***Drosophila* polypyrimidine-tract binding protein (PTB) functions specifically in the male germline**

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The mammalian polypyrimidine-tract binding protein (PTB), which is a heterogeneous ribonucleoprotein, is ubiquitously expressed. Unexpectedly, we found that, in *Drosophila melanogaster*, the abundant PTB transcript is present only in males (third instar larval, pupal and adult stages) and in adult flies is restricted to the germline. Most importantly, a signal from the somatic sex-determination pathway that is dependent on the male-specific isoform of the *doublesex* protein (DSX^M) regulates PTB, providing evidence for the necessity of soma–germline communication in the differentiation of the male germline. Analysis of a *P*-element insertion directly links PTB function with male fertility. Specifically, loss of *dmPTB* affects spermatid differentiation, resulting in the accumulation of cysts with elongated spermatids without producing fully separated motile sperms. This male-specific expression of *PTB* is conserved in *D. virilis*. Thus, *PTB* appears to be a particularly potent downstream target of the sex-determination pathway in the male germline, since it can regulate multiple mRNAs.

Keywords: *hephaestus*/male germline/PTB/
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Introduction

In metazoans, the majority (85–95%) of genes contain intervening sequences or introns. A typical intron has several important splicing signals: the 5′ splice site, the 3′ splice site and the branch site. In higher eukaryotes, the polypyrimidine tract (Py-tract) upstream of the 3′ splice site is also an essential splicing signal (Burge *et al.*, 1999; Reed, 2000). In plants, uridine-rich sequences that are present throughout introns appear to play a role in defining intron–exon boundaries (Lorkovic *et al.*, 2000). Proper recognition of splicing signals is crucial for accurate splicing. Five small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6 snRNPs) and several other proteins recognize these signals and assemble onto a pre-mRNA to form a large complex called the spliceosome (Green, 1991; Moore *et al.*, 1993; Will and Luhrmann, 1997; Nilsen, 2002). The assembly of the spliceosome involves several rearrangements, which include RNA–RNA, protein–protein and RNA–protein interactions (Staley and Guthrie, 1998). In the spliceosome, two transesterification reactions catalyze the joining of exons and removal of the lariat intron (Nilsen, 2000).

Alternative splicing is a process by which multiple mRNAs are synthesized from a single primary transcript, providing an excellent source of functional diversity (Lopez, 1998; Graveley, 2001). Preliminary estimates indicate that >35% of predicted human genes and 10–15% of *Drosophila* genes undergo alternative splicing (Mironov *et al.*, 1999; Brett *et al.*, 2002). Alternative splicing involves combinatorial use of alternative 5′ splice sites, 3′ splice sites, introns and exons (Lopez, 1998; Black, 2000; Smith and Valcarcel, 2000; Graveley, 2001; Singh, 2002). Although alternative splicing controls many important biological processes (Valcarcel *et al.*, 1995), the somatic sex-determination pathway in *Drosophila* is thus far one of the best-understood examples of splicing regulation. In *D. melanogaster*, the key sex determining genes, *Sex-lethal* (*Sxl*), *transformer* (*tra*) and *doublesex* (*dsx*), are transcribed in an identical manner in both sexes. However, these transcripts are processed differently in male and female flies by alternative splicing (Cline and Meyer, 1996; Schutt and Nothiger, 2000). For example, in chromosomal males (XY), *SXL* and *TRA* proteins are not synthesized, due to in-frame translation stop codons in the mRNAs spliced by the default pathway. As a result, the male-specific isoform of the *DSX* protein (*DSX^M*) is synthesized. However, in females (XX), alternative splicing allows synthesis of *SXL* and *TRA* proteins, leading to the synthesis of the female-specific isoform of *DSX* (*DSX^F*). The *DSX^M* isoform activates male-specific genes and represses female-specific genes in male flies, and the *DSX^F* isoform activates female-specific genes and represses male-specific genes in female flies (Nagoshi and Baker, 1990; Burtis, 1993).

The Py-tract adjacent to the 3′ splice sites is an important target for splicing regulation (Smith and Valcarcel, 2000; Singh, 2002). For example, the *Drosophila* protein *SXL* blocks splice sites in target pre-mRNAs by binding to adjacent uridine-rich sequences or Py-tracts, leading to exon skipping in the *Sxl* pre-mRNA, 3′ splice site switching in the *tra* pre-mRNA and intron retention in the *msl2* pre-mRNA. In contrast, the *TRA* protein, along with cofactors, binds to an exonic enhancer sequence(s) and activates an otherwise weak Py-tract/3′ splice site in the *dsx* pre-mRNA. The mammalian polypyrimidine-tract binding protein (PTB), another Py-tract binding protein, is also known as heterogeneous nuclear ribonucleoprotein I (hnRNP I) (Ghetti *et al.*, 1992); the hnRNP proteins are ubiquitously expressed, associate with nascent transcripts and play various roles in RNA metabolism (Krecic and Swanson, 1999). In vertebrates, transcripts from the *PTB* and brain-enriched *PTB* (*brPTB*) genes are widely expressed in many cell lines and tissues (Patton *et al.*, 1991; Ashiya and Grabowski, 1997; Chan and Black, 1997; Polydorides *et al.*, 2000). PTB was originally identified as a splicing factor and has four RNA

recognition motifs (RRMs) (Gil *et al.*, 1991; Patton *et al.*, 1991). Subsequent studies, however, showed that the mammalian PTB has sequence-specific RNA-binding activity and can negatively regulate the splicing of a wide spectrum of pre-mRNAs, including muscle and neuronal transcripts (Mulligan *et al.*, 1992; Lin and Patton, 1995; Singh *et al.*, 1995; Ashiya and Grabowski, 1997; Chan and Black, 1997; Gooding *et al.*, 1998; Wagner and Garcia-Blanco, 2001, 2002). The muscle- and neuron-specific splicing regulation of certain genes has been suggested to result from generalized repression of these genes by PTB in other cell types. Although SXL and PTB both compete for the binding of the general splicing factor U2AF⁶⁵ to specific Py-tracts in repressing 3' splice sites (Lin and Patton, 1995; Singh *et al.*, 1995), the SXL protein, unlike PTB, is expressed only in the cell type or sex that shows alternative splicing. PTB is also known to control splice site choice by positive regulation of the pre-mRNA encoding the calcitonin/calcitonin gene-related peptide (CT/CGRP) (Lou *et al.*, 1999).

In addition to its role in splicing regulation, PTB can also regulate polyadenylation of the CT/CGRP pre-mRNA (Lou *et al.*, 1996) and translation of RNAs from picornaviruses via internal ribosome entry sites (Kaminski *et al.*, 1995; Valcarcel and Gebauer, 1997; Belsham and Sonenberg, 2000; Wagner and Garcia-Blanco, 2001). Previous studies with the vertebrate PTB were carried out using various assays *in vitro* or in cultured cells. Therefore, we analyzed PTB in *Drosophila*, where its function can be studied in a natural setting *in vivo*.

In this study, we have demonstrated, surprisingly, that an abundant *Drosophila* PTB (dmPTB) transcript is expressed only in the male germline and is regulated by the well-characterized somatic sex-determination pathway in a DSX^M-dependent manner. Furthermore, molecular genetic analysis reveals that dmPTB function is essential for spermatogenesis during spermatid differentiation.

Results

An abundant PTB transcript is expressed only in males

To analyze PTB function *in vivo* and complement studies with the vertebrate PTB, we investigated the *Drosophila* PTB. Unexpectedly, the *D.melanogaster* ortholog of PTB (dmPTB) was expressed in adult males but not females, as determined by northern analysis using the full-length cDNA probe (Figure 1, dmPTB); minor transcripts could be observed in females only upon longer exposure (data not shown). Since prior studies have not suggested that PTB has a sex-specific function or regulation, it remained possible that the abundant band in Figure 1 resulted from cross-hybridization via an RRM, a common highly conserved RNA-binding domain (Varani and Nagai, 1998). To exclude this possibility, we prepared several probes corresponding to divergent portions of the gene such as the 5' and 3' untranslated regions (5' and 3' UTRs) and the variable linker region between RRM (inter-RRM). Each of the probes showed an identical male-specific signal (Figure 1). Consistent with this finding, BLAST results show that there is only one sequence match to the dmPTB cDNA (*P*-value 6.7e-290) in the *Drosophila* genome. These results confirm that this abundant mRNA

expressed in adult males but not females is a genuine dmPTB transcript.

dmPTB function is essential for male fertility

Previously, a large-scale *P*-element insertion mutagenesis screen for male sterility identified the *hephaestus*² (*heph*²) mutation (Castrillon *et al.*, 1993), which was later mapped to the dmPTB locus by the *Drosophila* Genome Project (Spradling *et al.*, 1999) (Figure 2A); other *P*-element insertions into the dmPTB locus are homozygous lethal (Dansereau *et al.*, 2002). However, the molecular basis for the male sterility of the *heph*² mutant was not studied. Since homozygosity for the *heph*² allele causes sterility in male but not female flies (Castrillon *et al.*, 1993), we reasoned that this phenotype might be due to the absence of the abundant male-specific dmPTB transcript. To directly test this hypothesis, we analyzed the expression of dmPTB in *heph*² flies. The dmPTB transcript was present in both wild-type and *heph*² heterozygous males (Figure 2B, lanes 1 and 3) but absent in *heph*² homozygous males (lane 5). Thus, the *heph*² *P*-element insertion disrupts the expression of the male-specific dmPTB transcript.

To exclude the possibility that the *heph*² insertion affected male sterility or dmPTB expression indirectly through unintended mutations, we excised the *P*-element using a standard protocol (Robertson *et al.*, 1988). The excision of the *P*-element was confirmed by PCR analysis of the genomic DNA (see Supplementary figure 1 available at *The EMBO Journal* Online) and by the loss of the *ry*⁺ marker on the *P*-element. In the *heph*^{2-ex}/*heph*^{2-ex} male flies, excision of the *P*-element restored the male-specific expression of dmPTB (Figure 2C, lane 2). Consistent with previous results (Castrillon *et al.*, 1993), the *heph*² homozygous flies showed an enlarged tip of the testis (Figure 2D, *heph*²/*heph*²). However, the loss of the *P*-element reverted gonad morphology to normal (Figure 2D, *heph*^{2-ex}/*heph*^{2-ex}). In addition, excision of the *P*-element restored male fertility to the wild-type level (Figure 2D, bottom). These results confirm the authenticity of the abundant dmPTB mRNA and provide the first direct link between male fertility and PTB function.

The somatic sex-determination pathway regulates dmPTB expression

The male-specific expression of the abundant dmPTB transcript raised the possibility that it could be regulated by the well-characterized somatic sex-determination pathway (*Sxl*→*tra*→*dsx*) (Cline and Meyer, 1996; Schutt and Nothiger, 2000). To determine whether the somatic sex-determination pathway regulates dmPTB, we either ectopically expressed or used known mutations in *tra* or *dsx*. First, the ectopic synthesis of TRA in male flies, raised at 22°C using an *hsp70::tra* transgene, abolished expression of the male-specific dmPTB transcript (Figure 3A, top, lane 3); the presence of TRA was confirmed by somatic sexual transformation (McKeown *et al.*, 1988; Nagoshi *et al.*, 1988; Arthur *et al.*, 1998; data not shown) and by the appearance of the female-specific *Yolk protein 1* (*Yp1*) transcript (Figure 3A, middle, lane 3). Secondly, in *dsx*¹/*Df* male flies (Burtis, 1993), the expression of the male-specific transcript of dmPTB was significantly reduced (~90%) relative to *dsx*^{1/+} (Figure 3B, lanes 1

Fig. 1. An abundant male-specific transcript of *dmPTB* is expressed in adult male but not female flies. Northern analysis of *dmPTB* expression in wild-type (Oregon-R) male and female flies, using probes for either the full-length or various portions (5' UTR, 3' UTR, RRM1 and inter-RRM) of the *dmPTB* cDNA. For this and subsequent figures, XY and XX indicate chromosomal sex. Only relevant portions of the RNA blots are shown for the small probes. M, molecular size markers (kb); *rp49*, a loading control (the asterisk for the *rp49* blot represents a 0.46 kb transcript).

Fig. 2. *P*-element insertion in the *dmPTB* locus abolishes *dmPTB* expression. (A) Schematic of the *dmPTB* gene, adapted from GadFly. Boxes, exons; lines, introns; solid boxes, coding sequence; stippled/empty boxes, alternative 5' UTR exons. Position (P) of the *heph2* *P*-element is shown. (B) A *P*-element insertion abolishes *dmPTB* expression, based on northern analysis. Relevant genotypes are shown above the chromosomal sex. The *dmPTB* probe is inter-RRM (Figure 1). (C) Excision of the *P*-element restores *dmPTB* expression. Northern analysis of *dmPTB* expression in homozygous *heph2* (lane 1) and *heph2-ex* (lane 2) strains. (D) Excision of the *P*-element restores gonad morphology and male fertility. Nomarski micrographs for male testes of given genotypes are shown. Arrows point to tips of the testes. The numbers in parentheses represent an average number, with standard deviation, of progeny from three independent matings involving 10 wild-type virgin females and 10 males of given genotypes, as described previously (Castrillon *et al.*, 1993). Comparable *dmPTB* expression and fertility results were obtained from two additional excision lines.

Fig. 3. The somatic sex-determination pathway regulates *dmPTB* expression. (A) Ectopic expression of TRA in chromosomal males represses *dmPTB* expression. Relevant genotypes are shown above the chromosomal sex. The *dmPTB* probe is inter-RRM, and *Yp1* is a female-specific marker. (B) Loss of *DSX*^M in XY flies greatly reduces the expression of the male-specific *dmPTB* transcript. (C) Expression of *DSX*^M in XX flies is insufficient for activation of the *dmPTB* transcript, but feminization of the XY soma greatly reduces the *dmPTB* transcript in males. The DSX type indicates doses of the male-specific (M) and female-specific (F) forms of DSX. Both the *dsx*^M and *dsx*^D alleles synthesize only the *DSX*^M isoform, irrespective of chromosomal sex (Nagoshi and Baker, 1990). Lane 6 is a mixture of *dsx*^{M/Df} and *dsx*^{+/Df} genotypes.

and 5). However, the loss of *DSX*^F function in chromosomally female flies failed to activate the expression of *dmPTB* (Figure 3B, lane 6). These results show that the presence of *DSX*^M is important for the activation of *dmPTB*, that loss of *DSX*^F alone is insufficient for the activation and also argue against the possibility that a non-sex-specific factor alone activates *dmPTB* in males.

Next, we determined whether the male-specific function of *dsx* (*DSX*^M) is sufficient for the activation of *dmPTB*.

Surprisingly, expression of *DSX*^M in chromosomally female flies was insufficient to activate *dmPTB*. First, the *dsx*^M allele, which only expresses the *DSX*^M isoform, did not activate *dmPTB* in XX flies (Figure 3C, lane 5) despite causing sexual transformation and disappearance of *Yp1* (Figure 3C, middle) (Nagoshi and Baker, 1990; Burtis, 1993). Secondly, deletion of *tra*, which also mediates switching from the *DSX*^F to the *DSX*^M isoform in XX flies (McKeown *et al.*, 1988; Nagoshi *et al.*, 1988), also did not activate *dmPTB* (Figure 3C, lane 4). Finally, expression of *DSX*^F, which is known to antagonize *DSX*^M function (Burtis, 1993), did repress the *DSX*^M-dependent activation of *dmPTB* in male flies (Figure 3C, lane 7). Thus, expression of the *DSX*^M protein in XX flies is insufficient for the activation of *dmPTB*, and *DSX*^F can prevent *DSX*^M-dependent activation of *dmPTB*.

Expression of the abundant *dmPTB* transcript is restricted to the male germline

Our combined results show that the somatic sex-determination pathway in *Drosophila* controls *dmPTB* expression. Intriguingly, the XX flies that expressed the *DSX*^M isoform and became somatically masculinized failed to express *dmPTB* (Figure 3C, lanes 4 and 5). These observations suggested that the abundant *dmPTB* transcript might be expressed in the germline, rather than in somatic cells. To distinguish between these possibilities, we analyzed the progeny of *mago*^{1/Df} and *tudor*^{1/Df} flies, which lacked a germline (Boswell and Mahowald, 1985; Boswell *et al.*, 1991). Indeed, the *dmPTB* transcript was absent from the male progeny of *mago*^{1/Df} and *tudor*^{1/Df} flies (Figure 4A, lanes 3 and 7), indicating that the abundant *dmPTB* transcript is restricted to the male germline. Moreover, we directly confirmed by *in situ* hybridization that *dmPTB* was specifically expressed in primary spermatocytes in the wild-type (Figure 4B, i) and *heph*^{2-ex/heph}^{2-ex} testes (Figure 4B, vii) but not in the *heph*^{2/heph}² mutant testis (Figure 4B, iv). We conclude that the somatic sex-determination pathway controls the sex-specific expression of *dmPTB* in the male germline.

dmPTB is expressed from the late third instar larval stage through adult flies

To determine the developmental regulation of *dmPTB* expression, we analyzed RNA blots from various stages of development. Figure 5A shows that the abundant transcript of *dmPTB* was not detectable in the embryonic (lanes 1 and 2), the first instar larval (lane 3) and the second instar larval (lane 4) stages of development. However, *dmPTB* was expressed in the late third instar larval (lane 5) and pupal (lane 6) stages of development. The expression of *dmPTB* at the L3 and the pupal stages is male specific (data not shown). We conclude that the abundant *dmPTB* transcript is expressed from L3 to adult stages.

Loss of *dmPTB* affects spermatid differentiation

To determine how loss of *dmPTB* affects spermatogenesis, we analyzed wild-type and *heph*^{2/heph}² mutant testes. Figure 5B shows that there was no difference between the wild-type and the mutant germ cells in the larval stage L3, the earliest stage at which abundant expression of *dmPTB* was observed. In adults, all of the stages of spermatogenesis were affected.

Fig. 4. The abundant *dmPTB* transcript is restricted to the male germline. **(A)** Loss of the male germline results in disappearance of the *dmPTB* transcript. Northern analysis was carried out using the inter-RRM probe. **(B)** Excision of the *P*-element restores *dmPTB* expression in the male germline. *In situ* hybridization and indirect immunofluorescence with testes from given genotypes were carried out using antisense (i, iv and vii) or sense (x) *dmPTB* probe (a negative control). DNA was stained with DAPI (iii, vi, ix and xii).

genesis could be identified in the testes of wild-type (Figure 5B, iii) and excision flies (Figure 5B, iv). In particular, individual motile sperms were abundant. However, the *heph*²/*heph*² mutant accumulated cysts of elongated spermatids, and the enlarged tips of these testes were also filled with such cysts (Figure 5B, v). In addition, the mutant lacked fully individualized motile sperms (Figure 5B, v). In the *heph*²/*heph*² mutant, we could observe all of the morphogenetic stages up to elongated spermatids (for reference, see Fuller, 1993). We note that the majority of the elongated spermatids from the *heph*² mutant showed numerous bulges along their tails characteristic of a defect during the individualization process

(data not shown). Therefore, we conclude that loss of *dmPTB* affects spermatid differentiation.

The male-specific expression of *PTB* is conserved in *D.virilis*

Next, we asked whether the sex-specific regulation of *PTB* is unique to *D.melanogaster* or is conserved in other organisms. We analyzed the expression of *PTB* in *D.virilis*, a distant relative of *D.melanogaster*. Figure 6A shows that *PTB* is unambiguously expressed in a male-specific manner in *D.virilis* (lanes 3 and 4); the weaker signal in *D.virilis* likely reflects imperfect complementarity with the *D.melanogaster PTB* sequence that was used

Fig. 5. The *dmPTB* transcript is expressed from the third instar larval to adult stages and is important for spermatid maturation. (A) Northern analysis was carried out using the inter-RRM probe. 0–12 hr, early embryonic stage; 12–22 hr, late embryonic stage; L1, L2 and L3 are the first, second and late third instar larval stages, respectively. (B) The *heph2* mutation affects spermatogenesis at or prior to sperm separation. L3 and adult refer to testes from the larval stage L3 and adults, respectively. Genotypes are indicated above each panel. Arrows refer to primary spermatocytes (ps), cysts with elongated spermatids (cy) and motile sperms (ms).

for hybridization. We conclude that the sex-specific regulation of *PTB* has been conserved between *D. virilis* and *D. melanogaster*, which diverged 60 million years ago (Marin and Baker, 1998).

Although components upstream in the sex-determination pathway have significantly diverged, *dsx* and likely its downstream functions are conserved (Raymond *et al.*, 1998; Zarkower, 2002). For example, the male-specific isoform of *dsx* (*DSX^M*) can functionally substitute for the *mab-3* gene in directing male-specific neuroblast differentiation in the nematode *Caenorhabditis elegans* (Raymond *et al.*, 1998). Both proteins contain the conserved DNA-binding motif (DM domain) that is also found in the human and the murine orthologs: the human *DMT1* gene is expressed only in testes, and the murine *Dmrt1* gene is essential for testis differentiation but dispensable for ovarian development (Raymond *et al.*, 2000).

Since *dmPTB* responds to the function of the male-specific isoform of *dsx*, we asked whether the sex-specific expression of *dmPTB* is also conserved in *C. elegans* and mouse. Figure 6B shows that both hermaphrodite (XX) and male (XO) worms expressed comparable levels of *PTB* transcripts. In addition, *PTB* was equally expressed in the carcass (Figure 6C, lanes 1 and 2) and gonads (lanes 3 and 4) of both sexes in 13.5-day-old mouse embryos, the stage at which testes and ovaries can be easily distinguished. We conclude that, although the *PTB* mRNA is

equally expressed in both sexes in worms and mouse, the abundant *dmPTB* transcript is restricted to males in *D. melanogaster* and *D. virilis*.

Discussion

This study provides the first evidence that there is a major male-specific transcript of the *Drosophila PTB* that is regulated by the somatic sex-determination pathway. The sex-specific function of the abundant *dmPTB* transcript reported here is restricted to the male germline. Finally, we provide a direct molecular link between male fertility and *PTB* function, which offers a molecular basis for the male sterility of the *heph2* mutant.

Model for *dmPTB* regulation

We postulate that the somatic sex-determination pathway, in a *DSX^M*-dependent manner, provides a signal for the proliferation and differentiation of male germ cells, leading to the expression of *dmPTB* in the male germline (Figure 7). Since *tra* and *dsx* are dispensable within the germline (Marsh and Wieschaus, 1978; Schupbach, 1982), their effect from the somatic tissue is inductive in nature (Steinmann-Zwicky *et al.*, 1989; Steinmann-Zwicky, 1994; Heller and Steinmann-Zwicky, 1998; Waterbury *et al.*, 2000; Janzer and Steinmann-Zwicky, 2001). Accordingly, the *DSX^F* isoform in the female soma or lack of the *DSX^M* isoform in the male soma would fail to

Fig. 6. The male-specific *dmPTB* transcript is also found in adult *D. virilis*. (A) An RNA blot using *D. melanogaster* and *D. virilis* RNA from male and female flies was probed using the entire *dmPTB* cDNA probe. The weaker signal in *D. virilis* likely reflects imperfect complementarity with the *D. melanogaster* *PTB* sequence that was used for hybridization. (B) *PTB* is expressed in both sexes in *C. elegans*. XO, *him-8* males (lane 1); XX, N2 gravid hermaphrodites in which spermatogenesis has ceased (lane 2). Ribosomal protein gene (*rp21c*) is a loading control. (C) *PTB* is expressed in both sexes in mouse embryos. XY carcass (whole embryo lacking gonads) (lane 1); XX carcass (lane 2); XY gonads (13.5 days old) (lane 3); XX gonads (13.5 days old) (lane 4). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is a loading control.

provide an appropriate signal for the development of the male germ cells. Thus, *dmPTB* expression is indirectly regulated by *DSX^M* in the male germline.

There are several differences in the mechanism of sex determination between somatic cells and the female germline (Steinmann-Zwicky, 1992; Mahowald and Wei, 1994; Cline and Meyer, 1996; Schutt and Nothiger, 2000), e.g. the mechanism by which the X:A ratio is sensed is different between the two cell types. Furthermore, sexual differentiation is entirely cell autonomous in somatic cells but also requires a somatic inductive signal(s) in germ cells (Steinmann-Zwicky, 1994; Heller and Steinmann-Zwicky, 1998; Waterbury *et al.*, 2000; Janzer and Steinmann-Zwicky, 2001). We emphasize that, unlike other male-specific transcripts that are either functional in somatic cells or dispensable for germline sex determination and spermatogenesis (Cann *et al.*, 2000; Ahmad and Baker, 2002; Streit *et al.*, 2002), *dmPTB* function is necessary in the germline for spermatogenesis. Thus, *dmPTB* provides evidence for the necessity of soma-germline communication in the differentiation of the male germline.

Several interesting aspects of *dmPTB* regulation, however, remain to be addressed. For example, relatively little

Fig. 7. A model for the sex-specific function of *dmPTB* in the male germline. A *DSX^M*-dependent inductive signal from the male soma is necessary for germ cell proliferation or maintenance and thus *dmPTB* expression. The *dmPTB* transcript is abundant in primary spermatocytes. The *heph²* mutation affects spermatid differentiation, resulting in the accumulation of cysts with elongated spermatids but lacking fully separated motile sperms. Briefly, spermatogenesis involves division of stem cells to regenerate themselves and produce gonial cells. Each gonial cell mitotically divides four times to produce 16 primary spermatocytes enclosed in a cyst of two somatically derived cells. The primary spermatocytes undergo two meiotic divisions to generate 64 spermatids, which undergo a series of morphogenetic changes culminating in fully separated motile sperms (for a detailed description of spermatogenesis, see figure 9 in Fuller, 1993).

is known about the molecular nature of somatic- or germline-specific activation signals for *dmPTB* expression. Also, we cannot distinguish whether the relevant germline-specific signal is repressed in the female germline or is activated only in the male germline (Heller and Steinmann-Zwicky, 1998). Finally, the promoter element(s) that confer male germline-specific expression remains unknown.

Male-germline-specific versus non-sex-specific functions of *dmPTB*

The male-germline-specific function of the abundant *dmPTB* transcript reported here directly links *dmPTB* function to male fertility. Specifically, *dmPTB* is expressed in primary spermatocytes and affects spermatid differentiation, resulting in the accumulation of cysts with elongated spermatids, but fully separated motile sperms are not observed. This phenotype is reminiscent of the defect seen in late male-sterile mutants such as the individualization-deficient *clathrin heavy chain* (*Chc*) mutant (Fabrizio *et al.*, 1998), suggesting that *dmPTB* may control a component(s) of the cytoskeletal machinery. The expression pattern of *dmPTB* is consistent with the observation that the majority of transcription in germ cells is limited to the premeiotic stages, although protein synthesis and significant morphological changes occur during postmeiotic spermatid differentiation (Fuller, 1993). Accordingly, we favor that *dmPTB* is expressed early during spermatogenesis but affects either directly or indirectly the events that occur or manifest late during spermatid differentiation (Fuller, 1993). We emphasize that many male-sterile mutants are known to show secondary effects even though such mutations affect

processes early during spermatogenesis (for further discussion, see p. 115 in Fuller, 1993). Thus, dmPTB in the male germline may control multiple targets or steps during spermatogenesis. Consistent with the known RNA-binding functions of the mammalian PTB (Valcarcel and Gebauer, 1997), it could regulate the splicing, polyadenylation or translation of potential mRNAs that participate in spermatogenesis.

The male-germline-specific function of dmPTB is not necessarily inconsistent with the ubiquitous expression and multiple known targets of the vertebrate PTB (Valcarcel and Gebauer, 1997). To reconcile these differences, we favor that dmPTB performs an additional non-sex-specific function(s) vital for both sexes in *Drosophila*. First, the male-sterile *heph²* mutant also affects viability of both sexes (M.D.Robida and R.Singh, unpublished results). Secondly, other mutations in the dmPTB locus are homozygous lethal (Dansereau *et al.*, 2002). The most likely explanation for the different phenotypes of these mutations is that, whereas the *heph²* mutation perturbs the male germline function but partially supports the vital function, the *ema* mutation compromises both functions. Thirdly, based on *in situ* hybridizations, dmPTB transcripts are expressed in several cell lineages (Davis *et al.*, 2002), and minor transcripts are observed in females only upon longer exposure (M.D.Robida and R.Singh, unpublished results).

Given that the dmPTB locus is large (>135 kb) and that there is an indication of two distinct 5' UTRs (distal and proximal) (Figure 2A) (Davis *et al.*, 2002), the simplest interpretation for the two phenotypes is that the abundant male germline-specific transcript reported here corresponds to an mRNA that contains the distal 5' UTR (Figure 1, 5' UTR probe) and is likely transcribed from an upstream promoter (Figure 2A). Accordingly, we favor that a downstream promoter(s) possibly contributes low abundance transcript(s) that are expressed non-sex-specifically in many cell lineages (Davis *et al.*, 2002). This situation is reminiscent of two types of *Sxl* transcripts arising from a sex-specific establishment promoter (*Pe*) that is transiently active early during development (blastoderm stage) in females and from a non-sex-specific maintenance promoter (*Pm*) that is active in both sexes later during development (Keyes *et al.*, 1992). Although PTB transcripts are expressed in both male and female gonads in mice and worms (Figure 6B and C), we cannot exclude the possibility that the PTB protein is functional only in the male germline or regulates male-germline-specific mRNA(s) in the gonads of these organisms.

Biological significance

In conclusion, this study demonstrates that hnRNP proteins (Krecic and Swanson, 1999; Elliott *et al.*, 2000), which are generally widely expressed, can serve a sex-specific function *in vivo*. In addition, these studies provide molecular evidence that an appropriate somatic sex-determination signal is required for proper differentiation of the male germline, and dmPTB provides a useful reagent to define interactions between soma and the male germline for proper male germline differentiation. Finally, dmPTB, which is needed for fertility and spermatogenesis, is potentially an important downstream target of the sex-

determination cascade in the male germline, where it could regulate multiple mRNAs.

Materials and methods

Fly strains and culture

The *heph²/TM3*, *Df(3R)dsx³/TM3*, *dsx¹/TM3*, *dsx^M/TM1*, *tra¹/TM2*, *Df(3L)st⁷/TM3* & *betaTub85D^D/TM2* and *dsx^D/TM2* stocks were obtained from the Bloomington Stock Center. The *Df(3L)st⁷/TM6B* and *Df(3L)st⁷/hsp70:tra^F/TM6B* stocks were obtained from Barbara Taylor and the *Df(1)H3g⁶/FM7c*, *Df(2R)Pu³⁶/SM5* (deficiency of *mago*), *mago¹/SM6a*, *Df(2R)PF1/SM6a* (deficiency of *tudor*), *tud¹/CyO* and *D.virilis* stocks from Bob Boswell. The *dsx^M/dsx⁺* and *dsx^M/Df* flies were generated as follows: *dsx^M/TM1* males were crossed to *Df(3R)dsx³/TM3* females, and *dsx^M/TM3* male progeny were collected and mated to *Df(1)H3g⁶/FM7c*. The *Fm7c/Y*; *dsx^M/dsx⁺* male progeny were collected and mated to *XX*; *Df(3R)dsx³/TM3* flies. *dsx^M/dsx⁺* and *dsx^M/Df* progeny were phenotypically separated. The *dsx¹/Df* flies were obtained by crossing *B⁺Y*; *dsx¹/TM3* and *XX*; *Df(3R)dsx³/TM3*; the *tra^F/dsx^D* flies by crossing *XX*; *Df(3L)st⁷/hsp70:tra^F/TM6B* and *XY*; *dsx^D/TM2*; the *mago¹/Df* flies by crossing *Df(2R)Pu³⁶/SM5* and *mago¹/SM6a*; and the *tud¹/Df* flies by crossing *Df(2R)PF1/SM6a* and *tud¹/CyO*. The *B⁺Y*; *tra¹/TM3* stock was obtained by crossing *B⁺Y*; *dsx¹/TM3* with *XX*; *tra¹/TM2* flies. The *tra¹/Df* flies were obtained by crossing *B⁺Y*; *tra¹/TM3* with *XX*; *Df(3L)st⁷/TM3*. All *TM3* balancer chromosomes carried the *Sb¹* marker. The *heph²/heph²* larvae were obtained from a *heph²/TM6B*, *Tb* stock. Flies were raised on standard corn meal food at 25°C (Ashburner, 1989). The *heph²/TM3* flies were raised at 22°C, and those carrying the *tra^F* cDNA were raised at 22°C on Formula 4-24 Plain Instant *Drosophila* Medium (Carolina Biologicals, NC) containing 0.27 mg/ml geneticin (Invitrogen, CA).

Caenorhabditis elegans culture and isolation

Worm strains were as described previously (Sulston and Hodgkin, 1988). The XO *him-8* males were obtained by filtering through nylon screens and the N2 gravid hermaphrodites were isolated by a 35% sucrose float (Lewis and Fleming, 1995).

DNA clones and probes

The cDNA clones for *rp49* (GH27246), *Yp1* (GH28744) and dmPTB (LD03431) and the mouse *Ptb* (IMAGE 480448) were obtained from Research Genetics (CA). The *C.elegans ptb-1* clone (yk1128d03) was obtained from Dr Yuji Kohara. The *rp21c* DNA was obtained from Dr Tom Blumenthal. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) plasmid was obtained from Gennye Walker. The smaller dmPTB probes were prepared using the following sets of primers: distal 5' UTR, forward (5'-AGTGAATAAATTATCAACGTGTGC-3') and reverse (5'-TTTCTTTTCTCTCGTAGTGCAAC-3'); 3' UTR, forward (5'-AATCCGATGCGAAGTCAGGTTT-3') and reverse (5'-AATACGACTCACTATAGG-3'); CDS1, forward (5'-GCCAAGGCCTCAAAGTCATC-3') and reverse (5'-GTTGTGACCTTGGTCCGTCTT-3'); and CDS2, forward (5'-TGTTAAGTACAACAACGACAAATC-3') and reverse (5'-TCGAGTAACCTCGCATTGCAG-3').

Northern analysis

Total RNA was isolated using TRI reagent (Sigma-Aldrich, MO). Poly(A)⁺ RNA was isolated using the PolyATtract mRNA isolation system (Promega, WI). For each lane, ~0.5–1.0 µg of poly(A)⁺ RNA was separated by electrophoresis on a 1% agarose gel with formaldehyde. RNA was transferred to a Duralose-UV membrane (Stratagene, CA), hybridized with a ³²P-labeled probe at 42°C overnight, washed extensively and imaged on a PhosphorImager (Molecular Dynamics).

P-element excision

The P-element (BDGP:ms(3)10515) causing the *heph²* phenotype was excised using a standard transposase source (Robertson *et al.*, 1988). The excision lines were analyzed by PCR analysis of the genomic DNA using appropriate primer pairs. The primers used were: 1, 5'-CGGGAT-CCTTGTTCTTGTAAACAGCAATTGTTG-3'; 2, 5'-AACTGCAGTTA-GAGCGAAGTAGCGGAGAAT-3'; and 3, 5'-CAATCATATCGC-TGTCTCACTCA-3'. The PCR was performed as follows: 94°C for 5 min, 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 1 min and one cycle of 72°C for 7 min, using a 50 µl reaction containing 1 × MgCl₂-free PCR buffer (Promega), 2.5 mM MgCl₂, 0.4 mM dNTPs, 1 µM each primer and genomic DNA equivalent of two flies. The PCR product was separated on an agarose gel and stained with ethidium bromide.

In situ hybridization

Testes were excised from adult males and fixed in 4% formaldehyde in phosphate-buffered saline and Tween-20 (PBST: 0.137 M NaCl, 2.68 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄, 0.1% Tween-20). Testes were washed for 10 min in 20 mg/ml glycine with PBST and stored overnight at -20°C in methanol. The samples were rehydrated in PBST, permeabilized for 2 h in 1% Triton X-100 in PBST, post-fixed for 5 min, washed for 10 min in the glycine solution and then washed extensively with PBST. They were hybridized overnight in 50% deionized formamide, 5× SSC, 0.2 mg/ml sonicated salmon sperm DNA, 0.1 mg/ml tRNA and 0.05 mg/ml heparin at 65°C with the full-length dmPTB (antisense or sense) RNA labeled with fluorescein (Roche, Basel, Switzerland). Samples were washed with PBST, incubated for 1–2 h at room temperature with 1:100 dilution of an anti-fluorescein rabbit antibody (Molecular Probes A-889) in PBST/2% BSA, followed by 1:250 dilution of a goat anti-rabbit secondary antibody coupled to an Alexa 488 fluor (Molecular Probes A-11034) in PBST/2% BSA, and washed with PBST. For the detection of DNA, the testes were then incubated with DAPI (1 µg/ml) in PBST for 10 min and washed 6 × 10 min with PBST. Testes were mounted in 70% glycerol/PBS and viewed using a Leica Confocal microscope.

Testes squashes

Testes from L3 larvae or adults were dissected in 1× PBST and placed on a slide in a drop of 1× PBST. A cover slip was overlayed to squash the testes. The resulting squashes were viewed using phase-contrast microscopy on a Zeiss Axiophot microscope.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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