitation (ChIP) and *in-situ* hybridization assays.

Cloning of promoter lacZ reporter constructs

Promoter *lacZ* reporter constructs were generated by PCR using genomic DNA from *wild-type* flies and appropriate primers with linked *EcoRI* and *BamHI* restriction sites. The PCR products were inserted into the transformation vector *pChab sal*, which supplies the 3' UTR from SV40 (Thummel et al., 1988). Transgenic *Drosophila* lines were established by injection of DNA as described by Michiels et al. (1993). For *protB*, three promoter *lacZ* constructs were generated: *pc-protB1* (−329 to +135, and the first intron in addition), *pc-protB2 Int1* (−105 to +94), and *pc-protB2 Int1+ Ex1* (−105 to +4). For *Mst77F*, the promoter *lacZ* constructs *pc-77F1* (−278 to +172), *pc-77F3* (−89 to +172), and *pc-77F3 5'UTR* (−89 to +38) were generated.

Cloning and monitoring expression of ProtamineB-eGFP under the control of the UAS–GAL4 system and the β2-tubulin (Tub85B) control region

For expression under the control of the UAS–GAL4 system, the complete ORF of *protB* fused in frame C-terminal to *eGFP* was cloned into the transformation vector *pUASl* (Brand and Perrimon, 1993), which contains the basic promoter and the 5' UTR of the *hsp70* gene and the 3' UTR of SV40 with a polyadenylation signal. Transgenic fly lines were established in a *w¹* background. The expression of *UAS-ProtB-eGFP* in male germ cells was driven by *bam-GAL4* (Chen and McKearin, 2003).

To promote the expression of ProtB under the control of $\beta 2$ -tubulin ($\beta 2t$), a construct containing the regulatory region of $\beta 2t$ (from 677 bp upstream of the ATG start codon) and the ORF of *protB* (all but the stop codon) with a C-terminal *eGFP* was cloned into the transformation vector *pChab sal lacZ*, which provides the SV40 3' UTR. The presence of the fusion gene in transgenic lines was verified by PCR using the primers *EcoRI- $\beta 2t$ -Prsen* (5' GATGAATTCTCATTGTAGGAGCCAGAG 3') and *ProtB-BamHI-as* (5' GATGGATCCCTTGCAAATCCG 3'). As a control, the same $\beta 2t$ regulatory region was fused to *eGFP* and cloned into *pChab sal lacZ*, which provides the SV40 3' UTR. At least two independent lines for *protB* constructs were analyzed and revealed the same expression pattern.

Adult testes were dissected, and then the eGFP signals were examined using a Zeiss microscope (AxioPlan2) equipped with appropriate fluorescence filters. Images were individually recorded and figures arranged with Adobe Photoshop CS2/CS5. Schemes designed with Adobe Illustrator CS5.

Cloning and monitoring expression of Mst77F-eGFP or Mst77F-mCherry and C-terminal truncated and Mst77F full-length under the control of the $\beta 2$ -tubulin (Tub85B) regulatory region

For expression of Mst77F under the control of the $\beta 2$ -tubulin ($\beta 2t$) promoter and the 5' UTR (Santel et al., 2000) a construct containing the regulatory region of $\beta 2t$ (from 677 bp upstream of the ATG) and the ORF of *Mst77F* (all but the stop codon) was cloned into the transformation vector *pPWG* (see The Drosophila Gateway (TM) Vector Collection), which resulted in a C-terminal in-frame fusion to eGFP. The 3' UTR and polyadenylation signal were from KC10.

To generate C-terminal truncated *Mst77F* constructs, sequences from -448 to +712 (*Mst77F 60C-eGFP*) or -450 to +394 (*Mst77F 141C-mCherry*) were cloned in frame C-terminal to eGFP or mCherry in *pChab sal lacZ* containing the SV40 3' UTR and polyadenylation signal.

Transgenic fly lines were established in a *w¹¹¹⁸* background. As *pChab sal lacZ* integrates randomly into the genome, three to five independent transgenic lines were analyzed for each construct, and all lines of each construct had the same expression pattern.

Antibodies and immunofluorescence staining

Hoechst staining was used to visualize chromatin. All antibodies were used in immunofluorescence stainings of squashed testis carried out essentially as described in Hime et al. (1996) and Rathke et al. (2007). To follow the fate of the histones, an anti-histone antibody from Chemicon, Millipore (MAB052; 1:1200) was used. This antibody recognizes H1 and all core histones.

Cy5-conjugated anti-mouse (Dianova; 1:100) was used as secondary antibody. Immunofluorescence, eGFP and Hoechst signals were examined using a Zeiss microscope (AxioPlan2) equipped with appropriate fluorescence filters. Images were individually

recorded and figures arranged with Adobe Photoshop CS2/CS5. Schemes designed with Adobe Illustrator CS5.

In-situ hybridization and β -Galactosidase assay

Whole-mounts of adult testes were hybridized *in-situ* according to Morris et al. (2009) with minor modifications such as pre-hybridization, hybridization and washes in hybridization buffer at 55 °C instead of 65 °C. DIG-labeled RNA probes were generated using 500–800 bp fragments of the corresponding ORFs amplified by PCR from genomic DNA and cloned into the *pCR[®]II-TOPO[®]* vector (Invitrogen). β -Galactosidase activity was visualized by a histochemical reaction using the chromogenic substrate X-Gal. Adult testes were fixed in 0.7% glutaraldehyde at room temperature for 15 min, and larval testes were fixed for 5 min. The enzymatic staining reaction was incubated at 37 °C for 10 min (adult testis), 30 min or overnight (ovn, larval testis). After a 30-min time interval the *w¹* or *w¹¹¹⁸* host strains show no background activity.

Chromatin immunoprecipitation and quantitative PCR analyses

Chromatin immunoprecipitation (ChIP) and quantitative PCR analyses were performed as previously described in Chen et al. (2005) using anti-Sa antibody. Each ChIP assay contained chromatin of 50 pairs of testes dissected from either *w¹¹¹⁸* or homozygous *can¹²*-mutant flies. Input DNA and DNA immunoprecipitated with specific antibodies (ChIP-DNA) were analyzed by real-time PCR using the gene-specific primers as listed below and TaqMan probes with the Universal PCR Master Mix (Applied Biosystems Inc. #58003365-01) in an ABI 7300 Real-time PCR system. Each PCR reaction was performed in duplicate, and the Ct numbers for each reaction were collected and averaged. The amount of PCR product was quantified using the absolute quantification method with a standard curve. At least three independent ChIP reactions, each from an independent biological replicate, were performed for each data point. *CycA* was used as an internal control (Chen et al., 2011). The ChIP DNA was first normalized with the input DNA amount (Input %), and then the raw input % data were converted to data expressing the fold change over the constitutively expressed *CycA* gene from the same sample. The relative enrichment normalized to *CycA* was then averaged, and the two different genotypes, *wt* and *can¹²*, were compared. The gene-specific primers used for PCR were as follows: *protB*: Forward, CAAAACCTACGCCAACTATATGGAATAA; Reverse, CGTAACCACCTAACAGAAGGATGT; and FAM, TATCCGCC;GGCGTCTATCCGCCGGCGTC; *Mst77F*: Forward, AGCTAGTCGGATTGCAAACAGAATAT; Reverse, TTTCTTGATCAGTATCAGTCGAGCTG; and FAM, ACGGACGGACGAATAT; *Mst87F*: Forward. GTCAAACCGATATACCTGTGCGTAA; Reverse, ATGTGTTTCAGGCCGAAAGGA; and FAM, CCAGATTTTGTATCATTATTATTG; and *CycA*: Forward, CAACAGCAAGAAGGCAACGA; Reverse, GAGTCCGATTATGCTCTGCTCTT; and FAM, CCCTTCCTTCTCTCTTTCTC.

Results

Transcription of *protB* and *Mst77F* in primary spermatocytes requires tTAFs

tTAF function was required for normal expression of *protB* and *Mst77F* transcripts in spermatocytes. *In-situ* hybridization to *wild-type* testes using antisense RNA probes for *protB* and *Mst77F* detected transcripts in spermatocytes (Fig. 1A, C, arrows) and in elongating spermatid stages, consistent with data of Jayaramaiah Raja and Renkawitz-Pohl (2005). *In-situ* hybridization to *can¹²*-mutant testis showed dramatic reduction in *protB* transcripts at all spermatocyte stages (Fig. 1B) in comparison to *wild-type* testis (Fig. 1A). Also the level of transcripts hybridizing with the *Mst77F* probe was dramatically reduced in the spermatocyte region of the *can¹²* mutant testis (Fig. 1D, arrow), although some signal cross hybridizing with the *Mst77F* probe was still detected (Fig. 1D, arrowhead), which contains late-stage arrested spermatocytes and/or dying cysts in the mutant testis. Consistent with the *in-situ* hybridization data, a comparison of transcript levels in *wild-type* versus tTAF (*sa^{-/-}*) mutant testes from microarray data of Chen et al. (2011) indicated that the level of *protB* transcripts was 12-fold lower and the level of *Mst77F* transcripts was over 4-fold lower in the tTAF (*sa^{-/-}*) mutant than in *wild-type* testes (Fig. 1E).

The reduction in *Mst77F* transcript levels in tTAF mutants compared to the *wild-type* observed by *in-situ* hybridization and transcriptome analysis using microarray data may underrepresent the actual effects of the tTAF (*sa^{-/-}*) mutation on *Mst77F* transcription. Both the *in-situ* probe for *Mst77F* and the microarray assay likely detected signal from transcripts of the 18 *Mst77F* pseudogenes (*Mst77Y-1* to *Mst77Y-18*) found on the Y chromosome in *Drosophila melanogaster*, in addition to the signal from transcripts of the autosomal *Mst77F* for which all our reporter constructs and fusion genes were designed. Compared to *Mst77F*, these Y-linked, possibly functional *Mst77* copies have an additional 12 bp within their 5' UTRs (Krsticevic et al., 2010; Russell and Kaiser, 1993). According to Krsticevic et al. (2010), 72% of the *Mst77F*-like mRNA expressed in *wild-type* testes arises from the autosomal *Mst77F* gene, while the remaining 28% arises from the *Mst77Y* genes. If this significant contribution of *Mst77Y* transcripts is independent of TAFs, it may account for both the only modest 4-fold apparent reduction (25% remaining transcripts) in *Mst77F* transcripts observed in the tTAF-mutant testes by microarray (Fig. 1E) as well as the transcripts detected by *in-situ* hybridization in the basal region of tTAF-mutant testes (Fig. 1D).

Chromatin immunoprecipitation (ChIP) assays indicated that *protB* might be a direct target of the tTAF Sa (Fig. 1F). Since the level of Sa protein does not change in *can¹²*-mutant testes (Chen et al., 2005), the occupancy of the tTAF Sa at promoters of target genes in testes from flies with a tTAF *can* mutation can be analyzed by ChIP using antibodies against Sa. In ChIP assays with anti-Sa, the genomic region containing the *protB* promoter in *wild-type* testes was enriched ~3-fold more than in *can¹²*-mutant testes (Fig. 1F). This compares favorably with a positive control tested in the same experiment, the *Mst87F* gene promoter (Chen et al., 2005), which was enriched 4.1-fold more in *wild-type* than in *can¹²* mutant testes. The *Mst77F* locus in *wild-type* testes was enriched only modestly, i.e., ~1.8-fold more than in *can¹²* mutant testes (Fig. 1F) in the same experiment.

Short upstream sequences regulate cell-type-specific expression of *ProtB* and *Mst77F*

Deletion analyses of the region proximal to the transcription start site of *protB* and of *Mst77F* demonstrated that a very short upstream region was sufficient to ensure transcription of each gene in male germ cells. Our previous studies with *protB-eGFP* gene fusions indicated that a genomic fragment from 1683 bp upstream to the ATG encoding the translational start, i.e., a fragment containing the 5' UTR and 655 bp upstream of the predicted transcriptional start site, was sufficient for a high level of cell-type- and stage-specific ProtB-eGFP expression (Jayaramaiah Raja and Renkawitz-Pohl, 2005). Similarly, studies with *Mst77F-eGFP* gene fusions indicated that a genomic fragment from 450 bp upstream to the ATG, i.e., a fragment containing the 5' UTR plus 278 bp upstream of the predicted transcriptional start site, was sufficient (Jayaramaiah Raja and Renkawitz-Pohl, 2005). Further deletion analysis using transgenes in which regulatory regions drove expression of β -Galactosidase (summarized in Table 1) revealed that a genomic fragment containing sequences from 105 bp upstream of the predicted transcriptional start site of *protB* plus the first 94 bp of the 5' UTR region (*pc-protB2 Int1*) was sufficient for β -Galactosidase expression in male germ cells (Fig. 2A, C). β -Galactosidase activity appeared only in elongated spermatid bundles (Fig. 2C; double arrowhead), whereas early haploid spermatids and younger stages revealed no β -Galactosidase activity (Fig. 2C; white arrow and arrowhead). The level of β -Galactosidase expression from *pc-protB2 Int1* was comparable to that from the full-length promoter construct *pc-protB1* (Fig. 2B, C; testes were incubated in parallel under the same conditions). Likewise, deletion analyses of similar reporter constructs for *Mst77F* (Fig. 2D) revealed that a region including from 89 bp upstream of the predicted transcriptional start site plus the 172 bp 5' UTR was sufficient to drive strong expression of β -Galactosidase from a reporter transgene (*pc-77F3*) in spermatids, similar to a full-length promoter-*lacZ* construct (*pc-77F1*; Fig. 2E, F).

Further deletion of sequences encoding parts of the 5' UTR of *protB* and *Mst77F* (bottom constructs in Fig. 2A, D) substantially reduced the level of reporter expression in spermatids (Fig. 3 respectively). Although the *pc-protB2 Int1+ Ex1* and *pc-77F3 5'UTR* reporters had the same regulatory regions upstream of the predicted transcriptional start sites as *pc-protB2 Int1* and *pc-77F3*, respectively, the testes from flies bearing the *pc-protB2 Int1+ Ex1* and *pc-77F3 5'UTR* constructs (Fig. 3C, G) showed much less β -Galactosidase activity in spermatids than testes from flies bearing the larger reporter constructs incubated in parallel under the same conditions (Fig. 3A, E). It is possible that the further 5' UTR deletions might have removed or damaged part of the core promoter, an enhancer, or a transcript-stability-mediating element. Alternatively the efficiency of the translation might be reduced. *In-situ* hybridization with a *lacZ* probe (same probe, same time of staining reaction in parallel) revealed that the constructs with the complete 5' UTR, having a high β -Galactosidase activity (Fig. 3A, E) also had high transcript level (Fig. 3A, F). The constructs from which a substantial segment of the 5' UTR had been deleted on the other hand had a low β -Galactosidase activity (Fig. 3C, G) and a low *lacZ* transcript levels (Fig. 3D, H). These data argue against a role of the 5' UTR in translational efficiency and instead favor a role of these sequences in the regulation of the transcript levels of both *Mst77F* and *protB*. In the presence of the 5' UTR the *lacZ* transcripts accumulate in spermatocyte stages (Fig. 3B, F), which is apparently not the case if substantial segments of

the 5' UTR are deleted (Fig. 3D, H). The transcript level in spermatocytes appears less effected in *Mst77F* constructs than in Protamine constructs. This suggests that the 5' UTR of *Mst77F* mRNA mainly affects transcript stability while the 5' UTR of *protamines* mRNA effects transcript level in spermatocytes and transcript stability in spermatids.

The 5' UTR of *protB* mRNA contains sequences that mediate efficient translational repression in spermatocytes

Comparison of transcripts detected by *in-situ* hybridization, which were visible in both spermatocytes and spermatids (Fig. 1A, C), with the expression of β -Galactosidase from the reporter constructs, visible only in elongating spermatid bundles (Fig. 2B, C, E, F; double arrowheads), raised the possibility that translational control elements (TCEs) or translational repression elements (TREs) in the UTRs might limit protein expression in spermatocytes. For all the reporter constructs of *protB* and *Mst77F* (Fig. 2A, D) and of fusion genes (Jayaramaiah Raja and Renkawitz-Pohl, 2005) tested so far, the 3' UTR (including the polyadenylation site) was derived from SV40, making the 5' UTR rather than the 3' UTR of *protB* and *Mst77F* a likely candidate for the localization of TCEs active in spermatocytes. Because in adult testes cytoplasmic β -Galactosidase activity from the reporters in elongating spermatids was already very strong after 10 min of reaction (Fig. 3A, D) and spermatid flagella extend apically far into the spermatocyte region, it was difficult to evaluate whether spermatocytes contain some β -Galactosidase activity. To focus on levels of reporter expression in spermatocytes, we analyzed larval testes lacking spermatids to address translational regulation.

Deletion analysis revealed that sequences within the first 94 bases of the 5' UTR of the *protB* locus were required for efficient translational repression of reporter constructs in spermatocytes (Fig. 4). To assay for premature expression of the reporter, testes of third-instar larvae, which contain stages of germ cell differentiation up to onset of the meiotic divisions but normally lack spermatids, were analyzed to focus on expression in spermatocytes. β -Galactosidase reactions were incubated for either 0.5 h or ovn in a single experiment (Fig. 4) to allow different levels to be compared within the linear range of the assay. In reporter constructs containing the intact *protB* 5' UTR (Fig. 4A, C), β -Galactosidase activity was not detected in the spermatocytes in larval testes after 0.5 h of staining, similar to larval testes carrying the positive control, a reporter construct *dj-like* +TRE (Fig. 4M), which we had previously shown mediates translational repression in spermatocytes (Hempel et al., 2006). In contrast we found larval testes incubated from flies bearing the construct *pc-protB2 Int1+ Ex1*, *i.e.*, having a 90-bp deletion within the 5' UTR, stained for 0.5 h of incubation were positive for β -Galactosidase activity in primary spermatocytes (Fig. 4E, F). Together these findings indicate that a region of the 5' UTR of *protB* is required for effective translational repression of the mRNA in spermatocytes. We note that significant β -Galactosidase activity was detected in reporter constructs containing the intact *protB* 5' UTR (Fig. 4B, D) after prolonged incubation of the staining reaction ovn, unlike for *dj-like*+TRE (Fig. 4N), suggesting that the *protB* 5' UTR is not sufficient for full translational repression. However, stronger β -Galactosidase activity was detected after ovn incubation of testes from flies carrying *pc-protB2 Int1+ Ex1* (Fig. 4F), which lack the 5' UTR, than for testes from flies carrying *pc-protB2 Int1* (Fig. 4D), which contain the 5'

UTR, consistent with sequences within the *protB* 5' UTR contributing to strong translational repression in spermatocytes.

In contrast, the 5' UTR of *Mst77F* did not appear to contain sequences sufficient to repress translation of *Mst77F-lacZ* fusions in spermatocytes (Fig. 4). Larval testes showed significant β -Galactosidase activity in spermatocytes already after only 0.5 h of staining (Fig. 4G, I) and enhanced activity after staining ovn (Fig. 4H, J), similar to constructs from *dj-like* from which the well-characterized, translational repression element (TRE) (Hempel et al., 2006) had been removed (Fig. 4O, P). These observations suggested that the 5' UTR of *Mst77F* was not sufficient for translational repression of *Mst77F* mRNA in spermatocytes, at least in larval testes (see Fig. 2D TR?). We detected no significant differences between reporter constructs that retained the *Mst77F* 5' UTR (Fig. 4G–J) and those from which the bulk of the *Mst77F* 5' UTR had been deleted (Fig. 4K, L) after the respective incubation times. Therefore we addressed the question whether the ORF is responsible for translational repression (see below).

Sequences within the ORF regulate accumulation of the Mst77F and ProtB proteins

Comparison of reporter transgenes that contained different amounts of the *Mst77F* protein-coding region fused in frame to *eGFP* indicated that the accumulation of the Mst77F protein in elongating spermatids was regulated either at the level of translational repression or by protein turnover by sequences in the ORF. We detected no expression of eGFP in larval testes (data not shown) carrying reporter transgenes with the full-length *Mst77F* protein-coding region up to but not including the stop codon fused in frame to *eGFP* (Jayaramaiah Raja and Renkawitz-Pohl, 2005). The construct included the 62-bp intron, and the stop codon at the end of the GFP was followed by a 3' UTR and polyadenylation signal from SV40 (*Mst77F-eGFP* in Fig. 5A). In adult testes, the Mst77F-eGFP fusion protein was only detected in condensing nuclei in late-stage elongated spermatids (Fig. 5B). Similar results were obtained when an Mst77F-eGFP fusion protein was expressed under control of the promoter and 5' UTR of the testis-specific tubulin gene $\beta 2t$ ($\beta 2t$ -*Mst77F-eGFP*, Fig. 5C). Deletion analysis identified a region of the ORF required for localization of Mst77F to the nucleus in elongated spermatids. The NLS (nuclear localization signal) of Mst77F is predicted to lie within the 60 C-terminal amino acids (Nakai and Kanehisa, 1992). Consistent with this prediction, a reporter transgene with a truncation that removed the 3' most 180 bp (60 codons) of the *Mst77F* ORF (*Mst77F 60C-eGFP*, Fig. 5A) showed Mst77F-eGFP in flagella of elongated spermatids but not in spermatid nuclei (Fig. 5D). No GFP signal was detected in spermatocytes. Similarly, when portions of the *Mst77F* ORF carrying only the first 222 bp (thus lacking the last 141 amino acids and the 62-bp intron) was fused in frame to *mCherry* (*Mst77F 141C-mCherry*, Fig. 5A), the mCherry localized to the flagella of late elongating spermatids, not to the nuclei. Furthermore expression of the Mst77F-mCherry fusion protein was detected solely in late spermatids (Fig. 5E), indicating that sequences located in the first 222 bp of the *Mst77F 141C-mCherry* ORF restrict accumulation of Mst77F protein in spermatocytes and early spermatids, either by specifying translational repression or protein turnover.

Similar reporter analysis indicated that the *protB* ORF also contains sequences that block protein expression or accumulation in spermatocytes and early elongating spermatids. For reporter constructs in which *eGFP* was expressed under the control of the promoter and 5' UTR of the testis-specific tubulin gene $\beta 2t$, with the 3' UTR and polyadenylation signal from SV40 (Figs. 6A, $\beta 2t$ -*eGFP*), eGFP was detected in adult testes in male germ cells at all stages from spermatocytes to elongated spermatids (Fig. 6B), reflecting the expression of the transcript detected by *in-situ* hybridization with a probe specific for the *eGFP* component of the mRNA (Fig. 6C). However, when sequences containing the protein-coding *protB* ORF (all but the stop codon) and including the 49 bp long second intron were introduced into the reporter construct ($\beta 2t$ -*protB-eGFP*, Fig. 6A), the ProtB-eGFP fusion protein was mainly only detected in late spermatids (Fig. 6E), in a pattern reminiscent of ProtB-eGFP, expressed under the control of the endogenous *protB* promoter. In contrast, the transcript for *protB-eGFP* was detected from early spermatocytes to late elongating spermatids (Fig. 6F), as for the $\beta 2t$ -*eGFP* reporter transcript (Fig. 6C). While in whole-mount preparations, the ProtB-eGFP fusion protein was detected solely in the nuclei of elongated spermatids (Fig. 6E, arrows and insert), closer inspection of squashed preparations of testes from $\beta 2t$ -*protB-eGFP* transgenic flies showed a very weak eGFP signal in spermatocytes (Fig. 6H). However, strong fluorescence of eGFP in the squashed preparations appeared only in elongating spermatids, where it localized to nuclei at the canoe-stage, the time when histones were gradually vanishing (Fig. 6J). The $\beta 2t$ -ProtB-eGFP expressing male flies were fertile. Similar results were obtained with the corresponding *Mst77F* constructs ($\beta 2t$ -*Mst77F-eGFP*, Fig. 5C). Thus, for both *protB* and *Mst77F*, the genomic region containing the ORF plus a small intervening intron was sufficient for the proper spatiotemporal accumulation of the protein. Both the translationally repressed *protB* mRNA and *Mst77F* mRNA seemed mainly localized in the cytoplasm (Jayaramaiah Raja and Renkawitz-Pohl, 2005). Splicing mainly takes place in the nucleus. Testing whether these translationally repressed mRNAs might be stored unspliced, we performed RT-PCR with primers flanking the introns of *protB* and *Mst77F* using purified mRNA from testes. We found that the vast majority of the stored endogenous mRNAs were spliced (Fig. S1); thus, participation of the intron in translational control in the cytoplasm is unlikely.

Strikingly, when *protB-eGFP* was expressed in testes under the control of the binary UAS-GAL4 system (Brand et al., 1994) using a heterologous promoter and 5' UTR from *hsp70*, and the 3' UTR and polyadenylation signal of SV40 (*UAS-ProtB-eGFP*, Fig. 6A), and the *bam-GAL4* driver, the ProtB-eGFP fusion protein was strongly detected in late spermatogonia, most likely because *bam-GAL4* drives transcription earlier than either the $\beta 2t$ -*protB* or *Mst77F* promoters. However, expression of the ProtB-eGFP fusion protein dropped abruptly as cells became early spermatocytes (Fig. 6L), despite the detection of the corresponding transcripts during the spermatocyte growth phase and up to the early spermatid stages (Fig. 6M), consistent with a cell-type-specific mechanism present in early spermatocytes but not in late spermatogonia that either blocks translation or ensures rapid turnover of ProtB protein. The same was observed with *UAS-Mst77F-eGFP* expressed under the control of *bam-GAL4* (Fig. S2I). No nuclear ProtB-eGFP was detected in late spermatids in testes from *bam-GAL4;UAS-ProtB-eGFP* flies, perhaps because, as indicated by the *eGFP* transcript distribution, the *protB-eGFP* transcripts expressed under control of the $\beta 2t$

regulatory sequences are present in spermatids later than the *protB-eGFP* transcripts expressed under control of the *bam-GAL4-UAS* system (compare Fig. 6F,M, double arrows).

Together these data suggested that accumulation of ProtB-eGFP and Mst77F-eGFP is prevented in spermatocytes, either by rapid protein turnover or by translational repression directed by sequences within the protein-coding region of the *protB* and *Mst77F* loci.

Discussion

Expression of sperm chromosomal proteins is regulated at three levels

Protamines and Mst77F are the major chromatin components of mature sperm. The genes encoding ProtB and Mst77F are both transcribed in primary spermatocytes, and the mRNAs are translationally repressed until post-meiotic stages. Here we presented evidence that expression of one or both of the sperm head proteins ProtB and Mst77F is regulated at three levels: 1) transcription dependent on the tTAFs, 2) translational repression dependent on sequences in the 5' UTR and/or ORF and 3) stage-specific protein accumulation regulated by sequences in the ORF. The results of transgenic fly assays are summarized in Table 1.

Transcription of *protB* and *Mst77F* is regulated by short upstream sequences and transcription of both depends on tTAF function

Reporter gene assays revealed that very short genomic regions proximal to the transcription start site were sufficient for proper tTAF-dependent transcription of *protB* (–105 bp) and *Mst77F* (–89 bp). We propose that as yet unknown proteins may cooperate with tTAFs to selectively activate transcription of chromatin condensation-relevant genes, complementary to our recently finding that selective cooperation of a bromodomain protein tBRD1 with tTAFs is important for nuclear shaping and nuclear positioning in spermatids bundles but not for chromatin condensation (Leser et al., 2012).

In addition, sequences in the 5' UTR of both *protB* and *Mst77F* favored high expression levels of β -Galactosidase reporter constructs. However, we found no obvious sequence similarities between *protB* and *Mst77F* in their promoter proximal upstream regions or 5' UTRs. We showed here that the tTAF Sa occupies the promoter of *Mst77F* and *ProtB* as was known only for three other genes *fzo*, *dj* and *Mst87F*. However, the canonical generally expressed TAFs are recruited by TBP, the DNA-binding part of the TBP complex. Sa has no proposed DNA-binding site (Hiller et al., 2004). The presumptive promoter region of *Mst77F* and *ProtB* is AT-rich, thus, we cannot exclude that TAF1-2, a splice variant of TAF1, binds to this region, as TAF1-2 binds to AT-rich sequences (Metcalf and Wassarman, 2006). TRF2, a variant of TBP expressed in spermatocytes, might be involved (Kopytova et al., 2006). TRF2 was proposed to act as core promoter-selective vector (Hochheimer et al., 2002). In addition, we did not find known promoter elements or other regulatory elements, such as the INR (initiator) or DRE (downstream regulatory element). Transcription of *protB* and *Mst77F* starts in early spermatocytes and depends directly or indirectly on the function of the testes-specific TAFs. In the case of *protB*, at least, the requirement for tTAF function appears to be direct, as binding of the tTAF Sa to the *protB* promoter. In the case of *Mst77F*,

interpretation of results is made more complex by the 18 transcribed pseudogenes on the Y chromosome (Krsticevic et al., 2010). Krsticevic et al. (2010) showed that 28% of the *Mst77* transcripts in testes derive from *Mst77Y*, for which the upstream sequences are not known (FlyBase), while 72% originate from *Mst77F*. This correlates well with our transcriptome data, in which 25% of the *Mst77F* transcripts were tTAF independent and 75% were tTAF dependent. Thus, we hypothesize that the autosomal *Mst77F* might directly depend on tTAFs, while expression of the Y chromosomal copies may be tTAF independent.

Efficient ProtB accumulation depends on the 5' UTR and the ORF, while accumulation of Mst77F protein in spermatids is mainly regulated by sequences within the ORF

During spermiogenesis, numerous stored, translationally silent mRNAs are translationally activated in a precise temporal sequence. In *protB* mRNAs, TCEs that strongly restrict expression in spermatocytes were located in the 5' UTR just downstream of the transcriptional start site. However, full repression of protein accumulation required sequences beyond the 5' UTR, within the *protB* ORF or internal introns (see Table 1 for an overview of reporter constructs and their expression), raising the possibility that sequences within the ORF participate in translational repression and/or regulate protein turnover. In comparison to β -Galactosidase activity of 5' UTR containing *lacZ* reporter genes in spermatids, however, the expression in spermatocytes was low, raising the possibility that the 5' UTR of *Mst77F* confers higher efficiency of translation in spermatids than in spermatocytes.

For *Mst77F* mRNA, the 5' UTR appeared to be of minor importance for translational repression. In this case, protein accumulation was mainly controlled by the ORF, as has been reported earlier for *Mst84Db* (Gigliotti et al., 1997). Although most of our *Mst77F* fusion gene reporters contained the intron of *Mst77F*, *Mst77F 141C-mCherry*, which lacked the intron, also conferred proper translational repression and/or protein accumulation, making it unlikely that key sequences responsible for translational repression are located in the small intron. Likewise, the *UAS-protB-eGFP* tested under control of *bam-GAL4* lacked the intron but was abruptly down regulated in early spermatocytes, indicating that the ORF is sufficient for repression of *protB* protein accumulation in spermatocytes.

The transcription of *protamine* genes in mice as controlled by short upstream sequences (–113 bp) (Zambrowicz et al., 1993) and *protamine* mRNA is also translationally regulated. In mice, the *protamine* genes are transcribed in round spermatids and translated several days later in elongating spermatids (for a review, see Kleene (2003)). This translational repression depends on an 18-bp TCE in the 3' UTR (Zhong et al., 2001), although *in vivo* RNA-binding proteins that regulate this repression have not yet been identified. In mammals, recent evidence points to microRNA levels as an important molecular control mechanism for translational repression (Dai et al., 2011). In *Drosophila* oogenesis, maternally provided mRNAs for early embryogenesis are stored and translationally repressed until the appropriate time during embryogenesis. Here, the 3' UTR is responsible for translational repression by recruiting proteins, which, for example, prevent assembly of the cap-binding complex, or bind a cap-binding protein that cannot interact with eIF4G (Lasko, 2011, in press).

In *Drosophila* spermatogenesis, however, our data point toward translational repression elements in the 5' UTR of *protamine* mRNA. This is a typical feature for translational control of certain transcripts during spermatogenesis in *Drosophila* (for a review, see Renkawitz-Pohl et al., 2005). The mRNAs that encode other post-meiotic chromatin components, such as Don Juan and Don Juan-like have also been shown to be translationally regulated by sequences in the 5' UTR (Blumer et al., 2002; Hempel et al., 2006; Rathke et al., 2007). In addition, mRNAs encoding sperm-flagellar outer dense fiber proteins have been shown to be regulated by a conserved translational control element (TCE) of 12 nucleotides in the 5' UTR in the *Mst(3)CGP* gene family (Kempe et al., 1993; Kuhn et al., 1988; Schäfer et al., 1990). In sharp contrast, chromatin-relevant mRNAs of *Drosophila* (*dj*, *dj-like*, *protB*, *Mst77F*, *tpl^{94D}*) do not share this or another conserved sequence motif in the 5' UTR. However, the 5' UTR of the *dj* and *dj-like* mRNAs is characterized by predicted, extensive stem-and-loop structures (Hempel et al., 2006). Those structures might be binding sites for translational repression-mediating proteins.

Spermatocytes contain the machinery to repress translation and/or accumulation of ProtB and Mst77F

The *bam-GAL4-UAS* system drives transcription starting in late spermatogonia. With this system, we observed an initial burst of ProtB-eGFP and Mst77F-eGFP protein expression in early germ cells in a small distance from the apical tip of the testis. These proteins were not detected when transcription of *protB-eGFP* or *Mst77F-eGFP* was driven under the control of the $\beta 2$ -*tubulin* promoter and 5' UTR, which initiates transcription and translation in spermatocytes. We speculate that the initial burst of ProtB-eGFP and Mst77F-eGFP expression in late spermatogonia in testes from *bam-GAL4; UAS-ProtB-GFP* or *UAS-Mst77F-eGFP* occurred because spermatogonia lack trans-acting factors required for translational repression or rapid protein turnover that normally restrict ProtB and Mst77F expression in spermatocytes and elongating spermatids. The abrupt decline in ProtB and Mst77F levels observed in young spermatocytes may reflect the onset of cell-type-specific expression of components of such trans-acting regulatory mechanisms.

The *protB* and *Mst77F* ORFs are required for translational activation and/or protein accumulation in late spermatids

Our results suggested that cell-type-specific mechanisms present in spermatocytes and early spermatids strongly inhibit accumulation of the ProtB and Mst77F proteins, acting at the level of translational repression, protein turnover, or both. These inhibitory mechanisms must be shut off or reversed to permit proper expression of the sperm chromatin proteins in late elongated spermatids. Translational activation of mRNAs at distinct time points is a general feature of post-meiotic protein expression during spermiogenesis. It remains to be elucidated whether accumulation of other sperm proteins synthesized from stored mRNAs also depends on sequences within the respective ORFs. We posit that specialized translational activation machinery mechanism(s) act at distinct time points of spermiogenesis (Fig. 7D), for example, to turn on protamine synthesis shortly before individualization. Indeed, we and others have previously characterized male sterile mutants of the *eIF4G2* gene, also known as *Off schedule* (*CG10192*), which encodes an alternate version of the core translation initiation machinery component eIF4G that is expressed in

spermatocytes and required for meiotic division and early spermatid differentiation in males. Among other effects, *eIF4G2*-mutant males have aberrant spermatids with large nuclei that arrest differentiation shortly after meiosis, long before ProtB and Mst77F synthesis starts (Baker and Fuller, 2007; Franklin-Dumont et al., 2007).

Translational control/activation is often regulated by cytoplasmic polyadenylation (Villalba et al., 2011). In *Drosophila*, both female and male germ lines contain cytoplasmic poly(A) polymerases (Benoit et al., 2008; Cui et al., 2008; Sartain et al., 2011). We showed that the 3' UTR of the *protamine* mRNA is dispensable for correct expression of protamines. This agrees with the finding that the length of poly(A) tracks of protamine mRNAs is indistinguishable between *wild-type* and mutants of the poly(A) polymerase *GLD-2*. Nevertheless, Prot-eGFP was not detected in *GLD-2* mutants that might arrest spermiogenesis too early to allow protamine translation/ accumulation. This agrees with our model that translation of *protamine* mRNAs takes place at a distinct developmental time point late in the spermatid elongation stage (Fig. 7A).

Thus, expression of ProtB and Mst77F in *Drosophila* male germ cells is controlled at least three levels. In spermatocytes, transcript expression is controlled by specialized cell-type-specific transcriptional machinery. In addition, *protB* and *Mst77F* mRNAs are translationally repressed until late spermatid stages, when ORF-dependent translational activation takes place. We expect this may also hold true for other stored mRNAs relevant for sperm morphogenesis. This regulation could involve cell-type-specific activation of translation, protein stabilization, or both.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.02.018>.

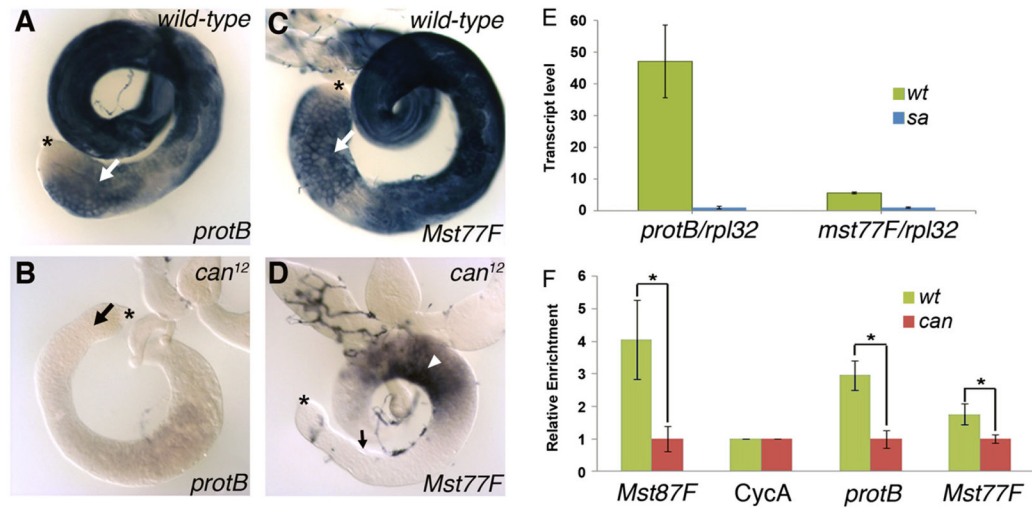


Fig. 1.

Transcription of *protB* and *Mst77F* depends on tTAFs. (A–D) *In-situ* hybridization of whole-mount wild-type (A, C) and *can¹²* (B, D) testes using *protB*-specific (A, B) and *Mst77F*-specific (C, D) probes. The staining reactions were incubated for the same length of time. Arrows point toward the spermatocytes; the arrowhead in D points toward the basal region; and asterisks indicate the tips of the testis, where germ-line stem cells reside. (E) Transcriptome analysis of transcript levels of *protB* and *Mst77F* in wild-type (*wt*) and tTAF (*sa^{-/-}*) (*sa*) mutant testes. The results are from three independent experiments; error bars indicate the sd. (F) Quantitative real-time PCR analysis of anti-Sa ChIP using wild-type and *can¹²* testes showing the enrichment of Sa at different targets. The results are from three independent experiments and are normalized to 1; error bars indicate the sd. All ChIP results were normalized to *CycA* within the same genotype, and then the results of the two genotypes were compared (*: *P* value < 0.05 using two-sample *t*-test).

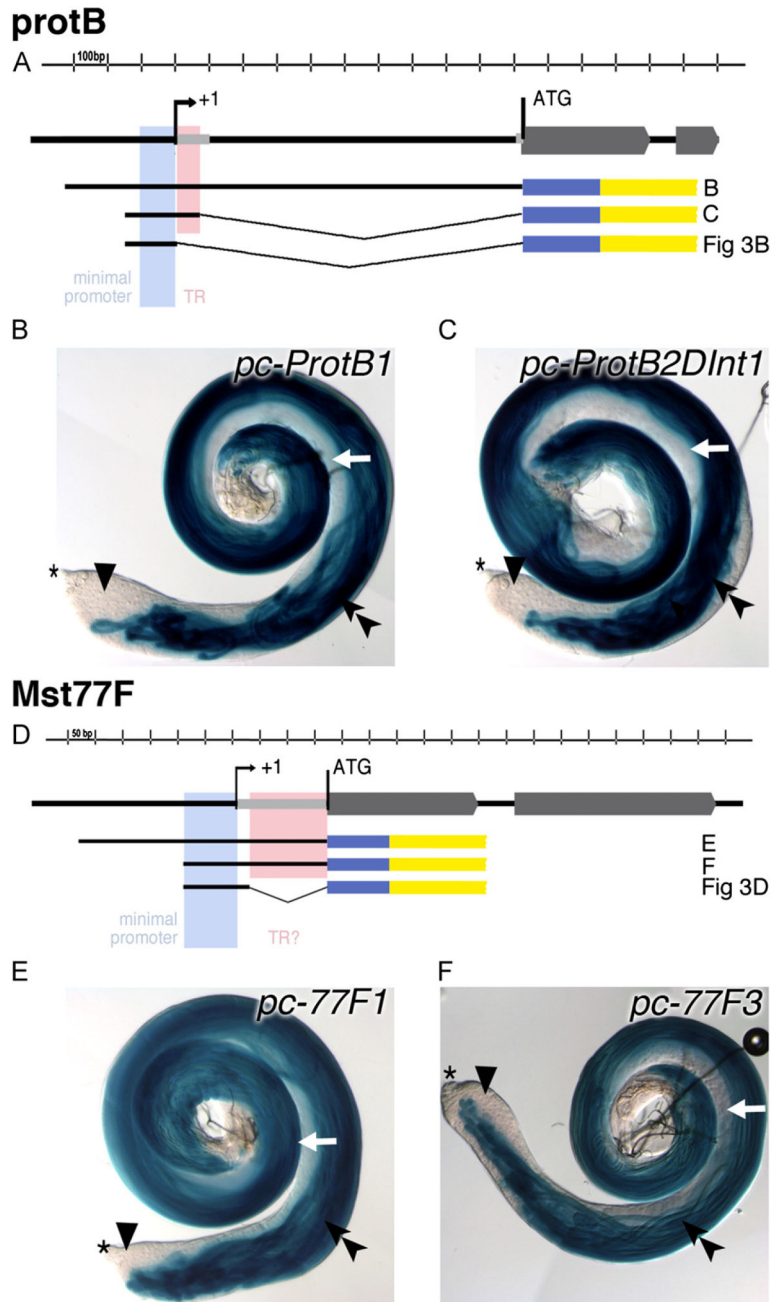


Fig. 2. Expression of ProtB and Mst77F is controlled by short upstream regions. (A, D) Schematic drawings of the genomic regions and the generated promoter-*lacZ* constructs of *protB* and *Mst77F*. Exons are depicted as gray block arrows, the minimal promoters are in light blue, and the regions tested for contributing to translational control (TR) are in pink. Black thick lines illustrate the regions contained within each promoter-*lacZ* construct; thin gray lines indicate the deleted regions. The reporter gene *lacZ* is indicated in blue; the SV40 3' UTR with a polyadenylation signal is indicated in yellow. (B, C, E, F) Analyses of β -

Galactosidase activity in the testis of transgenic flies bearing different *protB* (B, C) or *Mst77F* (E, F) promoter *lacZ* constructs after 10 min of staining reaction. Asterisk indicate the tip of the testis. Double arrowheads, elongated spermatid bundles; arrowheads, spermatocytes; and early spermatids, white arrows.

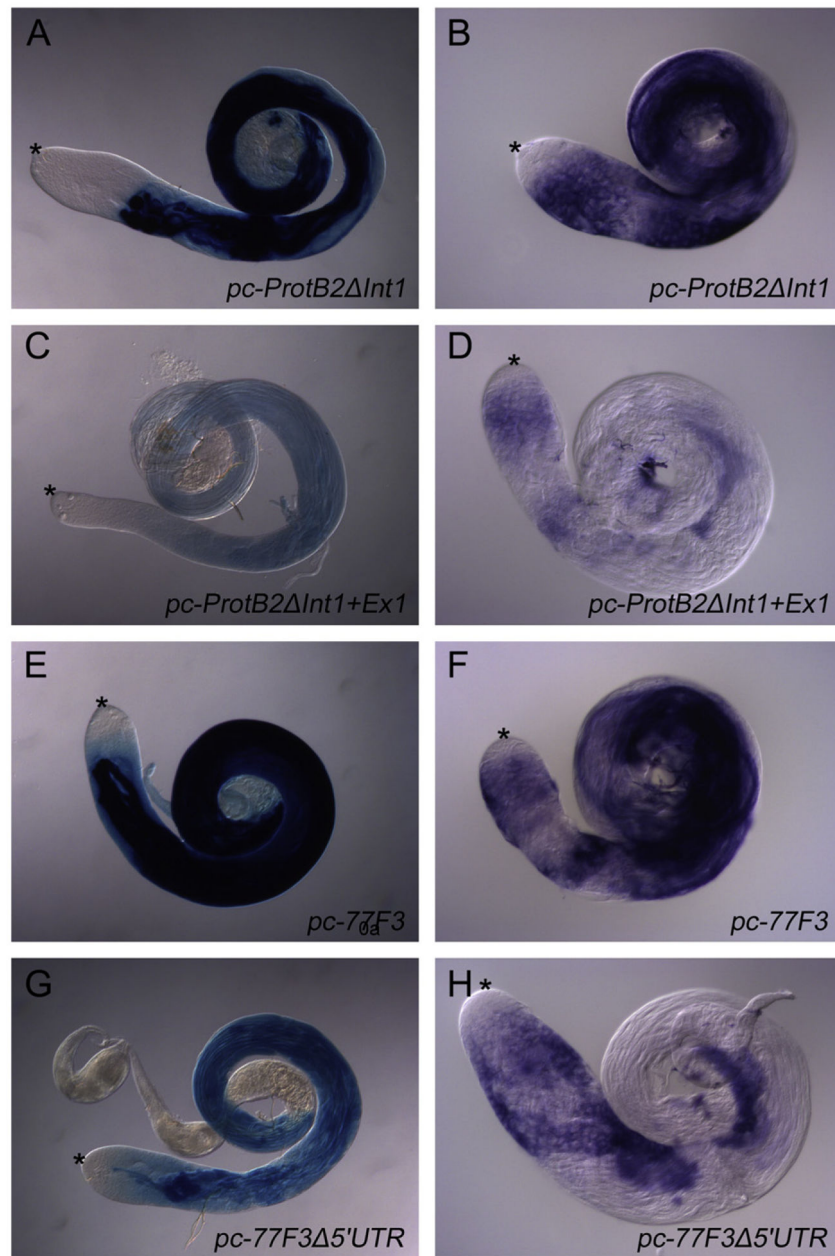


Fig. 3. The 5' UTR is essential for high expression of ProtB and Mst77F. (A, C, E, G) Adult testes of reporter lines were stained for β -Galactosidase activity in parallel for 0.5 h. *pc-protB2 Int* (A) and *pc-77F3* (E) include the 5' UTR, whereas *pc-protB2 Int1 Ex1* (C) and *pc-77F3 5'UTR* (G) lack most of the 5' UTR (see Fig. 2A, D). (B, D, F, H) Adult testes from these reporter lines were analyzed by *in-situ* hybridization with an antisense probe to *lacZ* RNA.

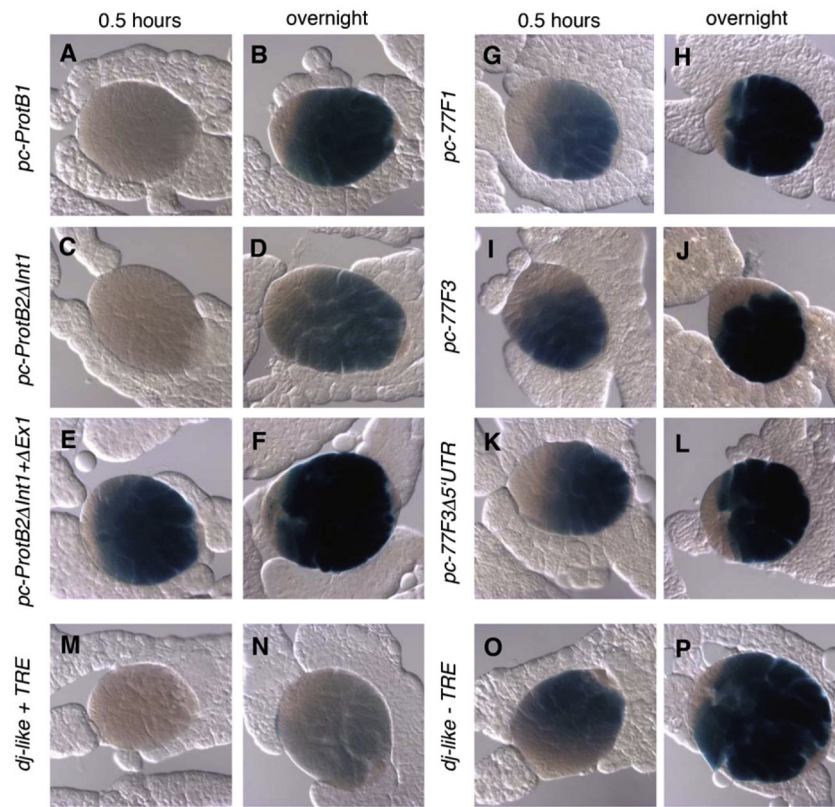


Fig. 4. The 5' UTR is not sufficient to block translational repression of β -Galactosidase in *protB-lacZ* and *Mst77F-lacZ* reporter gene assays. Larval testes of *protB* (A–F) and *Mst77F* (G–L) transgenic lines and *dj-like* reporter lines (M–P) were stained for β -Galactosidase activity in primary spermatocytes in parallel either for 0.5 h or ovn as indicated. (A–D, G–J, M, N) 5' UTR is present; (E, F, K, L, O, P) most of the 5' UTR is lacking.

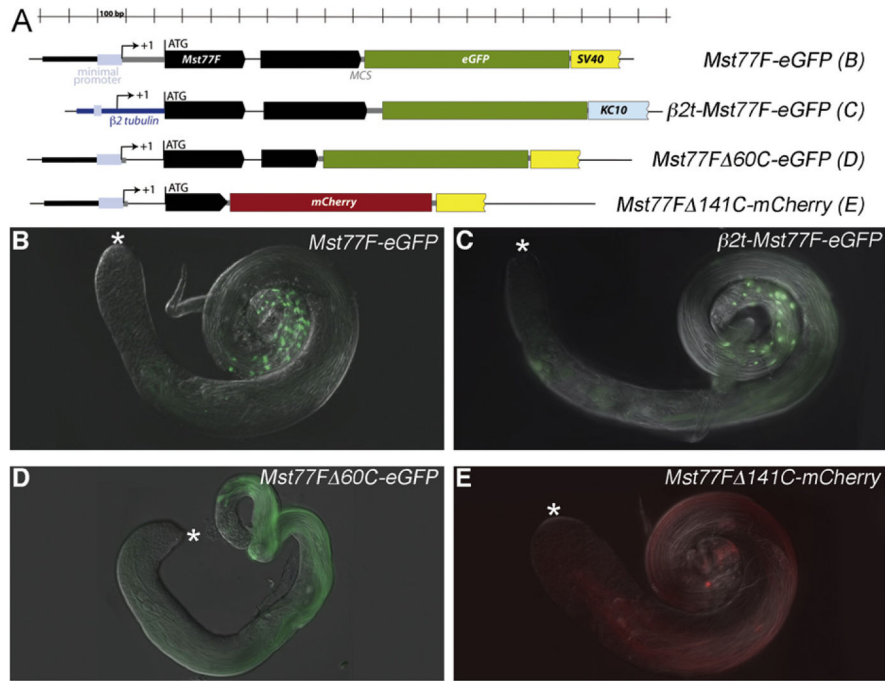


Fig. 5. Translational repression and activation of *Mst77F* mRNA depends on the *Mst77F* ORF. (A) Schematic drawings of the constructs used to test the *Mst77F* ORF for its regulatory capabilities. (B–E) eGFP fluorescence in adult testes of transgenic males carrying (B) *Mst77F-eGFP*, (C) $\beta 2t$ -*Mst77F-eGFP*, (D) *Mst77F* $\Delta 60C$ -*eGFP*, and (E) *Mst77F* $\Delta 141C$ -*mCherry*. Asterisk indicate the tips of the testis, where germ-line stem cells reside.

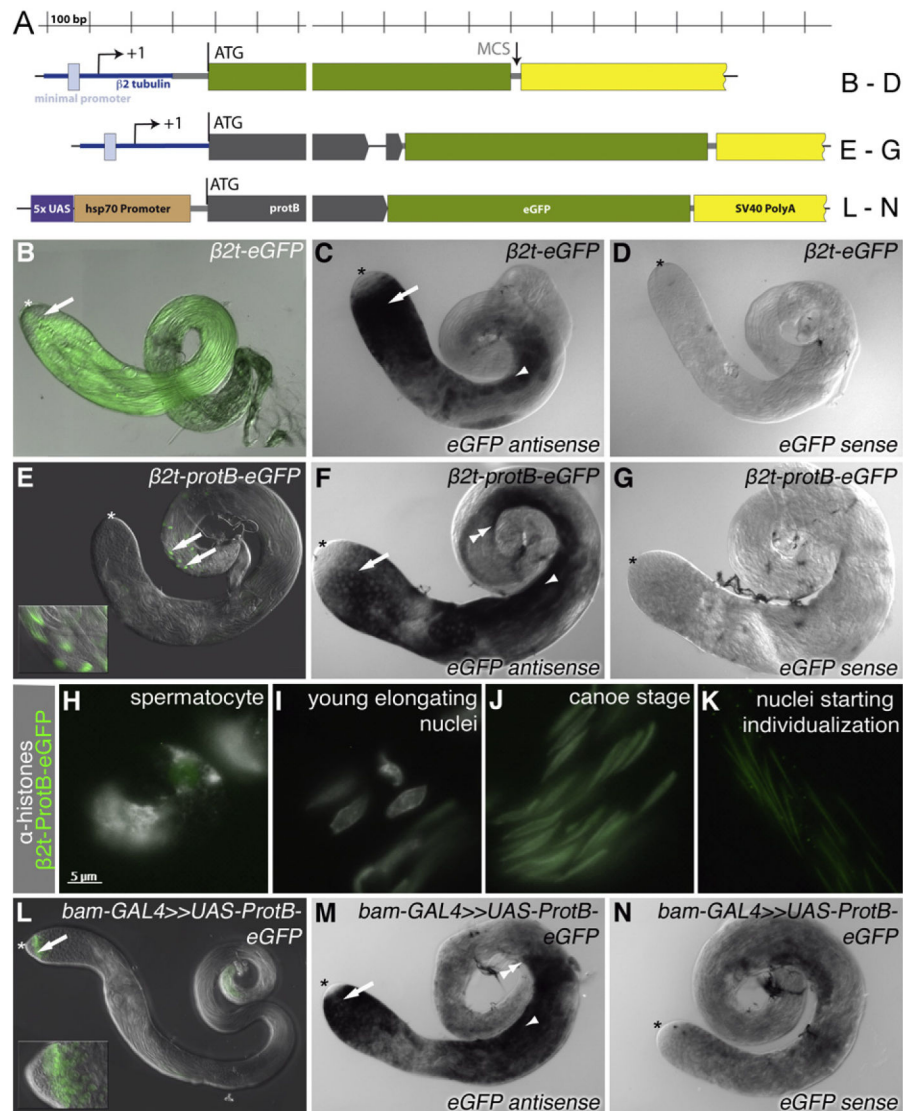


Fig. 6. The ORF of *protB* confers translation in late spermatids. (A) Diagram of the constructs. (B, E, H–L) eGFP fluorescence; (C, D, F, G, M, N) *in-situ* hybridization using an eGFP-specific probe. (B, C, D) *eGFP* under the control of the $\beta 2$ -*tubulin* promoter and 5' UTR ($\beta 2t$ -*eGFP*). (B) Whole mount of adult testis; arrow, late spermatids. (C) Hybridization with an *eGFP* antisense probe; arrow, early spermatocytes; arrowhead, early spermatids. (D) Hybridization with an *eGFP* sense probe. (E, F, G) *protB-eGFP* under the control of the $\beta 2$ -*tubulin* upstream control region and 5' UTR ($\beta 2t$ -*protB-eGFP*). (E) Whole mount of adult testis; arrows, late spermatids; inset, higher magnification. (F) Hybridization with an *eGFP* antisense probe; arrow, early spermatocytes, arrowhead, early spermatids; double arrowhead, indicates late signal in spermatids. (G) Hybridization with an *eGFP* sense probe. (H, I, J, K) Squash preparations of $\beta 2t$ -*protB-eGFP* testes, GFP autofluorescence and α -histone, (H) Spermatocytes, (I) Young elongating nuclei, (J) Canoe-stage nuclei. (K) Nuclei starting individualization (L, M, N) *bam-GAL4*-driven *UAS-ProtB-eGFP*. (L) Whole mount

of adult testis; Arrow, spermatogonia; inset, higher magnification of the tip of this testis. (M) Hybridization with an *eGFP* antisense probe; arrow, spermatogonia; arrowhead, spermatid stage; double arrow, *eGFP* transcripts hardly detectable distally to this point. (N) Hybridization with an *eGFP* sense probe; double arrow, spermatids. In (B–G, L–M), the asterisk marks the hub region of the testis.

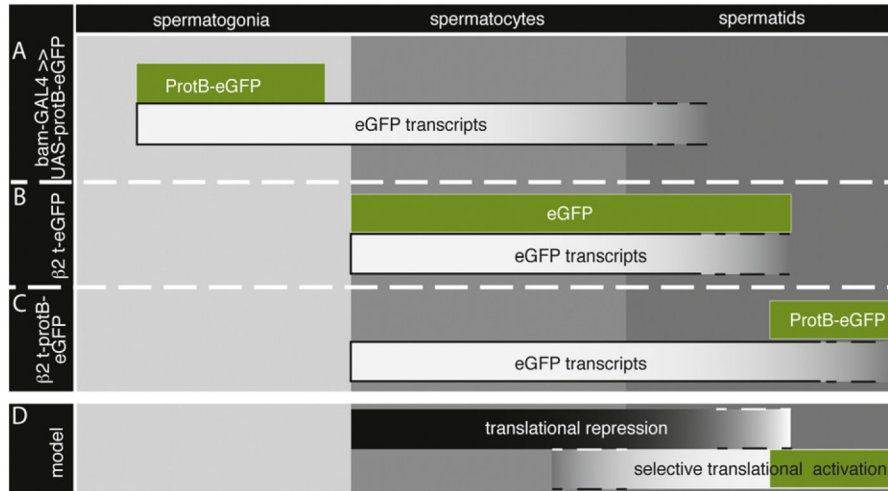


Fig. 7. *ProtB* mRNA is regulated by translational repression and stage-specific translational activation. The autofluorescence of the eGFP reporter is shown in green; the *eGFP* transcript is shown in white boxes relative to the stages of spermatogenesis. (A) ProtB-eGFP is limited to spermatogonia, while the corresponding *eGFP* transcript persists during the spermatocyte stage. (B) *β2t-eGFP* leads to overlapping *eGFP* transcripts and protein distribution in spermatocytes and spermatids. (C) *β2t-protB-eGFP* leads to transcripts present in spermatocytes and spermatids, while ProtB-eGFP is synthesized in late spermatids. (D) This model proposes that many mRNAs are synthesized in spermatocytes and selectively translated during the spermatid phase; ProtB-eGFP (in green) is depicted as an example.

Table 1

Summary of results of reporter gene assays and gene fusion expression of *Mst77F* and *protB*.

Construct name	Promoter	5' UTR	ORF	3' UTR including polyadenylation signal	Reporter expressed in larval testes (0.5h staining, β -Gal or eGFP life)	Reporter expressed in larval testes (ovn staining β -Gal)	Reporter expressed in spermatocytes in adult testes (10 min staining this work β -Gal or life eGFP)	Reporter expressed in spermatids in adult testes (10 min staining, β -Gal or life eGFP)
<i>protB-eGFP</i>	<i>protB</i>	<i>protB</i>	<i>protB-eGFP</i>	SV40	-	-	-	+late nuclear
<i>pc-ProtB1</i>	<i>protB</i>	<i>protB</i>	<i>lacZ</i>	SV40	-	+	-	+
<i>pc-ProtB2 Int1</i>	<i>protB</i>	Partial 5' UTR	<i>lacZ</i>	SV40	-	+	-	+
<i>pc-ProtB2 Int1+ Ex1</i>	<i>protB</i>	-	<i>lacZ</i>	SV40	++	+++	Likely	+
	$\beta 2t$	$\beta 2t$	<i>lacZ</i>	SV40	+	+	?	+++ Michels et al. (1989) Santel et al. (2000)
$\beta 2t$ -eGFP	$\beta 2t$	$\beta 2t$	eGFP	SV40	Not analyzed	Not analyzed	++	+++
$\beta 2t$ - <i>protB-eGFP</i>	$\beta 2t$	$\beta 2t$	<i>protB-eGFP</i>	SV40	Not analyzed	Not analyzed	-	++late nuclear
UAS- <i>ProtB-eGFP</i>	UAS-driven by <i>bam-GAL4</i>	<i>Hsp70</i>	<i>protB-eGFP</i>	SV40	Not analyzed	Not analyzed	eGFP in spermatogonia and fades in early spermatocyte stages	-(eGFP mRNA does not persist until late spermatids)
<i>Mst77F-eGFP</i>	<i>Mst77F</i>	<i>Mst77F</i>	<i>Mst77F-eGFP</i>	SV40	-	-	-	++ late nuclear or close to nuclei
<i>pc-77F1</i>	<i>Mst77F</i>	<i>Mst77F</i>	<i>lacZ</i>	SV40	+	++	-	+++
<i>pc-77F3</i>	<i>Mst77F</i>	<i>Mst77F</i>	<i>lacZ</i>	SV40	++	+++	-	+++
<i>pc-77F3 5'UTR</i>	<i>Mst77F</i>	-	<i>lacZ</i>	SV40	++	+++	-	+++
$\beta 2t$ - <i>Mst77F-eGFP</i>	$\beta 2t$	$\beta 2t$	<i>Mst77F-eGFP</i>	KC10	Not analyzed	Not analyzed	-	++ late nuclear or close to nuclei
<i>Mst77F 141C-mCherry</i>	<i>Mst77F</i>	<i>Mst77F</i>	<i>Mst77F 141-C-mCherry</i>	SV40	-	-	-	flagella of late spermatids
<i>Mst77F 60C-eGFP</i>	<i>Mst77F</i>	<i>Mst77F</i>	<i>Mst77F 60C-eGFP</i>	SV40	Not analyzed	Not analyzed	-	flagella of late spermatids
UAS- <i>Mst77F-eGFP</i>	UAS-driven by <i>bam-GAL4</i>	<i>Hsp70</i>	<i>Mst77F-eGFP</i>	SV40	Not analyzed	Not analyzed	eGFP in spermatogonia and fades in early spermatocyte stages	-