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The *Bmdsx* transgene including trimmed introns is sex-specifically spliced in tissues of the silkworm, *Bombyx mori*

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Abstract

Bmdsx is an orthologue of the sex-determining gene *doublesex* (*dsx*) and known to be sex-specifically expressed in various tissues of the silkworm, *Bombyx mori*. Its pre-mRNA is sex-specifically spliced and encodes female-specific or male-specific polypeptides. The open reading frame of *Bmdsx* consists of 5 exons, of which exons 3 and 4 are female-specific and its pre-mRNA was known to undergo default processing to generate the female-type mRNA. Previous reports have shown that the mechanism of splicing of the *doublesex* gene is different in *Drosophila melanogaster* and *Bombyx mori*. However, intron 4 is so long that it is difficult to identify the intronic cis-element(s) required for male-specific splicing of *Bmdsx* pre-mRNA using *Bmdsx* minigenes whose introns are shortened in various manners. As a first step toward discovery of the cis-element, the *Bmdsx* mini gene, which consisted of exon 1 and 5 and internally shortened introns 2 to 4, was constructed, and transgenic silkworms expressing this construct were generated. *Bmdsx* pre-mRNA transcribed derived from transgene was sex-specifically spliced. This result shows that the mini gene contained the information necessary for the correct regulation of alternative splicing.

Keywords: alternative splicing, *Bmdsx, Bombyx mori, piggyback,* long intron

Introduction

Bmdsx in *Bombyx mori* is a homologue of the sex-determining gene *doublesex* (*dsx*) of *Drosophila melanogaster*. Recent studies have shown that the *Bmdsx* gene is involved in somatic sexual differentiation in *Bombyx mori.* The ectopic expression of $Bmdsx^F$ in males activated the expression of two genes, vitellogenin and SP1, predominantly expressed in females (Tojo *et al.,* 1980; Mine *et al.,* 1983; Izumi *et al.,* 1988) and repressed the expression of the pheromone binding protein gene (Suzuki *et al.,* 2003), which is preferentially expressed in males. On the other hand, ectopic expression of $Bmdsx^M$ resulted in the repression of the vitellogenin gene. In addition, females ectopically expressing $Bmdsx^M$ had a well-developed 8th abdominal segment with scales which normal females do not have and structures whose shape looked like that of accessory gland in male (Suzuki *et al*., 2004).

Bmdsx pre-mRNA is spliced sex-specifically, as is *dsx* pre-mRNA (Suzuki *et al.,* 2001), but the mechanism of splicing at the *doublesex* gene is different in *D. melanogaster* and *B. mori* (Suzuki *et al.,* 2001). *dsx* pre-mRNA consists of 6 exons and 5 introns. Exons 5 and 6 are male-specific, and exon 4 is female-specific. The regulation of the female-specific splicing of *dsx* pre-mRNA requires the binding of serine-arginine-rich proteins and the splicing regulators TRA and TRA-2 to exon-enhancer elements to activate the weak female-specific 3' splice site (Hedley and Maniatis, 1991; Ryner and Baker, 1991; Tian and Maniatis, 1992). The activity of TRA and TRA-2 is not required for the processing of *dsx* pre-mRNA in males, therefore, this is considered to be a default form of splicing. On the other hand, the open reading frame of *Bmdsx* consists of 5 exons and 4 introns. Exons 3 and 4 are included into the mRNA only in the female (Suzuki *et al.,* 2001). The related sequences of TRA/TRA-2 binding motif are not present in the *Bmdsx* genomic sequence (Suzuki *et al.,* 2001). *Bmdsx* pre-mRNA undergoes default processing to generate the female *Bmdsx* mRNA (Suzuki *et al.,* 2001). This is the point that the female-specific splice acceptor sites of the *Bmdsx* gene were not weak (Suzuki *et al.,* 2001). In addition, one character of the *Bmdsx* pre-mRNA is that one of the introns (intron4) is extremely long (>40kb) (Suzuki *et al.* 2001).

In mammals, important examples of complex transcription units with very large introns include the dystrophin and cystic fibrosis genes, the neural development genes *quaking* and *reeler, ABLl,* and the retinoblastoma susceptibility gene (Lee *et al.,* 1987; Zielenski *et al.,* 1991; Ahn and Kunkel *et al.,* 1993; Chissoe *et al.,* 1995; Ebersole *et al.,* 1996; Royaux *et al.,* 1997). Large introns are also frequent among the developmental control genes of *D. melanogaster* (Scott, 1987). One of them, the homeotic gene

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Ultrabithorax (*Ubx*), has a 74kb intron. The mRNAs share the 5'- and 3'- terminal exons but differ in their inclusion of three units: the B element, which consists of 27 nucleotides between alternative 5' splice sites "a" and "b" for the 5'-terminal exon, and internal exons mI and mII. Splicing of either mI or mII to the "a" site of 5'-terminal exon regenerates a 5' splice site consensus sequence at the exon-exon junction. Thus, mI and mII may be joined constitutively to the 5'-terminal exon in the nascent transcript and subsequently removed (along with the downstream intron) by resplicing at the exon-exon junction (Hatton *et al.* 1998).

Unlike *Ubx*, *Bmdsx* exons 3 and 4 do not have consensus sequence of splice donor site. So the internal exons of *Bmdsx* are skipped in an unknown manner. As a first step toward discovery of the cis-element, the *Bmdsx* mini gene, which consisted of exon 1 and 5 and internally shortened introns 2 to 4, was constructed, and transgenic silkworms expressing this construct was generated. This experiment revealed that the mini gene contained the necessary information for the correct regulation of alternative splicing.

Materials and Methods

Animals

The *B. mori* non-diapause and white egg strain, pnd-w1, maintained in the National Institute of Agrobiological Sciences, was used. Larvae were reared on an artificial diet (Nihon Nosan) at 25° C. G2 animals were obtained from GFP-positive G1 adults mated to moths of the recipient strain.

Construct

A fragment containing the *B. mori* nucleopolyhedrovirus (BmNPV) *ie-1* promoter and exon 1 and exon 2 of *Bmdsx* was obtained by digesting pBac {ie1BmdsxM}(Funaguma 2003) at the *Xho*I sites following digestion at the *Fse*I and *Asc*I site. A genomic fragment that began at 875 nucleotides upstream of exon 5 and extends 7 nucleotides downstream of exon 5 was synthesized by PCR amplification using primers hspminiF (5'-GGGCCCTGCACCTGGCGTCTTATCCT-3') and miniA3R2 (5'-GTGATGACCTGACCGCAGTTTACCTGTATCGGCGC-3'). A fragment containing the polyadenylation site of the *B. mori* cytoplasmic actin gene *BmA3* was amplified using primers miniA3F (5'-GATACAGGTAAACTGCCCGACGGTCAGGTCATCAC-3') and AscIminiA3 (5'-GGCGCGCCGGGTCAAGACACAGACGCAT-3'). MiniA3R2 and miniA3F were designed to connect the 3' end of the above PCR fragment and the 5' end of the PCR fragment containing the *BmA3* polyadenylation site. To link the PCR products, PCR amplification was performed with primers hspminiF and AscIminiA3 using a mixture of the above PCR fragments. The resulting product (identified as miniC) was digested at the *Aor51H*I and *Asc*I sites. A *Bmdsx* mini gene (Suzuki *et al.,* 2001) was digested at the *Xho*I and *Aor51H*I sites. The above digested fragments were joined and inserted into the *Asc*I and *Fse*I site of a pBac[3xP3-EGFPaf] transformation vector (Berghammer *et al.* 1999; Horn *et al,* 2000; Horn and Wimmer 2000). The construct was identified as mini1. The nucleotide sequences of the resulting construct were confirmed by DNA sequencing. The *piggyBac* helper plasmid pHA3PIG (Tamura *et al.* 2000) was used as a source of transposase.

Embryo injection and transformed animals

Fertilized eggs at the preblastoderm stage were microinjected with construct DNA and the helper plasmid as previously described (Kanda and Tamura 1991; Tamura *et al.* 2000). After DNA injection, the embryos were maintained at 25° C in moist Petri dishes until hatching. G0 adults were mated within the same family or backcrossed to moths of the recipient strain, and the resulting G1 progeny were screened for EGFP fluorescence microscopy with an appropriate EGFP expression (Leica MZ FL III, www.leica-microsystems.com).

RT-PCR

Poly (A)+RNA was isolated individually using a Micro-Fast Track 2.0 mRNA-isolation kit (Invitrogen, www.invitrogen.com) according to the protocol provided by the manufacturer. RT-PCR was performed using the LA RNA PCR kit (Takara, www.takara-bio.co.jp) following the manufacturer's instructions. cDNA was produced by random priming. RT-PCR primers were as follows: endogenous *BmA3*, BmA3QPCR1F (5'-TACAATGAGCTGCGTGTCG-3') and BmA3QPCR1R (5'-CGGGCGTGTTGAATGTTTC -3'); and *Bmdsx* mRNA transcribed from the transgene, TGM2F (5'-ATTGGCGGGACACGATC-3') and TGM2R (5'-AGCGCTCCGTAGCACAA-3'). Primer TGM2F is specific for the transgenic construct. PCR conditions for *Bmdsx* were as follows: 94° C, 3 min followed by 35 cycles of 98° C, 20 seconds; 53.5° C, 30 seconds; and 72, 1 min. PCR products were analyzed on a 1.6 or 2% agarose gel. Individual PCR fragments were purified from gels and cloned into a pGEM-T easy vector (Promega, www.promega.com). These cloned DNA was sequenced with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 3100 Genetic Analyzer.

Results

Bmdsx mini gene system

To discover the cis-element(s) required for the sex-specific splicing of the *Bmdsx* pre-mRNA, a *Bmdsx* mini gene system was designed. When the *Bmdsx* mini gene, which consists of exons 2 to 5 and internally shortened introns 2 to 4, was incubated under splicing conditions in HeLa nuclear extracts, female-specific splicing was observed (Suzuki *et al.,* 2001). Therefore, it had the information required for constitutive splicing. We added *Bmdsx* exon1 to the Suzuki's *Bmdsx* mini gene in this experiment (see Materials and Methods and Fig.1). The kind of promoter used to express this mini gene in the transgenic silkworms is important. If the transcript of mini gene is in large excess, the pattern of splicing of pre-mRNA transcribed from the mini gene may be different from that of endogenous *Bmdsx* pre-mRNA (Nogués *et al.,* 2003). When *Bmdsx* female cDNA was under the control of a BmNPV *ie-1* promoter, the ratio of the $Bmdsx^F$ transcript to endogenous *Bmdsx* transcript was 1:2 in the fat bodies of transgenic animals (Suzuki *et al.,* 2003). Therefore, in this study the mini gene is under the control of the BmNPV *ie-1* promoter (Fig.1).

Transformation experiments

The *Bmdsx* mini gene under the control of the BmNPV *ie-*

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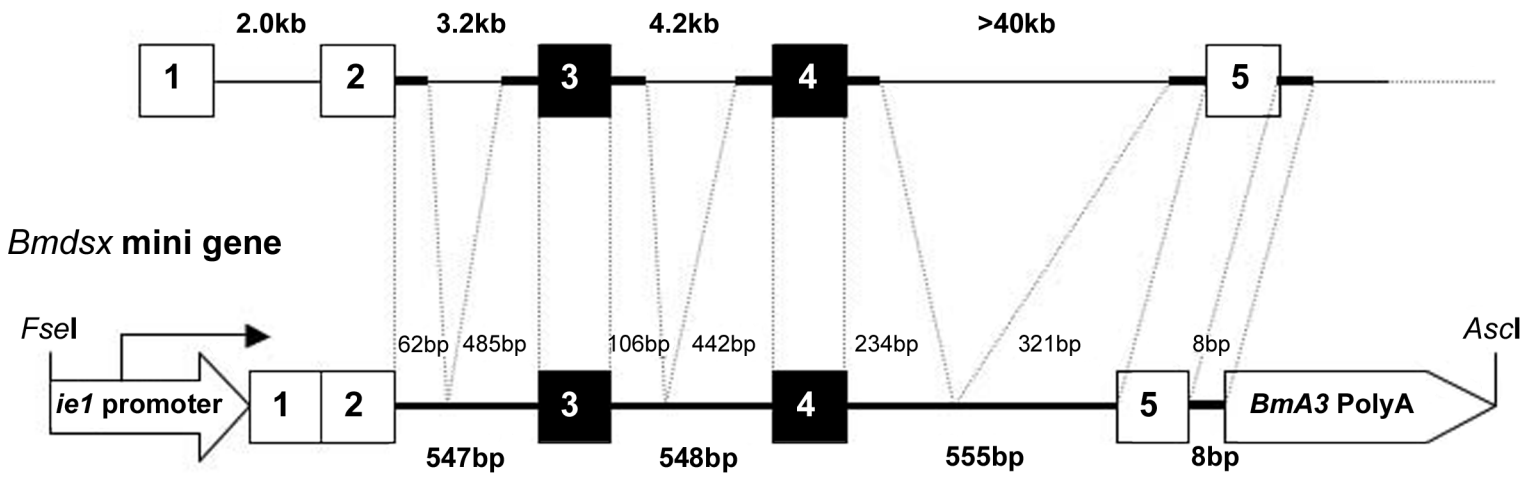

Figure 1. The *Bmdsx* mini gene system. The mini gene (not shown to scale) contains *Bmdsx* exons 1, 2, 3, 4, and 5 and encodes the entire open reading frame. Introns 2, 3, and 4 have been internally shortened. The mini gene has an 8bp fragment, which is the 5' end of intron5. The arrow indicates the site of transcription initiation within the *ie1* sequences. The length of each intron is indicated in nucleotides. Open boxes: common exons. Shaded boxes: female specific exons. Lines between boxes: introns.

Table 1. Results of a transformation experiment of the pBac{ie1mini1}into pnd-w1 embryos.

Number of injected embryos	Number of hatched embryos	Number of matings	Number of broods with EGFP-positive larvae
547	105(19.2%)	70	2(2.9%)

1 promoter was co-injected with a *piggyBac* transposase plasmid into eggs at the preblastodermal stage. Of 547 eggs injected with mini1, 105 larvae survived to the first larval stage. After sibiling mating, 2 of the G0 mating yielded progeny with EGFP eye fluorescence (Table 1).

Expression analysis of the mini1 transgene

The pattern of alternative splicing of the mini gene RNAs in the fat body of female or male 5th larvae was examined. If mini1 contains the necessary information for the correct regulation of alternative splicing, it should produce sex-specific products. RNAs were isolated individually from fat bodies of G1 transgenic animals. RT-PCR assays showed sex-specific products. When the mini gene was expressed in a transgenic female, the mRNAs detected were of an endogenous female-type (Fig. 2B). When the mini gene was expressed in a transgenic male, the mRNAs detected were of an endogenous male-type (Fig. 2B). When cDNA derived from fat bodies of non-transgenic silkworm was used as a template, corresponding PCR products were not detected (Fig. 2C). Previous studies have shown that *Bmdsx* pre-mRNA underwent default processing to generate the female *Bmdsx* mRNA and that some splicing factor(s) may inhibit the use of *Bmdsx* exons 3 and 4 in male animals (Suzuki *et al.,* 2001). Therefore, this *Bmdsx* mini gene may have an exonic splicing silencer (ESS) or an intronic splicing silencer (ISS) required for skipping *Bmdsx* exons 3 and 4 in males.

Discussion

A characteristic of the *Bmdsx* pre-mRNA is that its intron is very long (>40kb). The question of how a 5' splice site at the beginning of such a long intron can be accurately joined to the correct 3' splice site so far downstream, rather than to an intervening cryptic site, has puzzled scientists for a long time. This correct site is not only far away in the sequence but is also synthesized more than an hour after the 5' splice site (Black, 2003). This problem is often explained by the idea of recursive splice sites. The initial splice site may splice to intervening sites in a special manner because they regenerate a 5' splice site as they are joined to the original site. Thus, the 5' exon may hop along the long intron, being respliced several times at these ratcheting points, before being joined to the final correct site at the end of the long intron. This last site would presumably not regenerate a 5' splice site and, thus, would terminate the repressing process. There is evidence for the use of recursive splice sites in several long introns. Interestingly, a resplicing mechanism offers another point of control for altering the splice site choice. In the *Ubx* gene of *Drosophila,* a cassette exon containing a recursive splice site can be removed from the RNA even after joining to the upstream exon (Hatton *et al.,* 1998). However, unlike *Ubx* pre-mRNA, *Bmdsx* pre-mRNA does not have a splice site at the end of exons and cannot regenerate a splice site. Therefore, the mechanism of the sex-specific splicing of *Bmdsx* pre-mRNA is unique.

The need to regulate alternative splicing introduces an extra requirement for signals that must modulate splicing in a developmental and/or cell-type-specific fashion, and this complexity cannot be accommodated by the classical splicing signals (5' splice site, branch site, and 3' splice site) (Cartegni *et al.,* 2002). What is the cis-element required for the sex-specific splicing of *Bmdsx* pre-mRNA?

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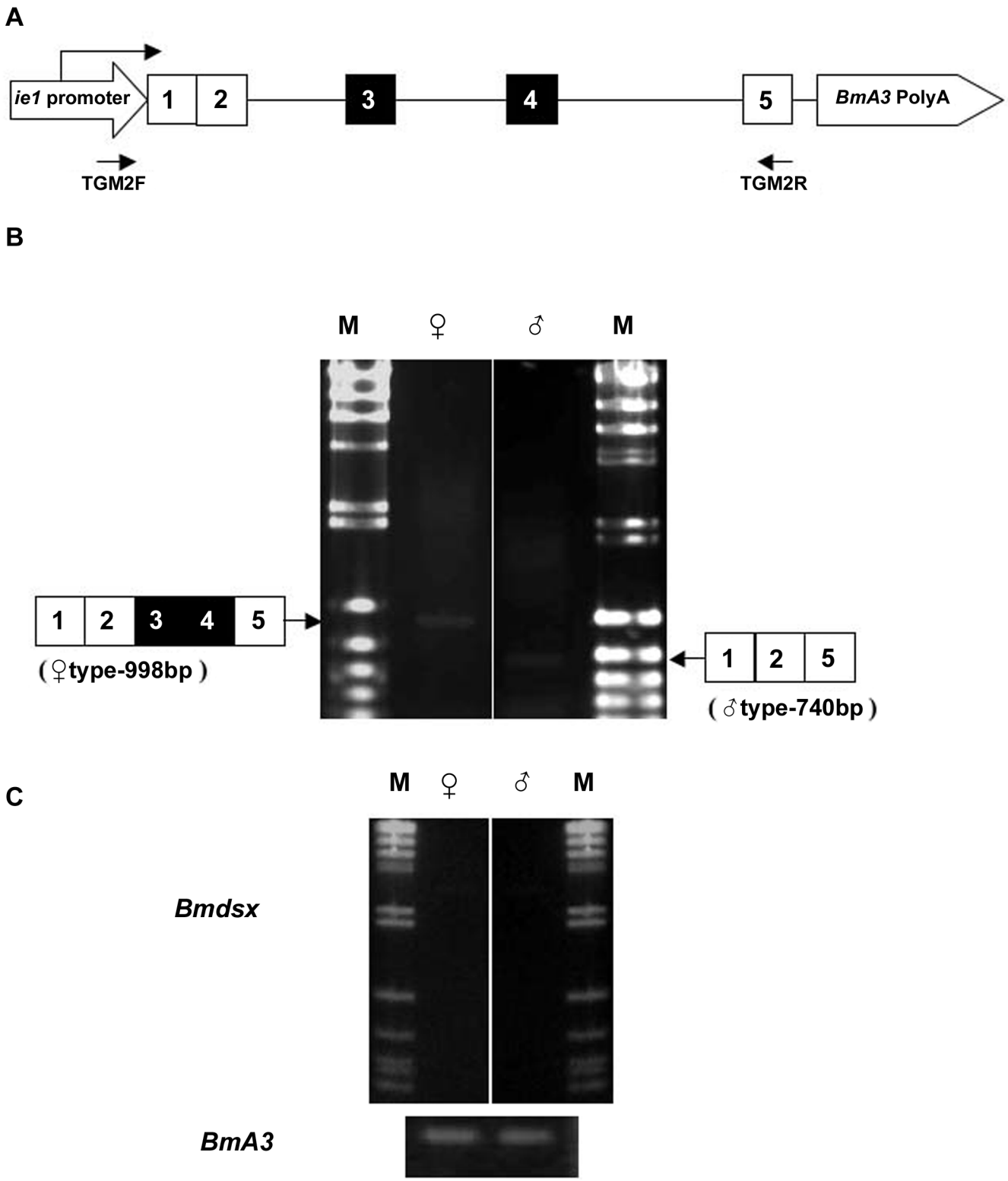

Figure 2. Pre-mRNA transcribed from the *Bmdsx* mini gene is sex-specifically spliced. (A) The diagram shows the structure of the *Bmdsx* minigene. Open boxes: common exons. Shaded boxes: female-specific exons. Arrows: primers for PCR. (B) Poly (A)+RNA was extracted from the fat body of transgenic silkworms. These RNAs were reverse-transcribed with random hexamer, and the cDNAs were PCR-amplified with primers TGM2F and TGM2R. Resulting products were separated on a 1% agarose gel and visualized with SYBR Green I (Molecular Probes) at a dilution of 1: 10,000. M represents the DNA marker(λ/*Hind*III+ ϕX174/*Hinc*II). The bands for the PCR products are schematically shown. (C) Poly (A)+RNA was extracted from the fat body. Upper lanes: these RNAs were reverse-transcribed with random hexamer, and the cDNAs were PCR-amplified with primers TGM2F and TGM2R. Lower lanes: these RNAs were reverse-transcribed with random hexamer, and the cDNAs were PCR-amplified with primers BmA3QPCR1F and BmA3QPCR1R. Resulting products were separated on a 1% agarose gel and visualized with SYBR Green I (Molecular Probes) at a dilution of 1: 10,000 (upper lanes) and visualized with ethidium bromide (lower lanes). M represents the DNA marker (λ/*Hind*III+ ϕX174/*Hinc*II). The bands for the PCR products are schematically shown.

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Table 2. Target sequences of hnRNP A1 and PTB (polypyrimidene tract binding protein) within the Bmdsx mini gene.

***Bmdsx* mini gene intron(length)**	**hnRNP A1**	**PTB**
2(547bp)	35-38nt	126-129nt
		191-194nt
		285-288nt
3 (548bp)		291-294nt
4 (545bp)	166-169nt	276-279nt
	300-303nt	

When the pre-mRNA transcribed from the *Bmdsx* mini gene used in this study was incubated under splicing conditions in HeLa nuclear extracts, female-type splicing was observed. It was concluded that the *Bmdsx* pre-mRNA underwent default processing to generate the female *Bmdsx* mRNA and that some splicing factor(s) may have inhibited the use of *Bmdsx* exons 3 and 4 in male animals (Suzuki *et al.,* 2001). However, it is also possible that the *Bmdsx* mini gene used in that study does not have the cis-element(s) required for male-type splicing. Recently the system for the germline transformation of the silkworm was developed using *piggyBac* vector (Tamura *et al.,* 2000). Therefore, we generated transgenic silkworms expressing the *Bmdsx* mini gene to determine whether the mini gene has the necessary information for male-type splicing. All splicing factors required for the sex-specific splicing of *Bmdsx* pre-mRNA are available in silkworms. As shown in Fig. 2B, pre-mRNAs transcribed from the mini gene were sex-specifically spliced, as was endogenous *Bmdsx* pre-mRNA. Therefore, this *Bmdsx* mini gene may have an exonic splicing silencer (ESS) or an intronic splicing silencer (ISS) required for skipping *Bmdsx* exon 3 and 4 in males. Whether there are previously identified silencer elements in this *Bmdsx* mini gene must be determined. The best characterized of the exonic splicing silencers was found to be bound by particular hnRNP proteins. The hnRNP proteins are a large group of molecules identified by their association with unspliced mRNA precursors. One of these proteins, hnRNP A1, has been implicated in pre-mRNA splicing (Black 2003). Some RNA binding sequences for hnRNP A1 binding have been previously described, namely, CUAGACUAGA in the ESS and AUAGAAGAAGAA in the Janus regulator of HIV tat exon 2 (Caputi *et al.,* 1999; Marchand *et al.,* 2002); UACCUUUAGAGUAGG in the ISS of human hnRNP A1 pre-mRNA (Chabot *et al.,* 1997); UUAGAUUAGA in the mouse hepatitis virus RNA transcription regulatory region (Li *et al.,* 1997); and UAGGGCAGGC in an ESS in the K-SAM exon of human FGF receptor 2 (Del Gatto *et al.,* 1995). Together with the SELEX winner sequence identified previously (Burd *et al.,* 1994), all of the reported sequences contain unique or tandem repeats of the sequence UAG (G/A) at their core (Guil *et al.,* 2003). One element, UAGA, is available in introns 2 and 4 of the *Bmdsx* mini gene used in this study (Table 2). Besides hnRNP A1, the other splicing repressor commonly found in associated with regulated exons is the polypyrimidine binding protein, PTB. PTB has been implicated in the repression of a wide range of vertebrate tissue-specific exons. *In vitro* selection experiments indicate that PTB optimally binds UUCU elements placed within a larger pyrimidine rich region. The three UUCU elements are available in intron 2 in the *Bmdsx* mini gene used in this study (Table 2). In the future, further experiments will be necessary to reveal whether their element is required for sex-specific splicing of *Bmdsx* pre-mRNA.

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