

Effect of RNAi-mediated knockdown of the *Bombyx mori transformer-2* gene on the sex-specific splicing of *Bmdsx* pre-mRNA

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ABSTRACT In *Drosophila melanogaster*, *transformer-2* (*tra-2*) is essential for female differentiation and is known to induce female-specific splicing of *doublesex* (*dsx*). The function of *Bmtra-2*, the *Bombyx mori* homolog of *tra-2*, on the other hand remains to be elucidated. As an initial step to learn about the biological function of *Bmtra-2*, we determined whether *Bmtra-2* is capable of inducing the female-specific splicing of *Drosophila dsx*. RNAi-mediated knockdown of *Bmtra-2* using *Bombyx* cultured cells transiently transfected with a *dsx* minigene revealed that *Bmtra-2* can induce female-specific splicing of *Drosophila dsx*. To examine the role *Bmtra-2* plays in regulating sex-specific splicing of *Bmdsx* pre-mRNA, we used an RNAi approach to reduce *Bmtra-2* expression in the early embryo. Embryos injected with dsRNAs or siRNAs targeted to *Bmtra-2* showed no variation in the sex-specific splicing pattern of *Bmdsx* pre-mRNA. RNAi knockdown of *Bmtra-2* in the early embryo caused abnormal testis formation. Taken together, these results indicate that *Bmtra-2* is required for normal testis development, but is not involved in regulating the sex-specific splicing of *Bmdsx* pre-mRNA, even though it is capable of inducing the female-specific splicing of *Drosophila dsx*.

KEY WORDS: *sex determination, Bombyx mori, transformer-2, alternative splicing, doublesex*

Introduction

In *Drosophila melanogaster*, *tra-2* is a key gene that acts on the cascades regulating both somatic sexual differentiation and male fertility (Belote and Baker, 1983). In female somatic tissues, the *tra-2* protein acts in concert with the *transformer* (*tra*) protein to bind to the *cis*-regulatory element (*dsxRE*) upstream of the *dsx* gene in the female-specific exon. This activates the weak 3' splicing site preceding the exon and generates the female-type Dsx protein (Dsx^F), which regulates the downstream genes that allow female development to proceed. Although the *tra-2* protein is expressed in males, the absence of male Tra activity means that *dsx* pre-mRNA is spliced by default to generate the male-type Dsx protein (Dsx^M), which in turn regulates genes necessary for male development (Ryner and Baker 1991). Moreover, *tra-2*, and *tra* also regulate the sex-specific splicing of *fruitless* (*fru*), the gene that controls sexual behavior and sex-specific nervous system differentiation

(Taylor and Knittel 1995). A similar function of *tra-2* has been reported in several dipteran insects. RNAi-mediated knockdown of *tra-2* in *Musca* (Burghardt *et al.*, 2005), *Ceratitis* (Salvemini *et al.*, 2009), and *Anastrepha* (Samo *et al.*, 2010) caused disruption of endogenous *tra-2* function in these species and the subsequent male-specific splicing of the endogenous *dsx* pre-mRNAs, leading to the transformation of chromosomally female embryos into adult pseudomales. These findings raise the possibility that *tra-2* function is conserved among dipteran insects.

tra-2 is also necessary for male germline function. Mutant males carrying loss-of-function *tra2* mutations are morphologically normal but display incomplete spermatogenesis and produce immotile sperm. As a result, these mutant males are sterile (Hazelrigg and Tu, 1994; Madigan *et al.*, 1996; Mattox *et al.*, 1996). The

Abbreviations used in this paper: Bmtra-2, *Bombyx mori* homolog of *tra-2*; *dsx*, *doublesex*; *fru*, *fruitless*; RE, regulatory element; RNAi, inhibitory RNA; *tra*, *transformer*.

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Supplementary Material (one figure) for this paper is available at: <http://dx.doi.org/10.1387/ijdb.120049ms>

Accepted: 4 July 2012. Final, author-corrected PDF published online: 12 November 2012. Edited by: Makoto Asashima.

testis-specific splicing of pre-mRNAs from two genes expressed during spermatogenesis, *exuperantia (exu)* and *alternative testis transcripts (att)*, are dependent on Tra-2 (Hazelrigg and Tu, 1994; Madigan et al., 1996). Mutations that affect the alternatively spliced 3' UTR of *exu* mRNA lead to a significant reduction in *exu* RNA accumulation in male germ cells. Null mutations in *exu* cause male sterility and lead to the formation of spermatids with defects in nuclear elongation (Hazelrigg and Tu, 1994).

In the silkworm, *B. mori*, the chromosomal sex determination mechanism is distinct from that of *D. melanogaster*, with female (ZW) being the heterogametic sex and male (ZZ) the homogametic sex. It has been shown genetically that female sex in *B. mori* is determined by the presence of a dominant feminizing factor on the W chromosome (Hashimoto, 1933). Moreover, no sex-specific regulatory *Sxl* homolog has been isolated from *B. mori* (Niimi et al., 2006). Also a *tra* homolog has not been found in the *Bombyx* genome (Mita et al., 2009). Despite these differences, a *B. mori dsx* homolog (*Bmdsx*) has been implicated in sex determination (Suzuki et al., 2005). *Bmdsx* produces alternatively spliced mRNA isoforms that encode sex specific transcription factors as observed in *dsx* (Suzuki et al., 2001). However, the underlying mechanism for sex-specific splicing is clearly different between *dsx* and *Bmdsx*. We have demonstrated that female splicing of *Bmdsx* pre-mRNA represents the default mode when tested in HeLa nuclear extracts, and also that the female exon is devoid of putative TRA/TRA-2 binding sites (Suzuki et al., 2001). A splicing inhibitor, BmPSI is involved in the regulation of male-specific splicing of *Bmdsx* (Suzuki et al., 2008). Since BmPSI does not exhibit any sequence relationship to known SR proteins, such as Tra and Tra-2, the regulatory mechanism of sex-specific alternative splicing of *Bmdsx* is distinct from that of *dsx*.

The *tra-2* homolog of *Bombyx mori* (*Bmtra-2*) has been identified, but its function remains to be elucidated (Niu et al., 2005). In the present study, we used *Bmtra-2* RNAi-mediated knockdown to determine whether *Bmtra-2* is required for regulating *Bmdsx* pre-mRNA sex-specific splicing. In addition, we provide evidence indicating a possible role for *Bmtra-2* in embryonic testis morphogenesis.

Results

Bmtra-2 can induce the female-specific splicing of *Drosophila dsx*

As an initial step to learn about the biological function of *Bmtra-2*, we examined whether *Bmtra-2* is capable of inducing the female-specific splicing of *Drosophila dsx*. For this purpose, RNAi-mediated knockdown of *Bmtra-2* was performed using *Bombyx* cultured cells (BmN cells) transiently transfected with a *dsx* minigene (Fig. 1A). In agreement with previous reports, female-type *dsx* mRNA was produced when the *dsx* minigene was co-transfected with both *tra* and *tra-2* cDNA constructs (Fig. 1B, lane 4). Similar results were observed when the *dsx* minigene was co-transfected with only the *tra* cDNA construct (Fig. 1B, lane 2). In this condition, the cells were subjected to RNAi experiments with *Bmtra-2* dsRNA. Although we were not able to completely block *Bmtra-2* expression, a significant reduction of female-type *dsx* mRNA was observed with a concomitant reduction in *Bmtra-2* expression (Fig. 1B, lanes 5 and 6). These results indicate that *Bmtra-2* can function in place of *Drosophila tra-2* to induce the female-specific splicing of *dsx*. Male-type *dsx* mRNA, which arises as the default in splicing, was

observed in all cases.

Downregulation of *Bmtra-2* by RNAi does not affect the sex-specific splicing of *Bmdsx*

To further test whether *Bmtra-2* plays a role in regulating sex-specific splicing of *Bmdsx* pre-mRNA, we utilized an RNAi approach to reduce *Bmtra-2* expression in *Bombyx* individuals. Microinjection of dsRNA into embryos has most often been used for *B. mori*, and in all cases successfully, although silencing levels vary (Terenius et al., 2011). Therefore, we decided to use embryos for RNAi experiments. Eggs during the early embryonic stage (6–8 hr after oviposition), a developmental period known to be sensitive to RNAi-mediated gene knockdown (Yamaguchi et al., 2011), were injected with *Bmtra-2* dsRNA. In addition, the effects of three siRNAs (si-29, si-30, and si-31) designed to target *Bmtra-2* were also examined. Total RNA was extracted from each egg four days after injection and subjected to RT-PCR analysis. As shown in Fig. 2A, real-time RT-PCR confirmed a significant reduction in *Bmtra-2* transcript levels in embryos injected with *Bmtra-2* dsRNA. Similarly, siRNA injections caused a significant reduction in *Bmtra-2* mRNA levels, but those effects were slightly lower than that observed

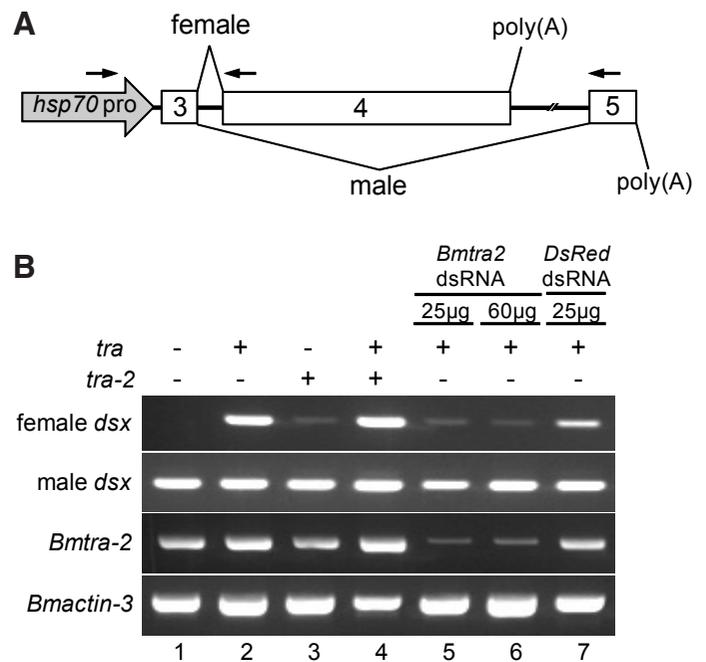


Fig. 1. Effect of RNAi-mediated *Bmtra-2* knockdown on the sex-specific splicing of a *dsx* minigene using *Bombyx* cultured cells. (A) Structure of the *dsx* minigene construct used for assaying the sex-specific splicing of *dsx* in BmN cells. Boxes represent exons. The numbers above the diagram represent exon labels. V-shaped lines above (skipping of the female-specific exon) and below (inclusion of the female-specific exon) the diagram represent the endogenous *dsx* splice variants observed in males and females. Polyadenylation sites are indicated by Poly A. The arrows indicate the approximate location of the primers that were used for RT-PCR in (B). **(B)** The *dsx* minigene was co-transfected into BmN cells with (+) or without (-) *tra* and *tra-2* cDNA constructs. Female- or male-specific splicing was detected by RT-PCR and analyzed on a 2% agarose gel. Labels to the left of the gel refer to female splicing (*dsx F*) and male splicing (*dsx M*). Lower panel shows the down-regulation of *Bmtra-2* expression in the dsRNA-treated cells.

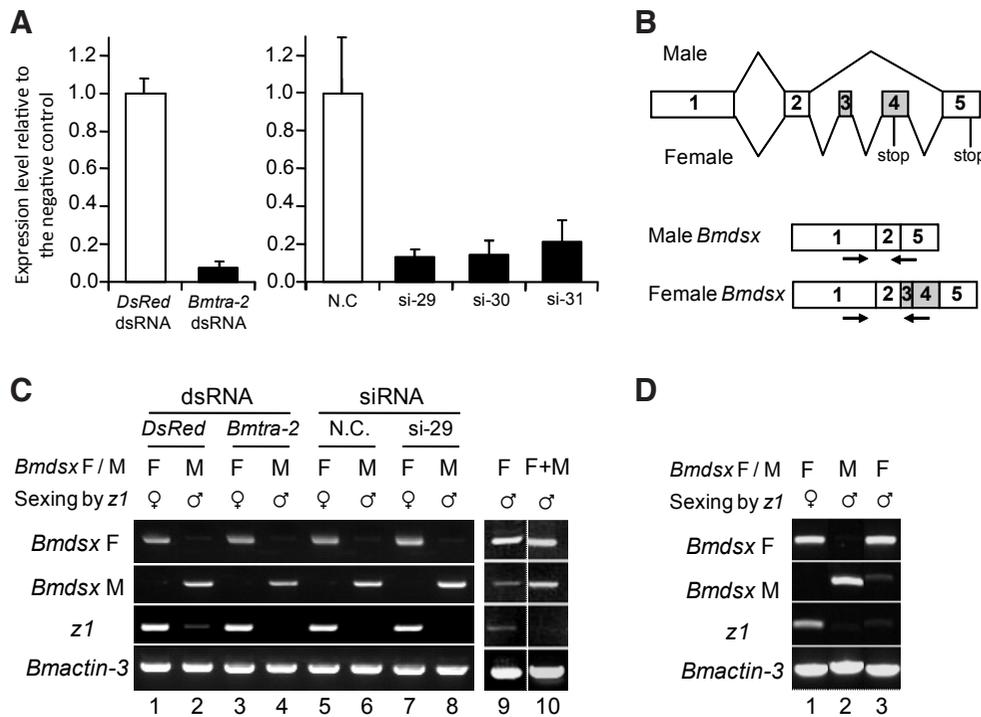


Fig. 2. Effect of RNAi-mediated *Bmtra-2* knockdown on the sex-specific splicing of *Bmdsx*. (A) Quantification of *Bmtra-2* mRNA level 4 days after injection using real-time RT-PCR. The elongation factor 2 (EF-2) was used as an internal control. Expression levels of *Bmtra-2* mRNA in eggs injected with *Bmtra-2* dsRNA or siRNAs were calculated relative to those of negative control eggs (shown as 1.00). Error bar, S.D.; n = 10 individuals. (B) Schematic diagram of alternative splicing in *Bmdsx* pre-mRNA. Boxes represent exons. The gray region indicates the female-specific exons. The numbers in the diagram represent exon labels. V-shaped lines above (skipping of alternative exons) and below (inclusion of alternative exons) the diagram represent the endogenous *Bmdsx* splice variants observed in males and females. Stop codons are indicated by stop. The arrows indicate the approximate location of the primers that were used for RT-PCR in (C). (C) Female- or male-specific splicing was detected by RT-PCR and analyzed on a 2% agarose gel. Labels to the left of the

gel refer to female splicing (*Bmdsx F*) and male splicing (*Bmdsx M*). Lower panel shows the results of molecular sexing by RT-PCR amplification of the transcript from the *z1* gene, which is located on the W chromosome. Bottom panel shows the results of RT-PCR amplification of the actin3 transcript, which served as a positive control for the RT-PCR reaction. (D) Abnormal expression of *Bmdsx* observed in untreated eggs.

with dsRNA. These results indicate that the injected dsRNA and siRNAs effectively silenced *Bmtra-2* in the embryo. However, in most cases (90%), downregulation of *Bmtra-2* did not affect the sex-specific splicing of *Bmdsx*, i.e. the expression pattern of *Bmdsx* was consistent with the sex of each embryo as estimated by expression of the W chromosome specific gene *z1* (Ajimura *et al.*, 2006) (Fig. 2C). Similar results were observed when the dsRNA mediated knockdown of *Bmtra-2* were performed using *Bombyx* male and female cultured cells (see Supplementary Figure S1). Although five of the 51 examined embryos (10%) showed abnormal expression of *Bmdsx* (i.e. the expression of female-type *Bmdsx* in male embryos) (Fig. 2C, lanes 9 and 10), this abnormality was also observed in untreated embryos (Fig. 2D, lane 3). This discrepancy may be explained by the accuracy of our molecular sexing. It was incomplete and sometimes the *z1* transcript was not amplified from embryos, which were identified as females by PCR using the female-specific SCAR marker. Its accuracy was approximately 97%.

Taken together, these results suggest that even though *Bmtra-2* can facilitate the female-specific splicing of *dsx*, it is not involved in the sex-specific splicing of *Bmdsx*.

The *Bmtra-2 B* isoform is predominantly expressed in testis

In *Drosophila*, *tra-2* pre-mRNA produces multiple mRNAs encoding three distinct isoforms of Tra-2 protein (Tra-2²⁶⁴, Tra-2²²⁶ and Tra-2¹⁷⁹) by alternative splicing (Mattox and Baker, 1991). Tra-2²²⁶ and Tra-2¹⁷⁹ are specifically expressed in the male germline with Tra-2²²⁶ essential for both testis-specific *exu* splicing and male fertility (Mattox *et al.*, 1996). *Bmtra-2* pre-mRNA can also yield six alternatively spliced mRNAs encoding six distinct isoforms of BmTra-2 protein (Niu *et al.*, 2005). To investigate which, if any, of these BmTra-2 isoforms are expressed in the male germline, RT-PCR analyses were performed with primer pairs reported by Niu *et al.*, (2005) that amplify specific portions of each isoform. We found that most of the isoforms were ubiquitously expressed

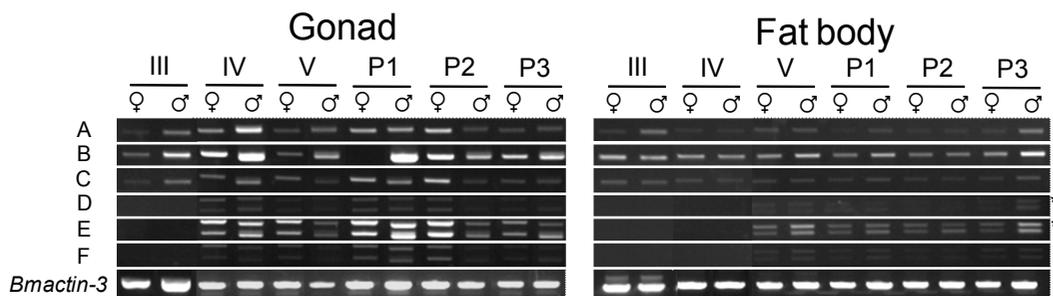


Fig. 3. RT-PCR analyses of the isoform-specific expression pattern of *Bmtra-2*. Total RNAs extracted from the gonad and fat body of males and females at different developmental stages were used as templates for RT-PCR reactions. Each of the isoforms (A–F) was amplified by RT-PCR using primer pairs reported by Niu *et al.*, (2005) that can amplify specific portions of each isoform. III, IV and V indicate 3rd, 4th and 5th instar larvae, respectively. P1, P3 and P5 indicate day-1, day-3 and day-5 pupae. RT-PCR amplification of the actin3 transcript was used as a positive control for RT-PCR reaction. Asterisks indicate unexpected transcripts that were not observed in the previous report described by Niu *et al.*, (2005).

instar larvae, respectively. P1, P3 and P5 indicate day-1, day-3 and day-5 pupae. RT-PCR amplification of the actin3 transcript was used as a positive control for RT-PCR reaction. Asterisks indicate unexpected transcripts that were not observed in the previous report described by Niu *et al.*, (2005).

in all of the tissues examined (Fig. 3). Isoform B mRNA, however, was predominantly expressed in the testis during the larval and early pupal stages with highest testis expression observed 1 day after pupation (P1). In addition, the expression levels of A, C, and E isoforms were relatively higher in the ovary as compared with those in the testis at 3 days after pupation (P3). These results may imply that *Bmtra-2* plays an important role in germline functions or gonadal development.

Downregulation of *Bmtra-2* in early embryo affects testis morphogenesis

To evaluate whether *Bmtra-2* does indeed play an important role in germline functions or gonadal development, we injected *Bmtra-2* dsRNA corresponding to a common region among the *Bmtra-2* isoforms into pupae just after pupation. However, no reduction in *Bmtra-2* transcripts was observed in pupae injected with the *Bmtra-2* dsRNA even when the amount of injected dsRNA per pupa was increased to 50 µg (data not shown). Therefore, we decided to use embryos for the RNAi experiments. *Bmtra-2* dsRNA was injected into eggs laid by the sex-limited *p^{Sa}* (sable larval marking) strain, which enables us to discriminate females from males by a larval phenotypic marker (Tazima, 1943). Since this strain produces diapausing eggs, dsRNA was injected into eggs about 1 day after oviposition due to the necessity of using hydrochloric acid treatment to break diapause. Sexing of each larva was carried out at the 3rd larval stage based on the sex-linked phenotypic marker, *p^{Sa}*. The gonads were dissected out and were observed with a stereomicroscope. As shown in Fig. 4, *Bmtra-2* RNAi caused abnormal testis morphologies in three of the 21 larvae examined. The number of testicular lobes decreased in all cases (Fig. 4B,

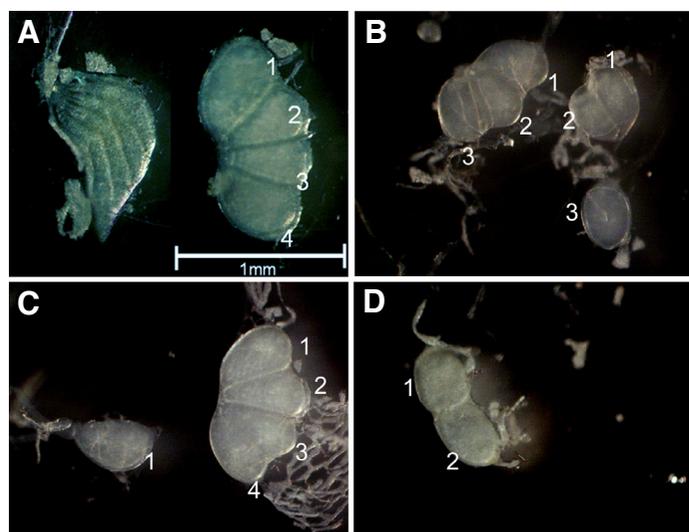


Fig. 4. Effect of RNAi-mediated *Bmtra-2* knockdown in the early embryo. dsRNAs were injected into eggs laid by the sex-limited *p^{Sa}* (sable larval marking) strain approximately 1 day after oviposition. Gonads were dissected out during the 3rd larval stage and observed with a stereomicroscope. (A) An ovary (left) and testis of a DsRed dsRNA injected individual. Note that a normal testis consists of four testicular lobes. Scale bar indicates 1 mm. (B–D) Abnormal testes observed in the *Bmtra-2* dsRNA injected individuals. The number of testicular lobes decreased in both testes (B). Although the right testis was normal, the left testis consisted of only one testicular lobe (C). The left testis consisted of only two testicular lobes while the right testis was non-existent (D).

C, and D). Moreover, one of the testicular lobes was dissociated from the others (Fig. 4B). In the most extreme case, the testis on one side was completely absent (Fig. 4D). The remaining larvae were reared to adult moths and their fertilities investigated. All of the examined male and female moths had nearly wild-type levels of fertility (data not shown). Similar results were observed when siRNA (si29) was used in the microinjections (data not shown). These results indicate that *Bmtra-2* has an important role in testis morphogenesis during the early embryonic stages.

Discussion

Here we have presented evidence that *Bmtra-2* is not involved in the sex-specific splicing of *Bmdsx* despite being able to facilitate the female-specific splicing of *dsx*. Taken together with our previous research, which found that female splicing of *Bmdsx* pre-mRNA represents the default mode when tested in HeLa nuclear extracts, and that the female exon is devoid of putative Tra/Tra-2 binding sites (Suzuki et al., 2001), it seems unlikely that *Bmtra-2* participates in the regulation of sex determination. This situation is similar to *tra-2α*, a human homolog of *tra-2*. *tra-2α* is not known to participate in the regulation of sex determination, but is capable of functionally replacing the endogenous Tra-2 protein when expressed in transgenic flies (Dauwalder et al., 1996). Previous studies have shown that the Tra-2α protein is a sequence-specific activator of pre-mRNA splicing (Tacke et al., 1998). Tra-2α binds to the exonic splicing enhancer on exon 4 of the gonadotropin-releasing hormone (GnRH) gene and promotes the neuron-specific excision of intron A from the GnRH pre-mRNA together with 9G8 and SRp30c (Seong et al., 2002). In this context, the protein level of Tra-2α is strikingly high in adult mouse brain, but low or barely detected in other tissues (Chen et al., 2005). The scavenger receptor class B (SR-B) pre-mRNA can produce two alternatively spliced mRNAs encoding two distinct SR-B protein isoforms (SR-B type I and type II) in response to estrogen stimulation. Tra-2α and, in particular, Tra-2β, another human homolog of *tra-2*, cause a shift from the SR-BI to SR-BII splice form (Zhang et al., 2007).

Tra-2β interacts with RBMY, which is a male germline RNA binding protein and potential alternative splicing regulator (Venables et al., 2000). This interaction modulates the splicing of several pre-mRNAs regulated by Tra-2β (Dreumont et al., 2010). For instance, two alternative exons in *CREB* and *Acinus* genes, which are efficiently included in the testis, are negatively regulated by Tra-2β and positively regulated by RBMY (Dreumont et al., 2010). Tra-2β also recapitulates testis-specific splicing of the homeodomain-interacting kinase *HipK3* gene in a concentration-dependent manner and binds specifically to a long purine-rich sequence in the *HipK3* testis-specific exon (Venables et al., 2005). In this context, the Tra-2β protein is most highly expressed in mouse and human testes (Venables et al., 2000, 2005). These findings that Tra-2β is expressed particularly well in testis and that it modulates the testis-specific splicing of several genes are reminiscent of the functions of testis-specific Tra-2 isoforms that modulate testis-specific splicing of pre-mRNAs from two genes (*exu* and *attf*) expressed during spermatogenesis. Therefore, an evolutionarily conserved role of Tra-2 protein seems likely to regulate testis-specific splicing of target pre-mRNAs.

Similar to *tra-2* and *tra-2β*, one of the *Bmtra-2* isoforms (B isoform) was predominantly expressed in testis during the larval and early

pupal stages (Fig. 3). Furthermore, downregulation of *Bmtra-2* in early embryos caused abnormalities in testis morphology (Fig. 4B, C and D). These results indicate that *Bmtra-2* has an important role in testis morphogenesis during the early embryonic stages. It is possible to hypothesize that the BmTra-2 B isoform may regulate testis-specific splicing of target genes that are crucial for testis morphogenesis. In the present study, the number of larvae showing abnormalities in testis was low (14.3%). As described above, in this experiment, dsRNA was injected into eggs about 1 day after oviposition due to the necessity of using hydrochloric acid treatment to break diapause. Previous work has demonstrated that siRNA injection 1 or 2 days after oviposition causes a drastic decrease in the effectiveness of RNAi (Yamaguchi *et al.*, 2011). Therefore, it seems possible that the low number of individuals with abnormal testis is due to the inappropriate timing of dsRNA injections.

Contrary to our findings that knockdown of *Bmtra-2* caused abnormalities in testis morphology, complete loss of Tra-2 function in *Drosophila* does not affect testis development (Unni *et al.*, 2003). Moreover, *tra-2* is only required in *Drosophila* for sexual differentiation such as female determination, male courtship behavior, and spermatogenesis, but is dispensable for general viability (Unni *et al.*, 2003). Differing from the *Drosophila* situation, functional orthologs of Tra-2 protein are thought to perform non-sex-specific functions essential for viability in both vertebrates (Seong *et al.*, 2002) and nematodes (WormBase web site, <http://www.wormbase.org>, release WS98). These findings suggest that the biochemical activities and RNA recognition specificities of Tra-2 proteins in the fly and other organisms may be similar while the functions of the targeted mRNAs differ. It is therefore more suitable to consider the conserved function of Tra-2 as a tissue-specific alternative splicing factor rather than as a sex determination factor. The recruitment of Tra-2 into the sex determination pathway in *Drosophila* may reflect the rapid evolution of sex-determining mechanisms. Although the proteins in the sex determination pathways often show high sequence similarities, their participation in sexual differentiation is often not conserved (Marin and Baker, 1998). In fact, the DM domain proteins, DSX- and MAB-3-related transcription factors (DMRTs), are the only known examples of proteins that control sex determination or sexual differentiation in a broad array of metazoan animals (Matsuda *et al.*, 2002).

As discussed above, an evolutionarily conserved role of the Tra-2 protein seems likely to regulate testis-specific splicing of genes expressed in testis or male germline. Consistent with this hypothesis, our findings in this study indicate that *Bmtra-2* has an important role in testis morphogenesis during the early embryonic stages. At present, we cannot exclude the possibility that *Bmtra-2* is also necessary for spermatogenesis since we were unable to successfully utilize RNAi-mediated knockdown to repress the expression of *Bmtra-2* during the larval and pupal stages. However, because *Bmtra-2* does not encode a protein isoform corresponding to the testis-specific Tra-2 isoforms, Tra-2²²⁶ and Tra-2¹⁷⁹, which are essential for spermatogenesis (Niu *et al.*, 2005), it can be assumed that *Bmtra-2* is not involved in *Bombyx* spermatogenesis. In addition, *Bmtra-2* may play an important role in female germline function or ovary development since the expression levels of A, C, and E isoforms were relatively higher in the ovary as compared with those in the testis at 3 days after pupation (Fig. 3). A full understanding of the function of *Bmtra-2* in the silkworm awaits further investigation.

Materials and Methods

Plasmid construction

The copia-dsx minigene, hsp-tra and hsp-tra-2 cDNA constructs were kindly provided by Prof. Kunio Inoue of Kobe University. The *dsx* genomic sequence was excised from the copia-dsx minigene construct and was cloned into the pHE-N vector multiple cloning site (Imai *et al.*, 2005) to allow expression of the *Drosophila dsx* gene in *Bombyx* cultured cells. 1 µg of the hsp-dsx minigene was transfected into BmN cells with or without 1 µg of hsp-tra or 1 µg of hsp-tra2 as shown in Fig. 1 according to the protocol described previously (Suzuki *et al.*, 2008).

Silkworm strains

The *Bombyx mori* non-diapause and white egg strain, pnd-w1, was kindly provided by Dr. Ken-ichi Moto of RIKEN, and the sex-limited *p^{sa}* (sable larval marking) strain, was provided by Dr. Takeshi Yokoyama from the Tokyo University of Agriculture and Technology. The developing eggs were incubated at 25 °C with sufficient humidity completely enclosed in a plastic case. Larvae were reared on an artificial diet (Nihon Nosan) at approximately 25 °C.

Preparation of dsRNAs and siRNAs

Sequence conserved between the *Bmtra-2* isoforms was amplified with primers TRA2dsRNAF2 (5'- CCG GAT CCT AAT ACG ACT CAC TAT AGG GCG ATT TGT GTC TTC AAC GTG C-3') and TRA2dsRNAR3 (5'- CCG GAT CCT AAT ACG ACT CAC TAT AGG GCG TAG GAG CGC TCC CGC TCG TAG -3'). Both primers contained a T7 promoter site (underlined). The amplified products were separated on a 2% agarose gel. A band of the appropriate size was excised and purified using a Wizard SV Gel and PCR Clean-Up System (Promega). The purified product was used as a template for *in vitro* transcription. Complementary RNA strands were transcribed using an AmpliScribe T7 Transcription Kit (CellsScript) and annealed by heating to 95°C for 1 min and then allowing to cool to room temperature. The quality of the dsRNA was examined by electrophoresis on a 2% agarose gel. The indicated amounts of dsRNAs were transfected into BmN cells according to the protocol described previously (Suzuki *et al.*, 2008).

Three siRNAs targeted to *Bmtra-2* (siRNA29, siRNA30, siRNA31) were designed using BLOCK-iT RNAi Designer (<https://rnaidesigner.invitrogen.com/rnaiexpress/>). The sequences of the siRNAs are as follows: siRNA29: sense- GCA CCG GAA UGG AAA UUG Att, antisense- UGA AUU UCC AUU CCG GUG Cat; siRNA30: sense- GCA GAU AAA CCA UAU CUU tt, antisense- AAA GAU AUG GUU UAU CUG Ctg; siRNA31: sense- CCG CGU CGA UUA UUC CAU Att, antisense- UAU GGA UUA AUC GAC GCG Gat. Each siRNA was synthesized using the Custom Select siRNA synthesis service provided by Ambion. Silencer Negative Control #1 siRNA (Ambion) was used as a negative control in the siRNA experiments.

Transfection of DNA and dsRNAs into cells

1 µg of the hsp-dsx minigene was transfected into BmN cells with or without 1 µg of hsp-tra or 1 µg of hsp-tra2 as shown in Fig. 1 according to the protocol described previously (Suzuki *et al.*, 2008). To examine whether *Bmtra-2* is capable of inducing the female-specific splicing of *Drosophila dsx*, the indicated amounts of *Bmtra-2* dsRNA were transfected into BmN cells as described previously (Suzuki *et al.*, 2008). Two days after dsRNA transfection, the cells were transfected with the hsp-dsx minigene (1 µg) together with hsp-tra (1 µg). Poly (A)+RNA was isolated 48 h after transfection using a Micro-FastTrack™ 2.0 mRNA-isolation kit (Invitrogen) according to the protocol provided by the manufacturer.

Injection of dsRNAs and siRNAs into eggs

dsRNAs and siRNAs were injected into eggs according as described by Yamaguchi *et al.*, (2011) with a slight modification. Briefly, eggs were aligned in the same direction and glued onto the surface of glass slides covered with liquid glue (Arabic Yamato, YAMATO). The eggs were perforated on the ventral side with a 0.5 µm diameter tungsten probe tip (Micro

TABLE 1

PRIMER SEQUENCES AND PCR CONDITIONS UTILIZED IN THIS STUDY

gene	primers	sequence	denaturation	annealing	elongation	n° cycles
<i>Bmtra-2</i>	BmTra2F1	ATGTCGTGATCGAGAGAGAAGTCGTTC	98°C 10 sec	55°C 30 sec	72°C 60 sec	26
	BmTra2R1	AGCGCTCCCGCTCGTAGCGTC				
<i>Bmdsx F</i>	FDSXF2	CGCCTTACCCGACAGGCGAG	98°C 10 sec	57°C 30 sec	72°C 60 sec	35
	E3E4R1	AATTTCCAGCATTTCTGGCG				
<i>Bmdsx M</i>	FDSXF2	CGCCTTACCCGACAGGCGAG	98°C 10 sec	57°C 30 sec	72°C 60 sec	35
	E2E5R	TGCATCATCCAATAACCTTCG				
<i>Bmactin-3</i>	BA3F1	AGATGACCCAGATCATGTTTCG	98°C 10 sec	55°C 30 sec	72°C 40 sec	26
	BA3R1	GAGATCCACATCTGTTGGAAG				
Female <i>dsx</i>	hspF1	CAACTACTGAAATCTGCCAAG	98°C 10 sec	57°C 10 sec	72°C 30 sec	30
	DmdsxFR2	ATTCACAACGTATTGGCCCTC				
Male <i>dsx</i>	hspF1	CAACTACTGAAATCTGCCAAG	98°C 10 sec	57°C 10 sec	72°C 30 sec	30
	DmdsxMR2	ATTTATTTCCACTCGAGCCTC				
<i>Bmtra-2 A</i>	Bmtra2L	GCAGAAAATATAGAATCCCATG	94°C 15 sec	55°C 30 sec	72°C 60 sec	26
	Bmtra2R11	CTAAGTTTACATTATAGAGGCGTG				
<i>Bmtra-2 B</i>	Bmtra2M	GCAGAAAATATAGAAGCCGACA	94°C 15 sec	55°C 30 sec	72°C 60 sec	26
	Bmtra2R11	CTAAGTTTACATTATAGAGGCGTG				
<i>Bmtra-2 C</i>	Bmtra2S	GCAGAAAATATAGAATCCCATC	94°C 15 sec	55°C 30 sec	72°C 60 sec	26
	Bmtra2R11	CTAAGTTTACATTATAGAGGCGTG				
<i>Bmtra-2 D</i>	Bmtra2L	GCAGAAAATATAGAATCCCATG	94°C 15 sec	55°C 30 sec	72°C 60 sec	26
	LBmtra2R22	GTCGGAAGATAAAGAATATGATC				
<i>Bmtra-2 E</i>	Bmtra2M	GCAGAAAATATAGAAGCCGACA	94°C 15 sec	55°C 30 sec	72°C 60 sec	26
	LBmtra2R22	GTCGGAAGATAAAGAATATGATC				
<i>Bmtra-2 F</i>	Bmtra2S	GCAGAAAATATAGAATCCCATC	94°C 15 sec	55°C 30 sec	72°C 60 sec	26
	LBmtra2R22	GTCGGAAGATAAAGAATATGATC				

Support Corp.). Injections were performed using a glass capillary (GN-1C, ST Science). Total RNA was extracted from each egg 4 days after injection using Isogen (Nippon Gene).

RT-PCR analyses

First-strand cDNAs were synthesized from 500 ng of poly (A)+RNAs extracted from the RNAi-treated cells using a PrimeScript cDNA synthesis kit (TaKaRa Bio Inc.) according to the protocol provided by the manufacturer with random hexamers as primers. Total RNA (1 µg) extracted from the RNAi-treated eggs was transcribed using the same cDNA synthesis kit with the oligo-dT primer included in the kit. An aliquot (1/20th of the resulting reaction) was used for PCR using EmeraldAmp PCR Master Mix (TaKaRa Bio Inc.). The primer sequences and PCR conditions utilized in this study are indicated in Table 1. To perform molecular sexing of each egg, the *W* chromosome specific gene *z1* was amplified using primers *z1-3* and *z1-11* (Ajimura et al., 2006). PCR products were analyzed on a 2% agarose gel and visualized with ethidium bromide.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR assays were performed using SYBR Premix Ex Taq II (TaKaRa Bio Inc.) on a Thermal Cycler Dice Real Time System (TaKaRa Bio Inc.) according to the manufacturer's instructions. QTBmtraF1 (5'- ATG TCT GAT CGA GAG AGA AGT CGT TC - 3') and QTBmtraR1 (5'- GCT GTG ACT ATG ACT GCG GCC GGA TG - 3') were used for the quantification of the *Bmtra-2* gene expression. BmEF-2F1 (5'- TCG TAC CGT GAG ACC GTA GCT - 3') and BmEF-2R1 (5' - ATG TCC TCT GGC AGA CCA TCA - 3') were used for the quantification of elongation factor-2 (*BmEF-2*) as an internal standard. The threshold cycle (CT) value was normalized with the CT value of the *BmEF-2* gene using Multiplate RQ software (TaKaRa Bio Inc.). The relative value of *Bmtra-2* expression against *BmEF-2* expression was obtained in triplicate and the ratio of *Bmtra-2* expression calculated.

Acknowledgements

We are grateful to Dr. J. Joe Hull for his helpful comments on the

manuscript. Prof. Kunio Inoue is gratefully acknowledged for providing the *copia-dsx* minigene, *hsp-tra* and *hsp-tra-2* cDNA constructs. We also thank Dr. Ken-ich Moto and Dr. Takeshi Yokoyama for providing the silkworm strains. We are grateful to Haruka Ito for technical supports of microinjections. This work was supported by Grant-in-Aid for Scientific Research (B), 23380032, 2011.

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