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Novel female-specific *trans*-spliced and alternative splice forms of *dsx* in the silkworm *Bombyx mori*

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ABSTRACT

The *Bombyx mori doublesex* gene (*Bmdsx*) plays an important role in somatic sexual development. Its pre-mRNA splices in a sex-specific manner to generate two female-specific and one male-specific splice forms. The present study investigated six novel *dsx* variants generated by *trans*-splicing between female *dsx* transcripts and two additional novel genes, *dsr1* and *dsr2*. Expression analysis indicated that *Bmdsx-dsr1* represented splicing noise, whereas *dsr2*, which *trans*-spliced with *dsx* to generate five variants, regulated the expression of the female-specific *B. mori dsx* transcript $Bmdsx^{F}$s. We unexpectedly found a novel exon 2n insertion during *Bmdsx* transcription, which did not influence the validity of the novel protein, $BmDSX^{F3}$. Ectopic expression of $BmDSX^{F3}$ repressed the pheromone-binding protein gene and the testis-specific gene *A2* in males, and activated of the storage protein 1 gene. Our findings suggest that *trans*-splicing is a novel regulatory function of *Bmdsx*, which participates in female sexual development by regulating the expression of three $BmDSX^{F}$ proteins.

1. Introduction

Sexual development involves a cascade of gene regulation. Despite differences among organisms in terms of the primary signal for sex determination, some key genes and functions lower down the cascade are relatively conserved [1,2], notably the *doublesex* gene. *dsx* in insects encodes a zinc-finger transcription factor of the *doublesex*/mab-3-related transcription factor (DMRT) family and is a homolog of the human divalent metal transporter 1 (*DMT1*) and mouse *Dmrt1* genes [3,4].

The sex determination mechanism in *Drosophila melanogaster* has been thoroughly examined, and a well-characterized genetic hierarchy (X: A > *Sxl* > *tra*/*tra2* > *dsx* and *fru*) has been shown to regulate somatic sexual differentiation and behavior [5–7]. *Dmdsx* alternatively splices into two sex-specific transcripts that encode two sex-specific proteins, $DmDSX^{F}$ in females and $DmDSX^{M}$ in males [8]. These two proteins have common amino termini but sex-specific carboxyl termini and act with other development-related genes to control somatic sexual differentiation and pattern dimorphisms, including the shape of sex-specific neurons [7], sex pheromone production [9], genital development [10,11], abdominal pigmentation [12,13], loss of the terminal male abdominal segment [14], and the origin and diversification of sex combs [15].

However, the hierarchy of sex determination in *Bombyx mori* has not been described in detail. *B. mori* demonstrates female heterogamety (ZW), which differs from that in female *Drosophila* (XX). Sex determination is initiated by the number of X chromosomes in *Drosophila* [16], whereas a putative feminizing factor (*Fem*) on the W chromosome is proposed to determine the femaleness of silkworms [17]. *Bmdsx*, the *B. mori* homolog of *Dmdsx*, was shown to splice in a sex-specific manner into two female forms ($Bmdsx^{F1}$ and $Bmdsx^{F2}$) and one male form ($Bmdsx^{M}$) [18,19]. The expression of $Bmdsx^{F1}$ in males or $Bmdsx^{M}$ in females can repress or activate the expression of three downstream genes (pheromone-binding protein, *PBP*; vitellogenin, *Vg*; and storage protein 1, *SP1*) [20,21]. However, limited knowledge of the regulatory mechanism of *Bmdsx* and the paucity of identified BmDSX downstream targets mean that our understanding of important aspects of sexual development in the silkworm is limited. In this study, we investigated the existence of *trans*-splicing forms of *Bmdsx* and their roles in the regulation of female-specific *dsx* mRNA translation.

2. Materials and methods

2.1. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from tissues (head, integument, hemocytes, midgut, fat body, trachea, Malpighian tubule, silk gland, gonads)

Abbreviations: *Bmdsx*, *Bombyx mori doublesex*; $Bmdsx^{F}$, female-specific splicing form of *Bmdsx*; $Bmdsx^{M}$, male-specific splicing form of *Bmdsx*; *dsr1* and *dsr2*, *Bmdsx*-related gene 1 and 2; ncRNA, non-coding RNA; pA, SV40 polyadenylation signal.

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from both sexes, and first-chain cDNA synthesis and RT-PCR analysis were performed as described previously [22]. PCR amplifications were conducted using the primer sets (Table S1) P9/P2 for *Bmdsx-dsr1*, P5/P6 for *Bmdsx-dsr2*, and P7/P8 for the novel exon 2n. P5, P7, and P9 bound specifically to the exon 1 common to both sex *Bmdsx* transcripts. P2 bound to *dsr1*, P6 bound to *dsr2*, and P8 bound to exon 2n.

2.2. Embryo injection and screening of transformed animals

The helper plasmid pHA3PIG [23] was mixed with the transgenic construct derived from the basic vector pBac[3 × P3-EGFPafm] [26] (Supporting Information). About 10–15 nl of a 1:1 mixture of construct and helper plasmids (400 ng/μl total DNA concentration) in Buffer EB (Qiagen, Germany) were micro-injected into each non-diapause egg (Table S2), which was prepared using the acid-treatment method after chilling. The embryos were maintained at 25 °C in moist petri dishes until hatching. G0 adults were backcrossed and the G1 progeny were screened for enhanced green fluorescent protein (EGFP) fluorescence in the embryo using an Olympus MVX10 fluorescence stereomicroscope (Olympus, Japan).

2.3. Inverse PCR analysis (iPCR)

Genomic DNA was extracted from G1 adults as described previously [23], digested with *Hae*III and circularized by ligation overnight at 16 °C. PCR was performed on the circularized fragments using the primer sets (Table S1) P16/P17 for the left-arm region and P18/P19 for the right-arm region. PCR fragments were cloned, and compared by BLAST analysis against the BGI assembly (http://www.silkdb.org/silkdb/genome/index_png.html) [24,25].

2.4. Southern blot analysis

Genomic DNA was digested with *Xba*I and the digested DNA (50 μg per lane) was separated on a 0.8% (wt/vol) agarose gel and transferred onto a nylon membrane (Roche, Germany) under a vacuum. The membrane was hybridized at 46 °C with a 720-bp digoxigenin-labeled EGFP probe and immersed in a solution containing alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche). The EGFP probe was synthesized using a PCR DIG Probe Synthesis Kit (Roche) and the hybridization signal was visualized using a chemiluminescent method with a Clinx Chemiscope Series Chemiluminescence imaging system (Clinx Science Instruments, China).

2.5. W chromosome-specific PCR

Transgenic animals were sexed by assaying for the presence of a W chromosome, using the W chromosome-specific primer set P20/P21 (Table S1). Genomic DNA was extracted as above and used as a template.

2.6. Real-time quantitative PCR (Q-PCR)

RNA was extracted from female fat bodies and male testes from G2 day-3 fifth-instar larvae and male antennae from G2 adults. cDNAs were produced as above. The primer sets P22/P23, P24/25 and P26/P27 (Table S1) were used to compare the expression levels of *SP1*, *PBP* and *tsA2*, respectively [20,27,28] between transgenic and wild-type silkworms. Q-PCR was carried out using SYBR® Premix Ex Taq™ II (TaKaRa), and amplifications were detected with the StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative expression levels were calculated after correction for expression of the translation initiation factor 4A (*eIF-4A*), which was chosen as a reference gene [29] and assayed in triplicate, in parallel with *SP1*, *PBP* and *tsA2*.

Before Q-PCR analysis, RNA from the same transgenic individuals was used to analyze the expression of the $Bmdsx^{F3}$ transgene by 3′ rapid amplification of cDNA ends (RACE), as described (Supporting Information), and RT-PCR was performed using the primer set P9/P28 (Table S1). Primer P28 was specific for the transgenic construct (Fig. S4).

3. Results

3.1. Trans-spliced variants between Bmdsx and two dsx-related genes, Bmdsr1 and Bmdsr2

Cloning of the 3′ end of *Bmdsx* produced two novel *trans*-spliced variants, *Bmdsx-dsr1* and *Bmdsx-dsr2* (Fig. S1). Structural analysis of the two variants suggested that *Bmdsr1* and *Bmdsr2* participate in the regulation of silkworm sexual development by *trans*-splicing with the female splice form $Bmdsx^{F1}$. We therefore investigated these potential *trans*-spliced variants by PCR amplification to determine whether the two genes also *trans*-spliced with another female splice form, $Bmdsx^{F2}$, and with the male-splicing form, $Bmdsx^{M}$. *Trans*-spliced variants of *Bmdsx* and *dsr2* were expressed only in the female integument, fat bodies, trachea, silk gland, ovary, and testis, whereas *Bmdsx-dsr1* expression was undetected (Fig. 1A). Cloning and sequencing all RT-PCR products demonstrated that the putative *Bmdsr2* gene was able to *trans*-splice with $Bmdsx^{F2}$ in several ways, but was unable to *trans*-splice with $Bmdsx^{M}$ (Figs. 1B and S7). Interestingly, two *trans*-spliced variants occurred in female-specific tissues: *Bmdsx-dsr2*a in the female fat

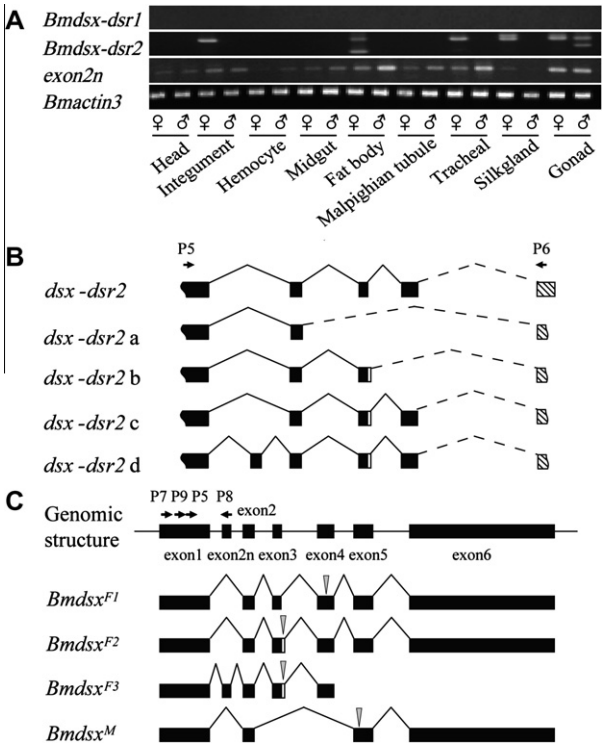

Fig. 1. *Trans*-spliced and alternative-spliced forms of *Bmdsx*. (A) Expression patterns of *Bmdsx-dsr1*, *Bmdsx-dsr2*, and the novel exon 2n in nine tissues from female and male silkworms. *Bmactin3* was used as control. (B) Structures of the five *trans*-spliced variants between *Bmdsx* and *Bmdsr2*. Solid bar represents *Bmdsx* exons, and the "V" line indicates introns. *Bmdsr2* exon is indicated by the slash box. (C) pre-mRNA of *Bmdsx* splices to produce four outcomes. Primers are indicated by horizontal arrows. Grey arrowhead shows the stop codon. Exon 2n represents the novel 81-bp exon.

body and *Bmdsx-dsr2*d in the female silk gland. The *Bmdsx-dsr2*a variant may generate a BmDSX^{F} protein that lacks the carboxyl-terminus. Despite the fact that *Bmdsx-dsr2*b expression was confined to the male testis, the presence of exon 3 indicated that this variant was derived by *trans*-splicing between *Bmdsx*^{F2} and *dsr2*. We unexpectedly discovered a novel 81-bp exon 2n between exon 1 and exon 2 of the *Bmdsx* fragment in *Bmdsx-dsr2*d (Figs. 1B and S7). PCR analysis revealed that exon 2n was common to both sexes (Fig. 1A).

3.2. Deduced protein encoded by *Bmdsx*^{F3}

The presence of *Bmdsx-dsr2*d and of the novel 81-bp exon 2n indicated the existence of a novel female-specific splice form *Bmdsx*^{F3}, which differed from *Bmdsx*^{F2} through the presence of the novel 81-bp exon 2n between exons 1 and 2 (Fig. 1C). The gain of the novel exon 2n inserts 27 amino acids (aa) between the DBD/OD1 and the DBD/OD2 domains. However, this insertion does not change the functional domains of BmDSX^{F3} (Fig. S3), suggesting that the deduced protein BmDSX^{F3} may participate in regulating the sex development of female soma, together with BmDSX^{F1} and BmDSX^{F2}. Consequently, the putative protein encoded by *Bmdsx*^{F3} (273 aa) is 27 aa longer than BmDSX^{F2} (246 aa).

3.3. Transformation experiment

To study the role of *Bmdsx*^{F3} in sex differentiation, the *Bmdsx*^{F3} transgenic line was established using the *piggyBac* vector containing a *Bmdsx*^{F3} cDNA under the control of a *BmA4* promoter. As shown in Table S2, one green fluorescent protein (GFP)-positive brood containing seven GFP-positive individuals was identified. Sexing the G1 GFP-positive larvae at the day-1 fifth-instar stage showed that G1 individuals were normal females and males, in roughly equal proportions (Table S2). The sex ratio and morphology of G2 GFP-positive adults were also normal (data not shown).

3.4. Insertion site determination and Southern blot analysis

To confirm successful transformation and that insertion of the transgene had not disrupted important genes related to sexual regulation, we examined the transgenic insertion site and gene copy number. Inverse PCR showed that the sequence of the two *piggyBac* arms was bordered by the characteristic TTAA sequence (Fig. 2B), and the transgene was inserted into chromosome 23 (nscaf3027) between the two genes, *BGIBMGA011555* and *BGIBMGA01156*, which are juvenile hormone-binding protein genes, not related to sexual regulation. Southern blot analysis showed that a single band was present in the two transgenic sexes, indicating a single integration in the *Bmdsx*^{F3} transgenic line (Fig. 2C).

3.5. Expression of the *Bmdsx*^{F3} transgene

Bmdsx^{F3} mRNA transcription from the transgene was detected by 3′ RACE analysis of RNAs from transgenic fat bodies. Sequencing analysis revealed a *Bmdsx*^{F3} transcript containing the 3′ untranslated region (UTR), which was part of the SV40 polyadenylation signal (Figs. S4A and B), proving that the exogenous *Bmdsx*^{F3} mRNA was transcribed from the transgene. This result was confirmed by RT-PCR analysis using a pair of primers that specifically recognized the transgenic *Bmdsx*^{F3} transcript, and not endogenous *Bmdsx* mRNA, using the same RNA as a template (Fig. S4C).

3.6. Molecular sexing of transgenic silkworms

To eliminate the possibility that sex transformation took place, we typed the transgenic silkworms by PCR using W chromosome-specific primers. PCR analysis confirmed that all the transgenic females and males examined were chromosomally females and males (Fig. 3A), respectively, demonstrating that males had not been transformed into females.

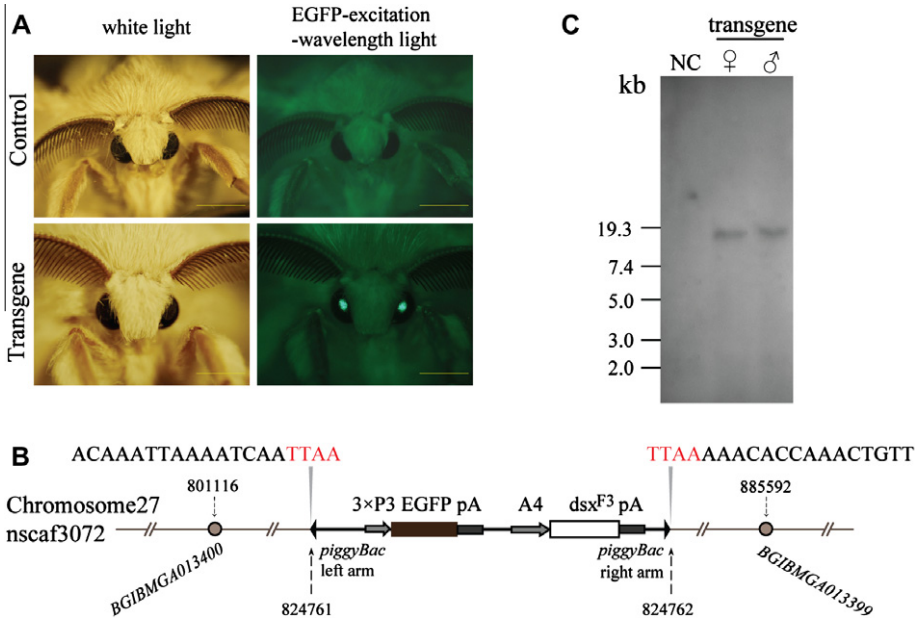

Fig. 2. Gain of the *Bmdsx*^{F3} transgenic line. (A) Transgenic silkworm positive for EGFP fluorescence in the eyes. (B) Genomic structure surrounding the *piggyBac* insertion, with the TTAA duplicated sequence appearing at all 5′ and 3′ insert boundaries. Grey arrowhead indicates insertion site. Numbers indicate the chromosomal location of insertion. Circle shows the surrounding genes. (C) Genomic Southern blot analysis to verify the transgene insertion. Genomic DNA from wild-type silkworms was used as a negative control (NC).

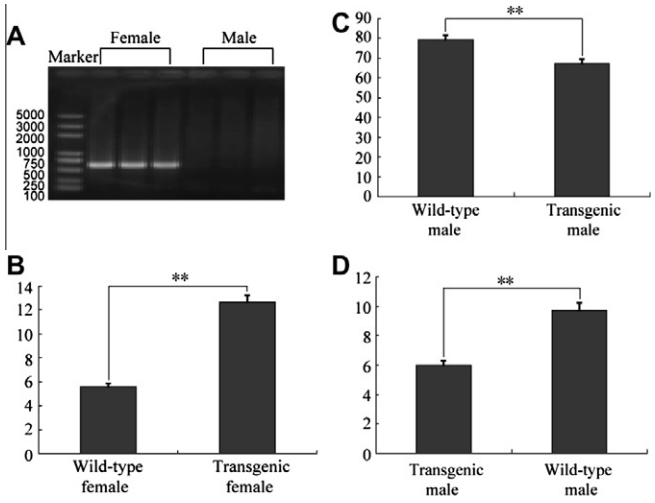

Fig. 3. Molecular sexing and expression analysis of *SP1*, *PBP* and *tsA2* in transgenic silkworms. (A) Molecular sexing by PCR using W chromosome-specific primers. (B) Relative amounts of *SP1* mRNA in female fat bodies from transgenic and wild-type silkworms. (C) Relative amounts of *PBP* mRNA in male antennae from transgenic and wild-type silkworms. (D) relative amounts of *tsA2* mRNA in the testes of transgenic and wild-type males. $^{**}P<0.01$, Student's *t*-test.

3.7. Effects of transgenic expression of $Bmdsx^{F3}$ on gene expression

SP1 is a downstream target gene of *Bmdsx* and has been used to evaluate the effect of sex regulation of *Bmdsx* [20,21,27]. To test if *SP1* was induced in the fat bodies of $Bmdsx^{F3}$-transgenic silkworms, *SP1* mRNA levels were determined by Q-PCR analysis. *SP1* was markedly induced and the levels of *SP1* mRNA in transgenic females were approximately 2.3 times higher than in wild-type females (Fig. 3B). We also determined the expression levels of *PBP* preferentially expressed in male antennae. A repressive effect of the $Bmdsx^{F3}$ transgene on *PBP* was also noted in transgenic males (Fig. 3C).

tsA2 is a testis-specific gene [28], and its promoter region has two potential BmDSX-binding sites (ACATTGT), suggesting that *tsA2* is a novel candidate target gene of *Bmdsx*. To test whether the $Bmdsx^{F3}$ transgene could repress *tsA2* expression in the testis, we compared *tsA2* mRNA levels between wild-type and transgenic silkworms. As shown in Fig. 3D, expression levels of this gene were decreased in transgenic males, to approximately 62% of that in wild-type males.

These results demonstrated that transgenic expression of $BmDSX^{F3}$ induced the expression of the storage protein 1 gene *SP1*, and also repressed the male antennae-specific gene *PBP* and testis-specific gene *tsA2*.

4. Discussion

Alternative splicing is a common splicing mechanism by which pre-transcripts of the sex-switch genes in insects are modulated into sex-specific forms to regulate the somatic sexual development of the two sexes. *Trans*-splicing differs from alternative splicing in the utilization of two distinct pre-mRNAs. *Trans*-splicing studies [30,31] have been reported in mammals [32–34], *Drosophila* [35], and *B. mori* [36], but the relationship between *trans*-splicing and sexual patterns in the silkworm remains unknown. This study provided direct evidence for *trans*-splicing during silkworm sexual development by modulating the translation levels of three $Bmdsx^{F}$ transcripts. Despite the extremely low abundance of *Bmdsx-dsr1*, the *trans*-spliced variants of $Bmdsx^{F}$ and *Bmdsr2* were obviously expressed in female tissues, especially in carcasses without gonads. Despite the identification of the *trans*-spliced variants *Bmdsx-dsr2*b and *Bmdsx-dsr2*d in male gonads, their structures indicated that they were derived from *trans*-splicing between *Bmdsr2* and two female-specific splicing forms ($Bmdsx^{F2}$ and $Bmdsx^{F3}$). In contrast, *Bmdsr2* did not *trans*-splice with the male-specific splice form $Bmdsx^{M}$. These results suggest that (1) *Bmdsr2* is mainly involved in female somatic sexual development by *trans*-splicing with female-specific transcripts of *Bmdsx*, and (2) *Bmdsr2* is a novel additional gene in silkworm sexual regulation. Cell transfection further demonstrated that *Bmdsr2* as a 3′ UTR influenced the negative regulation of female sexual development.

The presence of the female-specific exons 3 and 4 in $Bmdsx^{F3}$ contradicts the existence of the *trans*-spliced variant *Bmdsx-dsr2*d in the testis. The former indicates that $Bmdsx^{F3}$ is only expressed in female tissues, whereas the latter indicates the presence of $Bmdsx^{F3}$ mRNA in the male gonads. The results of the present study suggest that $Bmdsx^{F3}$ might not exhibit absolute sex specificity and might show low expression levels in the testis where it *trans*-splices with *Bmdsr2*d to generate *Bmdsx-dsr2*d. $Bmdsx^{F3}$ is similar to $Bmdsx^{F2}$, which exhibits a female-specific expression pattern and low expression levels in the male gonad [19]. The presence of $Bmdsx^{F2}$ and $Bmdsx^{F3}$ transcripts in male gonads does not influence testis development in normal males. It is possible that *Bmdsr2* suppresses the developmental effects of $BmDSX^{F2}$ and $BmDSX^{F3}$ in the testis by *trans*-splicing. Alternatively, males may lack female-specific cofactors that interact with $BmDSX^{F}$ to influence female sexual differentiation. This hypothesis is strongly supported by the fact that $DmDSX^{F}$ requires the Ix protein as a cofactor to affect female-specific development in *D. melanogaster* [37].

The *dsx* gene has been characterized in various organisms including the dipterans *Musca domestica* [38] and *Anopheles gambiae* [39], the hymenopterans *Apis mellifera* [40], *Nasonia vitripennis* [41], and the lepidopterans *Antheraea assama* [42], and *B. mori* [18,19]. In *B. mori*, *Bmdsx* is proposed to have six exons and three splicing forms, including two female forms and one male form. However, our results showed that *Bmdsx* has a novel 81-bp insertion (exon 2n) during *Bmdsx* transcription and a novel female-specific splice form, $Bmdsx^{F3}$. Moreover, changes in the levels of *SP1*, *PBP* and *tsA2* mRNAs induced by the $Bmdsx^{F3}$ transgene further confirmed a role for $Bmdsx^{F3}$ in sexual regulation; its ectopic expression may have had no effect on the development of male-specific morphological features because transgenic males lacked an Ix-like cofactor despite having endogenous *Bmdsx* alleles, and the level of $BmDSX^{F3}$ was unable to antagonize the induction effect of $BmDSX^{M}$. The presence of at least three female splice forms may also explain why ectopic expression of $Bmdsx^{F1}$ in males does not lead to visible gender transformation [20]. We propose a model (Fig. 4) to explain the pattern of action of the three female-specific DSX proteins and two splicing mechanisms during female sexual development. In females, *Bmdsx* generates three alternative female-specific forms, and *trans*-splicing then occurs to down-regulate the expression level of $BmDSX^{F}$ protein to allow female sexual differentiation.

Unlike the situation in lepidopteran insects, dipteran *D. melanogaster*, *M. domestica*, and *A. gambiae*, as well as hymenopteran *A. mellifera* and *N. vitripennis* have only one female-specific DSX protein. However, two female-specific DSX proteins were identified in the domesticated silkworm *B. mori*, as well as in the two wild silkmoth species *A. assama* and *A. mylitta*. Our study also showed that a third female-specific DSX protein is encoded in *B. mori*. Hence, *dsx* in these insects exhibits relative evolutionary conservation in terms of biological function despite variation in its expression. Whether the presence of more than one female-specific DSX protein is peculiar to lepidopterans, and whether the existence of more than two female-specific DSX proteins is a common feature among lepidopterans remains unknown. A deeper understanding

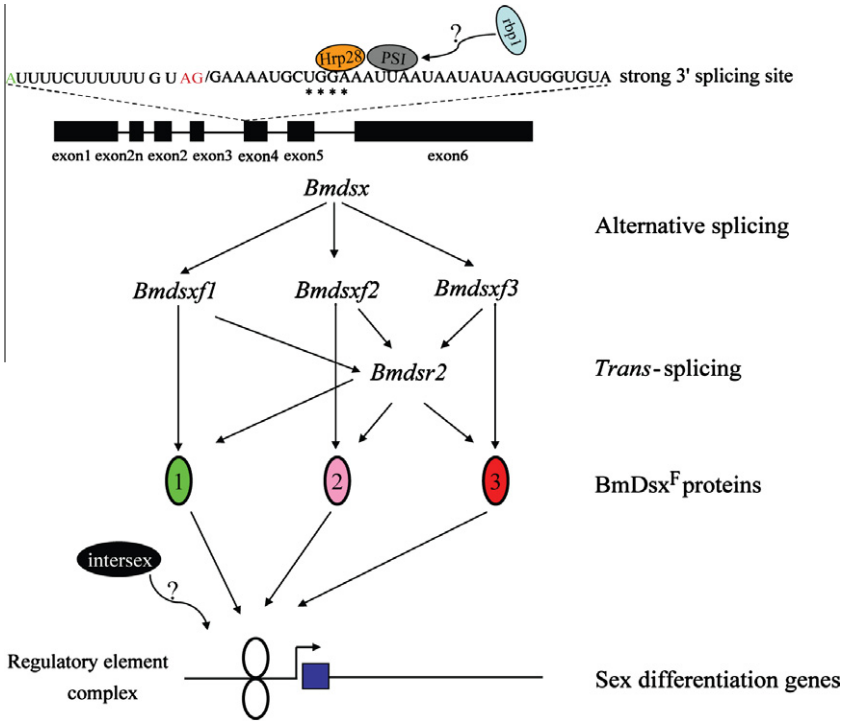

Fig. 4. Model explaining the pattern of action of three BmDSX^F proteins and two splicing methods during female sexual differentiation. In females, *Bmdsx* generates three alternative-spliced forms, and the *Bmdsr2* as a 3′ UTR then down-regulates the expression levels of BmDSX^F proteins by the *trans*-splicing method. Finally, the three proteins further influence the expression of sex-differentiation genes, together with the BmDSX^F homodimers or heterodimers, in a female-specific manner.

of the degree of divergence that can be tolerated while maintaining functional conservation is expected to present a challenge in the coming years.

In conclusion, the silkworm *doublesex* gene *Bmdsx* undergoes *trans*-splicing and alternative splicing. *Trans*-splicing is rare and variants may reflect splicing noise. Some variants, however, may have biological significance and thus warrant further studies. The roles of the splice forms of *Bmdsx* must be clearly determined to clarify its role in silkworm sex-specific morphological differentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.062.

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