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Identification of Novel Bombyxin Genes from the Genome of the Silkmoth *Bombyx mori* and Analysis of Their Expression

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Insulin family peptide members play key roles in regulating growth, metabolism, and reproduction. Bombyxin is an insulin-related peptide of the silkmoth *Bombyx mori*. We analyzed the full genome of *B. mori* and identified five novel bombyxin families, V to Z. We characterized the genomic organization and chromosomal location of the novel bombyxin family genes. In contrast to previously identified bombyxin genes, bombyxin-V and -Z genes had intervening introns at almost the same positions as vertebrate insulin genes. We performed reverse transcription-polymerase chain reaction and in situ hybridization in different tissues and developmental stages to observe their temporal and spatial expression patterns. The newly identified bombyxin genes were expressed in diverse tissues: bombyxin-V, -W, and -Y mRNAs were expressed in the brain and bombyxin-X mRNA in fat bodies. Bombyxin-Y gene was expressed in both brain and ovary of larval stages. High level of bombyxin-Z gene expression in the follicular cells may suggest its function in reproduction. The presence of a short C-peptide domain and an extended A chain domain, and high expression of bombyxin-X gene in the fat body cells during non-feeding stages suggest its insulin-like growth factor-like function. These results suggest that the bombyxin genes originated from a common ancestral gene, similar to the vertebrate insulin gene, and evolved into a diverse gene family with multiple functions.

Key words: bombyxin, brain, fat body, ovary, gene expression, insulin, insulin-like growth factor

INTRODUCTION

The insulin family consists of insulin, insulin-like growth factor (IGF), and relaxin, which exert a wide variety of physiological effects on growth, metabolism, and development of vertebrates. Insulin family members have also been identified in invertebrates, including insect species of different orders (Nagasawa et al., 1984, 1986; Iwami et al., 1989; Lagueux et al., 1990; Brogiolo et al., 2001; Riehle and Brown, 2002; Krieger et al., 2004; Wheeler et al., 2006; de Azevedo and Hartfelder, 2008; Li et al., 2008). Among these, bombyxin, a family of peptides produced in the brain of the silkmoth *Bombyx mori*, was the first insulin-related peptide isolated from insects (Nagasawa et al., 1984, 1986). Bombyxin has been considered as a critical factor regulating insect molting and metamorphosis (Nagasawa et al., 1984), tissue growth (Nijhout and Grunert, 2002), carbohydrate metabolism (Satake, 1997), and ovarian development (Fullbright et al., 1997). The exact physiological function of bombyxin in *B. mori* remains unclear, however, further study is necessary for the elucidation of its function (Iwami, 2000; Garde and Hoffmann, 2005).

Bombyxin genes are expressed predominantly in the brain (Kawakami et al., 1989; Iwami, 2000) in contrast to the insulin gene, which is expressed in the pancreas. Recently, insulin-like peptides of *B. mori* and *Drosophila melanogaster* have been identified and found to be highly produced in the fat body cells, suggested to be insect IGFs (Okamoto et al., 2009a, b; Slaidina et al., 2009).

Thus far, thirty-two bombyxin genes belonging to seven families have been isolated from the *B. mori* genome (Kondo et al., 1996; Iwami, 2000). These results suggests that a high degree of diversification in the structure and genomic organization of bombyxin genes has occurred throughout evolution (Kondo et al., 1996; Iwami, 2000; Bayazit, 2009).

It is still unknown, however, whether the genes encoding insulin and its structurally related peptides emerged from a single ancestral (insulin-type) gene (Smit et al., 1993). Due to sharp contrasts in the number of introns, multiple gene copies, and different expression sites in bombyxin genes, it has been thought that different mechanisms underlie the evolution of insulin family genes between vertebrates and

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invertebrates (Kondo et al., 1996; Iwami, 2000). Previous reports indicated that more bombyxin genes remain to be found in the *B. mori* genome (Kondo et al., 1996; Iwami, 2000) and thus we aimed to identify all bombyxin genes using genomic analysis.

We analyzed bombyxin genes in the *B. mori* genome database and identified five novel family genes. The present paper is the first report of a comprehensive survey of insulin-related genes in *B. mori* genome after the completion of the genome project. We present here the characterization of these genes and show that two family genes have introns at almost the same positions as human insulin gene. We found the novel bombyxin genes are expressed in various tissues and developmental stages using reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization. In addition, characterization of structure and genomic organization of bombyxin genes will provide an important basis to elucidate evolutionary relationships of insulin family members in insects.

MATERIALS AND METHODS

Gene identification, structure, and alignment analysis

Nucleotide sequences of new bombyxin genes were identified from the *B. mori* genome (The International Silkworm Genome Consortium, 2008). The *B. mori* genome database KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/) (Shimomura et al., 2009) was searched for additional genes encoding bombyxin and insulin-related peptide using nucleotide and peptide sequences of previously identified bombyxin genes. The deduced amino acid sequences of significant matches were examined for insulin-like characteristics, including the presence of signal peptide, B chain, C-peptide, and A chain and dibasic processing sites flanking the C-peptide. Signal peptides were predicted using SIG-pred (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) (Zhang et al., 2009), and endoprotease cleavage sites for C-peptides were determined according to the procedures outlined by Seidah and Chretien (1997). Intron-exon boundaries were confirmed by comparison between the genomic and cDNA sequences. The cDNA sequences used are sequences of representative nos. in the CYBERGATE database (http://150.26.71.213/cgi-bin/main_MX): *V1*, fcaL21f12f; *V2*, fcaL20e07f; *X1*, ovS329C05f; *Y1*, ovS326C11f; *Z1*, fe100P26_F_N03. The cDNA sequence for *W1* was identified in this study. Geneious Pro 4.8 (Biomatters, Auckland, New Zealand) was used for nucleotide and amino acid sequence analysis and for phylogenetic analysis with default parameters.

Experimental animals

Eggs of a racial hybrid of *B. mori*, Kinshu × Showa, were obtained from Ueda Sanshu (Ueda, Japan), and larvae were reared on an artificial diet (Silkmate II, Nihon Nosan Kogyo, Yokohama, Japan) at 25 ± 1°C under a 12-h light/12-h dark photoperiodic cycle (Sakurai et al., 1998). Newly molted fifth instar larvae were fed from the beginning of the photophase following the scotophase during which they molted to fifth instars. The 24 h period of the photophase following the scotophase during which the fourth instar larva molted was designated day 0 of the fifth instar (V0). Similarly, the 24 h period of the photophase following the scotophase during which the fifth instar larva pupated was designated day 0 of the pupal stage (P0), and that of pupa eclosed was designated day 0 of adult stage (A0). Days 2, 4, and 8 after V0 and days 2 after P0 were thus denoted as V2, V4, V8, and P2, respectively. Day 2 after A0 was denoted as A2.

RNA isolation, cDNA synthesis, and PCR amplification

Total RNA was extracted from various tissues using the acid guanidinium thiocyanate phenol-chloroform method with minor modifications (Tsuzuki et al., 2001). RNase-free DNase I-treated (Promega, Madison, WI, USA) RNA (1 μg) was used as a template for cDNA synthesis using ReverTra Ace (Toyobo, Osaka, Japan) and an oligo(dT) primer in a 20 μl reaction mixture. The reverse transcription products were diluted with 80 μl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The resulting cDNA was used as a template to amplify DNA fragments using PCR in a 10 μl mixture containing GoTaq Green Master Mix (Promega) and gene-specific primers (Table 1). As control, cDNA samples were prepared without adding reverse transcriptase to demonstrate the absence of genomic contamination. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/), with default criteria (Rozen and Skaletsky, 2000). Because bombyxin genes belong to a multigene family, the gene-specific primers were designed from the signal peptide and C-peptide sites, which are the least common sites among these genes, to avoid non-specific amplification. Amplification was performed in a thermal cycler (Mastercycler ep384, Eppendorf, Hamburg, Germany), with denaturation at 94°C for 30 s, annealing as mentioned in Table 1 for 30 s, and extension at 72°C for 30 s. RT-PCR reactions were performed at least three times and gave reproducible results.

Cloning, plasmid purification, DNA sequencing, and sequence analysis

The *B. mori* genome contains multiple bombyxin gene copies having at most 56% and 85% similarity between and within families, respectively (Iwami, 2000; Kondo et al., 1996). To evaluate the risk of amplifying highly similar non-target genes and to confirm whether targeted gene transcripts were expressed, cDNAs of the transcripts from the identified bombyxin genes were sequenced. The PCR products of respective bombyxin gene primers were cloned into the pGEM-T vector (Promega) and sequenced. For sequence determination, at least three individual clones were analyzed.

In situ hybridization

Whole-mount in situ hybridization: Dissected brains were washed in 10 mM phosphate-buffered saline (PBS, pH 7.4) with 0.1% Tween 20 (PBT) and fixed in a solution of 85% ethanol, 4% formaldehyde, and 5% acetic acid on ice for 45 min, as described previously (Hossain et al., 2006, 2008). Tissues were deproteinized with proteinase K (50 μg/ml in PBS) at 37°C for 30 min and re-fixed in 4% (w/v) paraformaldehyde in PBT at room temperature for 20 min. After incubation in prehybridization solution (50% (v/v) formamide, 5X standard saline citrate (SSC), 1 μg/ml yeast t-RNA, 50 μg/ml heparin, 0.1% TritonX, 0.1% CHAPS (3-{(3-cholamidopropyl) dimethyl ammonio}-propanesulfonate), 5% dextran sulfate, 5 mM EDTA, and 2% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany)) at 50°C for 90 min, the samples were hybridized with riboprobes (1 ng/μl each) at 50°C overnight. Probes for each bombyxin gene were prepared using the plasmid where the RT-PCR products were cloned. Digoxygenin (DIG)-labeled RNA probes were synthesized using a DIG RNA Labeling Mix (Roche). Sense probes were used as negative controls. After being washed several times with a series of wash buffers (50% formamide, 5X SSC, and 1% SDS; 2X SSC and 0.1% CHAPS, and 0.2X SSC and 0.1%

Table 1. Primers used in PCR and in situ hybridization.

Gene	Forward	Reverse	Annealing temperature (°C)
bombyxin-V1	TTCACGGTGATGATAGTGCTG	CTTGTAGCGGGTCCAGTCAG	56
bombyxin-W1	TCTACGACCGCGTTAGAACAA	TTTCTGGATGCGGTGCTC	55
bombyxin-X1	TGACGGCAAAACTTTATTTCG	CCTGGATCTTTGCTTTGCAG	53
bombyxin-Y1	TGAAATTTTCTGCGGTTTTTG	CGGACAGCCATGACCAGT	54
bombyxin-Z1	TTCTGCTGTATTTCCTGATCGT	TCTTGGCGTACTGGGAAGAC	55
RpL3	AGCACCCCGTCATGGGTCTA	TGCGTCCAAGCTCATCCTGC	58

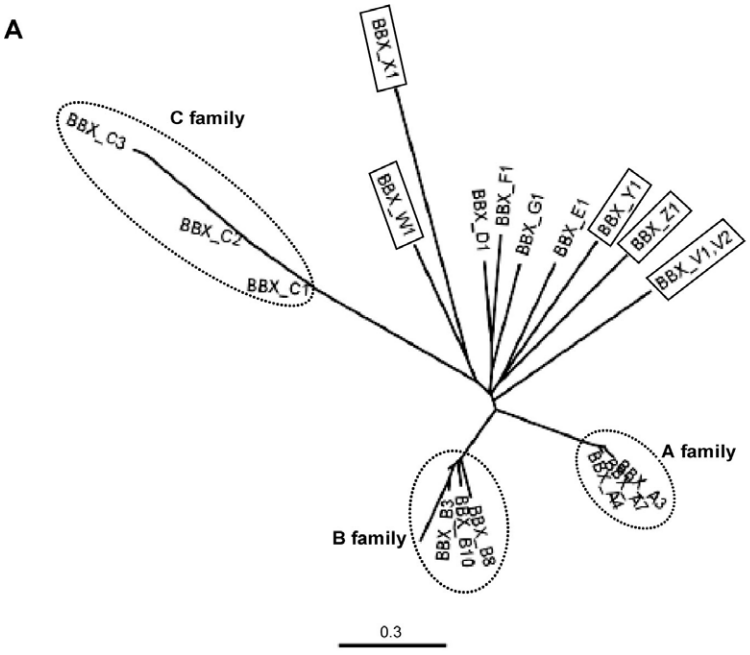
CHAPS) at 50°C and KTBT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, and 0.1% Triton X-100) at room temperature, the tissues were blocked in 1.5% blocking solution for 90 min. The tissues were incubated with an anti-DIG antibody conjugated with alkaline phosphatase (Roche) at a 1:2500 dilution at 4°C overnight followed by extensive washing in KTBT. Samples were incubated in staining buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$ and 0.1% Tween 20). Staining was developed using NBT/BCIP solution (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) (Roche). After dehydration in ethanol, brains were clarified with methyl salicylate and observed under a microscope (BX-50F, Olympus, Japan).

Section in situ hybridization: Freshly dissected tissues were embedded in Tissue-Tek O.C.T compound (Sakura Fintek, Torrance, CA, USA) and frozen on dry ice. Frozen tissues were sectioned at 10 μm using a cryostat at –15°C, mounted on slides, air dried, and fixed for 12–16 h at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Sections were then treated with proteinase K (10 μg/ml in 100 mM Tris–HCl, pH 8, and 50 mM EDTA) at 37°C for 20 min, re-fixed for 20 min, washed with PB, acetylated with 0.25 % acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min at room temperature, washed with PB, and dehydrated using a graded series of ethanol. The sections were hybridized with probes (2 ng/μl) in the presence of hybridization buffer (50% formamide, 10 mM Tris HCl, pH 7.6, 200 μg/ml yeast tRNA, 1X Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA) and incubated overnight at 37°C in a humidified chamber with 50% formamide. Slides were washed in 50% formamide and 2X SSC at 37°C for 30 min, treated with 10 μg/ml ribonuclease A (Sigma-Aldrich, St. Louis, MO, USA) in TNE (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 1 mM EDTA) for 15 min, and washed with 2X SSC and 0.2X SSC at 50°C. Staining was developed as previously mentioned.

RESULTS

Identification and analysis of novel families of bombyxin genes

We identified novel bombyxin genes, which we grouped into five families named bombyxin-V to -Z, by performing BLAST searches on the *B. mori* genome database using all known bombyxin family genes. The deduced amino acid sequences of these novel bombyxin genes showed 21% to 50% identity among the prepropeptides of bombyxin family members and 21% to 26% identity with human preproinsulin. Phylogenetic analysis revealed that the newly identified bombyxin genes did not belong to any known family of bombyxins and were thus considered as novel genes (Fig. 1A). Our newly identified bombyxin-Y1 gene was identical to the 8k-bombyxin-like peptide (8K-BLP) gene identified by Okamoto et al. (2009a). The open reading frames (ORFs) of the newly identified bombyxin genes encoded preprobombyxins with four domains in the order of signal peptide, B chain, C-peptide (domain), and A chain. The A and B chains of deduced bombyxins showed high similarity to those of previously identified bombyxins and other insulin members, while the C-peptide (domain) and signal peptide showed less similarity (Fig. 1B).



B

		Signal peptide	B chain (domain)
Bombyxin	V1	MKFTVMIVLMALAYVSA	HKEAVTLCGRNLANARVAVCYGAEYVD
	W1	MKTVLLIVLLLTMSTTA	LEQQKQARHYCGRFLALTLADLCWEY
	X1	MLLTAKLYFVLYVLLLLRESKG	DALTMNTRIRDMCSRALSNLIFDICTGTLPVTDLPPESLSKVRA
	Y1	MKFSAVFVILLVLLTVAVLS	ENAGRTYCGRYLARTLANLCSDAGQE
	Z1	MFLLYFLIVVALVSA	DVHDKELKIEENPRVYCGRHLANARMVLCYDTVE
	A1	MKILLAIALMLSTVMWVST	QQPQRVHTYCGRHLARTLADLCWEAGVD
Human Insulin		MALWMRLLPLLALLALWGPDPAAA	FVNQHLCGSHLVEALYLVCGERGFFYTPKT
Human IGF-I		MHTMSSSHLFYLALCLLTFTSSATA	GPETLCGAELVDALQFVCGDRGFYFNKPT

		C peptide (domain)	A chain (domain)
Bombyxin	V1	KRTSGNTLFDDFEDVDLHGDWPWTGRRGALSADWT RYKR	QGIVNECCFKPCTTDVLLKYC
	W1	KRSDDSYYDGNNQDLTEPPSPQTAEHR IQKR	GVADDCCLRACTLDVLLLYC
	X1	KRASLFSAKQ RSRR	VADECCLRSCTVSQLVEYCPETW
	Y1	KRGEDWSWLS ASGRK	DGAVTENGVANECCLHPCTLEVLLSYC
	Z1	KRAQSYLDANIISAGDLSSWPGLSSQYAKTRAFALAEKSKR	GPGLVDECCLKPCYTYDLLNYC
	A1	KRSGAQFASYGSAWLMPYSEG RQKR	GIVDECCLRPCSVDVLLSYC
Human Insulin		RREAEDLQVGQVELGGGPGAGSLQPLALEGSLQ KR	GIVEQCCTSICSLYQLENYCN
Human IGF-I		GYGSSSRRAPQT	GIVDECCFRSCDLRRLEMYCA–(D domain)

Fig. 1. **(A)** A phylogenetic tree showing the relationships among bombyxin family members. BBX = bombyxin. The scale bar indicates an evolutionary distance of 0.3 amino acid substitutions per position. Novel bombyxins are boxed. **(B)** Amino acid sequence comparison of novel preprobombyxins, preprobombyxin-A1 (Nagasawa et al., 1986), human preproinsulin (Bell et al., 1980), and prepro-IGF-I (Jansen et al., 1983) as representatives of the vertebrate lineage. *Bombyxin-V2* is not shown in the figure, as this ORF sequence is similar to that of V1. Gaps are introduced for maximum matching. Residues that are completely conserved among the prepropeptides are boxed. Inverted triangles indicate cysteine residues for disulfide bonds. The dibasic processing sites flanking the C peptide are underlined.

Organization of bombyxin genes: *bombyxin-V* and *-Z* have introns

We characterized the genomic structure and chromosomal location of the novel bombyxin genes. Figure 2 shows the exon–intron organization of the newly identified bombyxin genes. *Bombyxin-W1*, *-Y1* and *-Z1* were localized on chromosome 1, while chromosomes 9 and 11 harbored family V and X genes, respectively. The comparison of the genomic and cDNA sequences (data not shown) revealed that *bombyxin-V1* and *V2* had one intervening intron of 754 and 833 nucleotides, respectively, in the 5'-untranslated region (UTR), but could not be assigned on exact positions on chromosome 9. *Bombyxin-W1*, *-X1*, and *-Y1* lacked introns at the conserved intron sites of insulin-family genes and were thus considered traditional bombyxin genes. *Bombyxin-Z1* consisted of four exons and three introns. The first (154 bp) and second (327 bp) introns were in the 5'-UTR, and the third was in the C-peptide region (2905 bp).

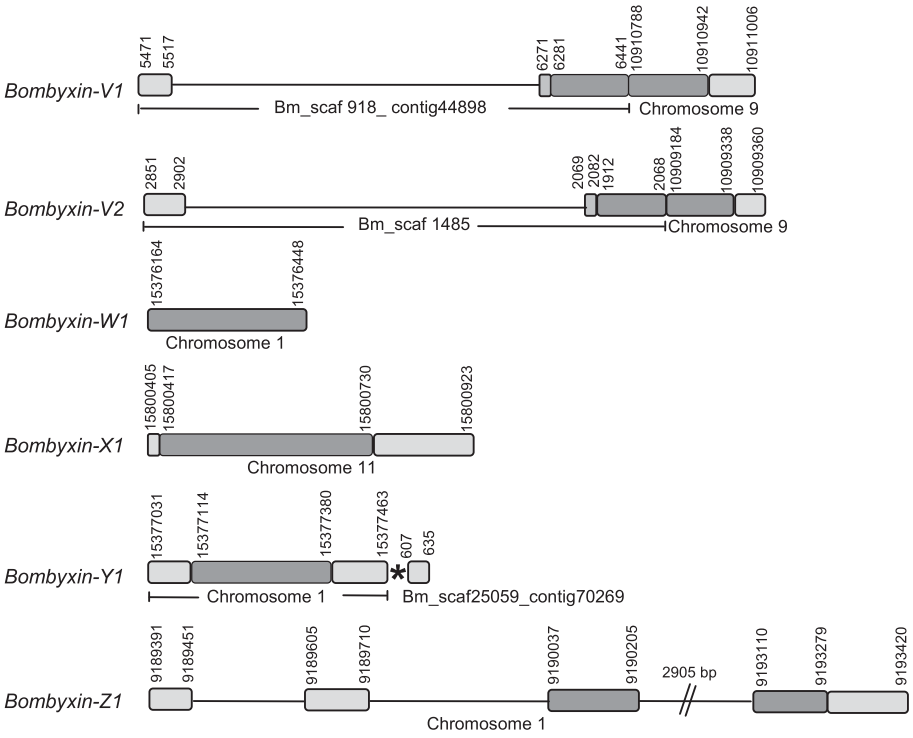


Fig. 2. Schematic representation of the intron-exon organization of newly identified bombyxin genes. Thick lines, exon; dark gray thick lines, ORF; light gray thick lines, UTR; thin lines, intron; "//", a break due to space limitation; "*", gap. Numbers above the gene structure indicate the chromosome/scaffold position (based on KAIKObase version 2) in the gene. Although bombyxin *V1* and *V2* have been assigned on the different positions on chromosome 9, the exact positions of the 5'-regions of them could not be assigned, while 3'-halves of *bomyxin-V1* and *-V2* are located at positions 10910788–10911006 and 10909184–10909360 on chromosome 9, respectively. Non-mapped portion of the chromosome is marked by scaffold and contig.

Expression of bombyxin genes in diverse tissues

To identify the tissues that express bombyxin genes, we performed RT-PCR expression analysis. Figure 3A shows tissue-specific expression profiles of the identified bombyxin genes in the life stages studied: V2, V8, P2, and A2.

Bombyxin-V1 (or *V2*) and *-W1* mRNAs were highly expressed in the brain at all four studied stages. High expression of *bombyxin-X1* was observed exclusively in the fat bodies in V8, P2, and A2. Analysis of *bombyxin-Y1* expression showed high levels of the transcript in the brain and ovary of larval stages. In P2, *Y1* was highly expressed in fat bodies and in A2, it showed the highest expression in the ovary. The expression pattern of *bombyxin-Y1* (identical to previously identified 8K-BLP gene) was partly presented by Okamoto et al. (2009a). Interestingly, *bombyxin-Z1* was mostly expressed in the female fat body and ovary in V2 and V8 and highly expressed in the ovary and fat body of P2, as well as in the ovary of A2.

Expression of bombyxin genes at different developmental stages

To profile the developmental expression of bombyxin genes, a survey of transcription was conducted semi-quantitatively using RT-PCR on the tissues in which the genes were highly expressed. Figure 3B shows developmental expression profiles. All genes except *X1* were expressed throughout V0, V2, V4, and V8. *X1* expression was low or undetectable at V0, V2, and V4 and began at V8 in the male fat body.

Spatial distribution of bombyxin gene transcripts

To specify the location of *bombyxin*-expressing cells, we performed in situ hybridization. *Bombyxin-V1* (Fig. 4A, B), *-W1* (Fig. 4D), and *-Y1* (Fig. 4F) mRNAs were localized to four pairs of the large medial neurosecretory cells in V2 brains. Section in situ hybridization showed the same expression pattern (Fig 4. C, E, G). We further confirmed the expression of bombyxin genes by identifying transcripts in V8 and P2 ovaries using in situ hybridization on frozen sections (Fig. 4H–K). *Z1* transcripts were predominantly and selectively detected surrounding the egg cells of the ovariole, which we assumed to be follicle cells. *Y1* mRNA was also detected in a similar location in the ovary (data not shown). We then used frozen sections of V8 and P2 fat bodies to detect *bombyxin-X1* and *-Z1* transcriptional location, but we could not find any signal, possibly due to ubiquitous and low level expression at the cellular level.

DISCUSSION

In the present study, we identified six novel bombyxin genes. Together with the previously identified 32 genes (Kawakami et al., 1989; Kondo et al., 1996; Tsuzuki et al., 1997; Yoshida et al., 1998; Iwami, 2000), 38 bombyxin genes have been identified in the haploid *B. mori* genome and classified into 11 families, A to G and V to Z. Analyses of the genomes of other insects have revealed the presence

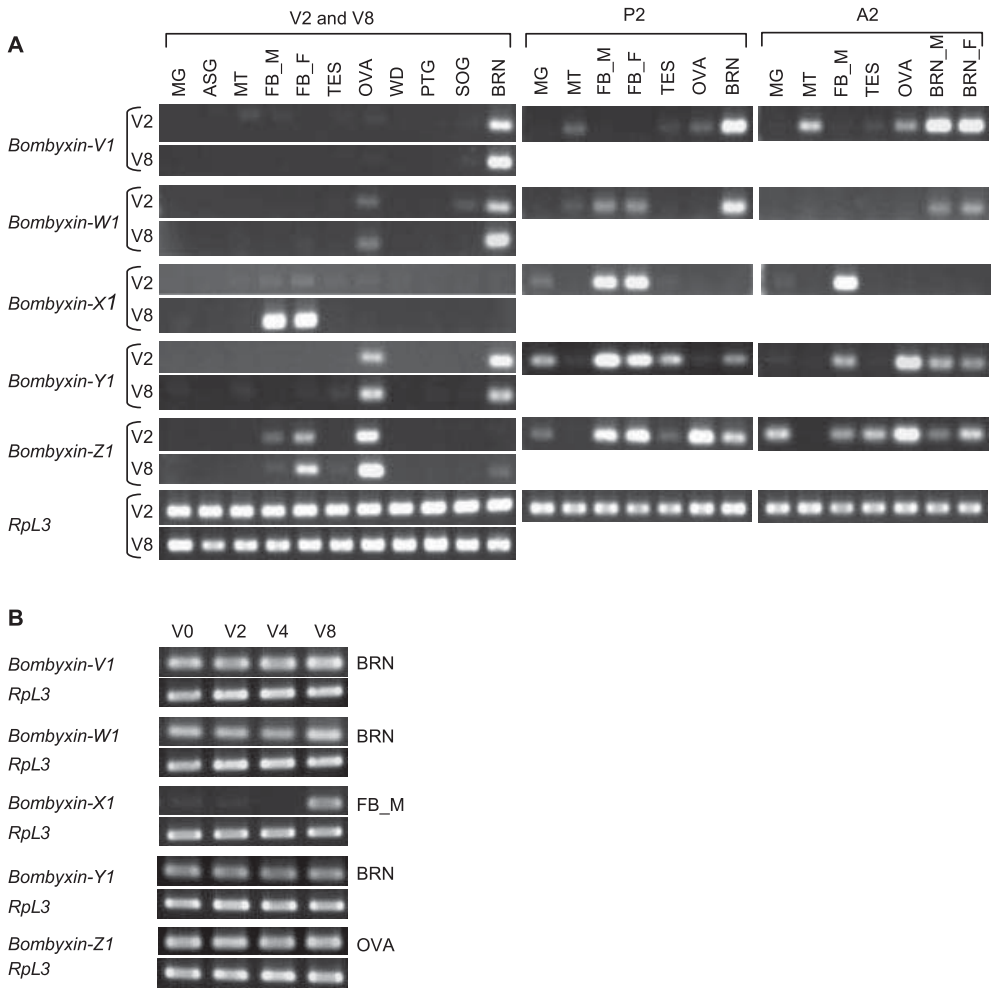


Fig. 3. **(A)** Expression analysis of newly identified bombyxin genes in various tissues and developmental stages. Tissues of V2 and V8 larvae, P2 pupa, and A2 adult were used. Abbreviations : MG, midgut; ASG, anterior silk gland; MT, Malpighian tubule; FB_M, male fat body; FB_F, female fat body; TES, testis; OVA, ovary; WD, wing disc; PTG, prothoracic gland; SOG, suboesophageal ganglion; BRN, brain of both male and female; BRN_M, male brain, and BRN_F, female brain. *RpL3* was used as a control. The number of PCR cycles was 27. There was no amplification without reverse transcriptase in the reaction, indicating no genomic DNA contamination. These data are representative of at least three experiments. **(B)** Developmental changes in bombyxin gene expression. Expressions in tissues of V0, V2, V4, and V8 stages were analyzed using RT-PCR. Tissues exhibiting high expression of the bombyxin genes were used for this experiment. Abbreviations used are the same as in (A). *RpL3* is shown to demonstrate an equal amount of cDNA in each sample. These data are representative of at least 3 experiments.

of multiple genes for insulin-related peptides: seven in *D. melanogaster* (Brogiolo et al., 2001), seven in *Anopheles gambiae* (Krieger et al., 2004), eight in *Aedes aegypti* (Riehle et al., 2006), two in *Apis mellifera* (Wheeler et al., 2006), four in *Tribolium castaneum* (Li et al., 2008), and at least six in *Samia cynthia ricini* (Kimura-Kawakami et al., 1992). The previously identified 25 bombyxin genes are clustered in a 50 kb range on chromosome 11 and six genes in a 15 kb range on an anonymous chromosome with unique distribution patterns: gene pairs, gene triplets, and single genes (Iwami, 2000). Three of the newly identified genes are located on chromosome 1, two on 9, and one on 11, without forming gene triplets or gene pairs. Gene clustering is usual for the bombyxin genes that have the highest number of gene copies. The *Drosophila* insulin-like peptide (DILP) 1-5 genes occur in a gene cluster within 26 kb on chromosome 3 and DILP6-7 on X chromosome (Brogiolo et al., 2001). *Anopheles gambiae* (Ag) ILP1-4 clustered within 12 kb of DNA and separated from 6–7 by 23 kb on chromosome 3, AgILP5 is located on chromosome 2 (Krieger et al., 2004).

All of the newly identified bombyxin genes apparently encode preprobombyxin, having a signal peptide, B chain, C-peptide (domain), and A chain, similar to other bombyxin genes (Iwami et al., 1989, 1990; Kawakami et al., 1989; Kondo et al., 1996; Tsuzuki et al., 1997; Yoshida et al., 1998), preprobombyxin-related peptides of *S. cynthia ricini* (Kimura-Kawakami et al., 1992; Brogiolo et al., 2001; Krieger et al., 2004), and preproinsulins (Steiner et al., 1985). Of the four domains of preprobombyxins, the highest similarities were seen in the A-chain, and the degree of amino acid conservation was A chain > B chain > C-peptide (domain) and signal peptide. The general structural features of vertebrate and non-vertebrate insulin-related genes (including novel bombyxin genes) are thus well conserved. Although two dibasic amino acids are present in the C-peptide (domain), there is possibility–that bombyxin-X1 and -Y1 remain as a single polypeptide chain, which is more like vertebrate IGFs in view of the previous analysis (Okamoto et al., 2009a).

All the previously identified bombyxin genes were lack of introns both in the 5' untranslated region and in the C-peptide region (Kondo et al., 1996; Iwami, 2000). The present study reveals for the first time that *bombyxin-V1*, *-V2*, and *-Z1* have intervening introns, unlike previously identified bombyxin genes. The most straightforward interpretation of not having introns in all bombyxin genes might be due to loss of the intron from intron-containing bombyxin genes. We do not exclude other possibilities, for example, *bombyxin-Z1* might have acquired novel introns after they branched from other bombyxin genes. Presence of introns has already been reported in some insulin-like genes of *Drosophila* (reviewed in Wu and Brown, 2006), *A. gambiae*

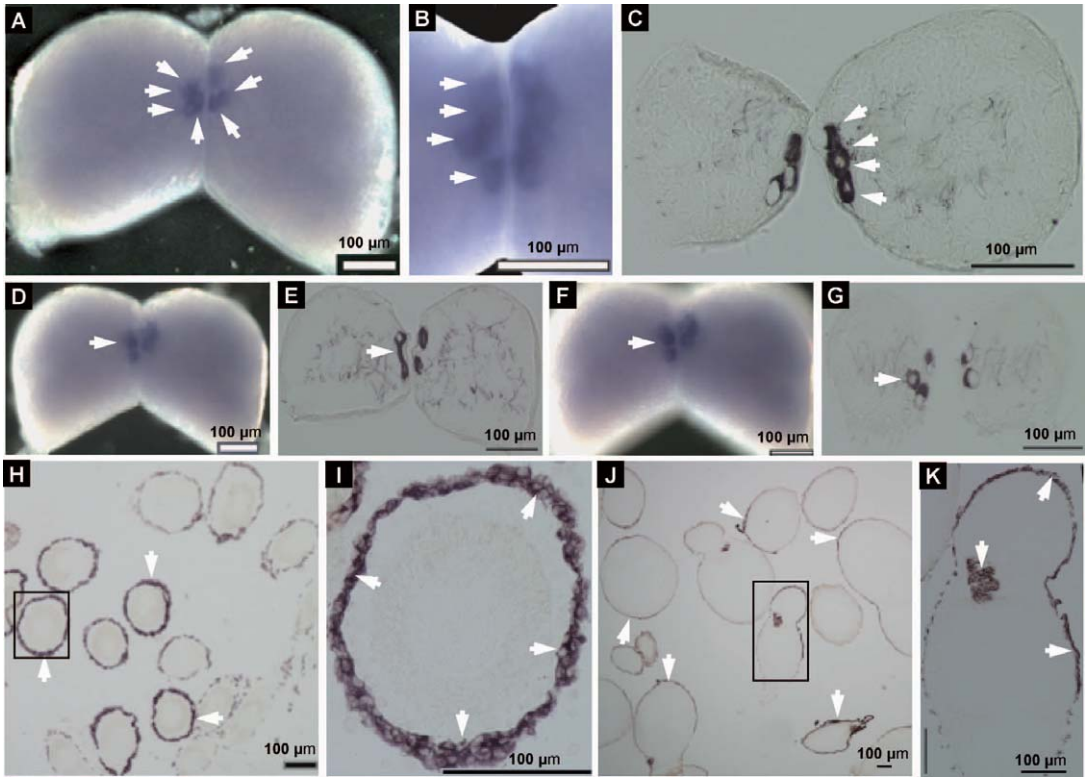


Fig. 4. Localization of bombyxin mRNAs in different tissues using in situ hybridization. **(A, C)** *Bombyxin-V1* expression in the medial neurosecretory cells (MNCs) of V2 brain. **(B)** Magnified micrograph of *V1* expression. **(D, E)** *Bombyxin-W1* expression in MNCs. **(F, G)** *Bombyxin-Y1* expression in MNCs. **(H, J)** *Bombyxin-Z1* expression in V8 and P2 ovaries, respectively. **(I, K)** Magnified micrograph corresponding to the areas shown in H and J, respectively. Staining is seen in the follicular cells of the ovary. Arrows indicate mRNA localizations. These data are representative of at least 10 hybridizations. No signal was detected in hybridizations performed with sense probes (data not shown). Scale bars = 100 μm.

(Krieger et al., 2004) and *A. aegypti* (Riehle et al., 2006). Structurally, the insulin protein has been highly conserved throughout vertebrate evolution. Vertebrate insulin genes, including human insulin genes, contain two introns: one within the 5'-UTR and the other in the C-peptide region (Steiner et al., 1985). Due to the presence of intron in the C-peptide region in insulin, IGFs, and relaxin genes, Crawford et al. (1984) suggested that these genes had evolved from a common ancestral gene-by-gene duplication. From our findings, the presence of intron at almost the same position in *bombyxin-Z1* indicates that bombyxin and vertebrate insulin genes share a common ancestral gene.

The newly identified bombyxin genes were expressed in a tissue-specific manner, suggesting a specific function. We found that three genes were predominantly expressed in four pairs of MNCs in the brain. Previously identified bombyxin genes of all seven families were exclusively expressed in the same cells (Iwami, 2000). Analogous findings have also been reported for other lepidopterans (Van de Velde et al., 2007) and other insects (Brogiolo et al., 2001; Ikeya et al., 2002; Krieger et al., 2004).

The expression of bombyxin and insulin-related genes is low in a number of tissues other than brain (Iwami et al., 1996; Brogiolo et al., 2001; Riehle et al., 2006). In the present study, we found that three of the newly identified genes were highly expressed in tissues other than brain. *Bombyxin-Z1* was expressed in various tissues in P2 and A2 including the brain, fat body, and midgut. Interestingly, it was highly expressed in the ovary throughout the stages examined. Invertebrate insulin and IGFs assumed to have effects on oocyte maturation (El-Etr et al., 1979), embryogenesis (Froesch et al., 1985), and ovarian development (Sim and Denlinger, 2009). Bombyxin induces meiosis in the ovary (Orikasa et al., 1993). Brown et al. (2008) found that an insulin-like peptide in *A. aegypti* regulates egg maturation and metabolism. In many insects, insulin-related genes are involved in oogenesis (Kozlova and Thummel, 2000; Sun et al., 2002). The mosquito insulin receptor has been reported to localize in the cell membrane of the follicle cells that produce ecdysteroids (Riehle and Brown, 2002). The follicular cells of the ovary have been reported as an expression site of ecdysteroid-regulated genes in insects (Carney and Bender, 2000; Paul et al., 2006; Takeuchi et al., 2007). The transcript of *bombyxin-Z1* was localized to the follicular cells of the ovary, the same site of transcripts of the ecdysteroid-regulated genes. From these facts, it is tempting to hypothesize that bombyxins, at least those of a particular family and possibly bombyxin-Z1, control the oogenesis and embryogenesis of *B. mori*, similar to the mammalian hormone relaxin, another member of the insulin family (Wu and Brown, 2006).

Vertebrate insulin genes are expressed in the pancreatic cells, and this has been a major criterion to differentiate vertebrate and invertebrate insulins or insulin-related peptides

(Iwami, 2000). Our study revealed that *bombyxin-X1* was predominantly expressed in the fat body during the molting period. Ecdysone reduces food consumption and then induces starvation during molting and pupation in *B. mori* (Wang et al., 2010). DILP6, one of the insulin-like peptides of *D. melanogaster*, is specifically produced and required for growth during this period (Slaidina et al., 2009). Growth of the isolated wing imaginal disks of *M. sexta* in vitro requires both bombyxin and ecdysone to achieve normal growth (Nijhout et al., 2007). The functional class of IGF has recently been described in *B. mori* (Okamoto et al., 2009a) and *D. melanogaster* (Okamoto et al., 2009b; Slaidina et al., 2009). Considering these findings, we hypothesize that bombyxin-X1 might have an IGF-like growth function and may play an important role in metamorphosis. Whether *bombyxin-X1* actually exhibits such activity requires further investigation.

In conclusion, five novel bombyxin family genes were identified. Our results clearly indicate that the identified bombyxin genes are expressed in a tissue-specific and, in some cases, developmental stage-specific manner. They are predominantly expressed in the brain, fat body, and ovary. Similarities in the structure, location of intervening introns, and high gasteroenteric expression between bombyxin and insulin genes suggest that the bombyxin genes might have originated from a common ancestral gene similar to the vertebrate insulin gene. Based on the sequence diversity and expression specificity, it is tempting to think that, similar to members of the insulin family in humans, each of the bombyxins also serves a distinct function. Further detailed studies might provide clues to clarify the functions of bombyxin families.

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