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Full Length Research Paper

Molecular characters and expression analysis of a new isoform of the myocyte enhancer factor 2 gene from the silkworm, *Bombyx mori*

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Using reverse transcriptase- polymerase chain reaction (RT-PCR), the cDNA encoding a new isoform of the myocyte enhancer factor-2 (BMEF2) was cloned from the brain of the strain "Liangguang 2" of *Bombyx mori*. The open reading frame (ORF) of the new isoform shows 12 nucleotides absence of (GCACTCACAAAG) and three nucleotides mutation to the former BMEF2 (GenBank no. AB121093). Thus, we named it BMEF2B (GenBank no. EF100967). Using the SWISS-MODEL Prediction Server, the 3-D structure of BMEF2B was modeled. There are two β-turns and three α-helixes predicted in BMEF2B, with the pattern of α-β-β-α-α. The BMEF2B mRNA was detected not only in the central nervous system (CNS), including brain, suboesophageal ganglion (SG), thoracic ganglion (TG), and abdominal ganglion (AG), but in the non-neural tissues, such as the midgut (Mg), fat body (FB), and epidermis (Ep), muscle (Ms) and ovary (Ov). BMEF2B mRNA content in the brain was measured using the combined method of quantitative RT-PCR and Southern blotting, which kept consistently high from larvae to adults.

Key words: Myocyte enhancer factor-2, new isoform, tissue expression, developmental expression, *Bombyx mori*.

INTRODUCTION

The myocyte enhancer factor-2 (MEF2) family of transcription factors plays a critical role in the development of skeletal, smooth, and cardiac muscle (McKinsey et al., 2002). All MEF2 factors show homology within a conserved MADS (MCM1, Agamous, Deficiens, serum response factor SRF) domain as well as an adjacent domain known as the MEF2 domain (Black and Olson, 1998). The MEF2 are suggested to interact with tissue specific cofactors to control gene expression in phenotypically different muscles. Furthermore, MEF2 has been shown to function synergistically with myogenic bHLH proteins to activate the myogenic program in vertebrate skeletal muscles (Black et al., 1998). MEF2 also functions in collaboration with PAR domain protein-1 in *Drosophila* skeletal muscles (Lin et al., 1997). Recently, MEF2 had been identified as a factor to enhance PTTH gene expression in *Bombyx mori*, and BmMEF2 may participate in several other cellular processes that regulate prothoracicotropic hormone (PTTH) secretion through signaling pathways (Shiomi et al., 2005).

Using molecular techniques, four MEF2 genes, designnated MEF2A, -B, -C and -D, had been cloned and sequenced from humans and mice (McKinsey et al., 2002). Based on the whole genomic sequence data, seven MEF2 factors, MEF2A-F, were identified in *Drosophila melanogaster* (Lilly et al., 1994; Hoskins et al., 2007). Shiomi et al. (2005) identified a MEF2 factor from *B. mori* (BMEF2). BmMEF2 appears to be particularly important in the brain where it is responsible for the differentiation of lateral neurosecretory cells, including the enhancement of PTTH gene expression, which is the first report to identify a target gene of MEF2 in the invertebrate nervous system. PTTH and other neuropeptide, such as eclosion hormone (Wei et al., 2008), allatotropin (Yin et al., 2005) and diapause hormone (Sun et al., 2003; Wei et al., 2004), playing a central role in the endocrine network controlling insect development (Harshini

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et al., 2003), growth, molting, metamorphosis and diapause.

To date, there are no reports about the other isoforms of MEF2 gene in *B. mori.* In this study, we cloned MEF2 cDNA from the brain of stain "Liangguang 2" of *B. mori* by reverse transcriptase- polymerase chain reaction (RT-PCR), which sequence showed some absence and mutations to the MEF2 from the stain "N4". Thus, we designated the new isoform of MEF2 as BMEF2B, and the other as BMEF2A. Using RT-PCR and southern blotting methods, the distribution and developmental expression of BMEF2B mRNA were demonstrated in *B. mori*.

METHOD AND MATERIALS

Insect

A commercially available strain (Liangguang 2, a bivoltine strain) of the silkworm, *B. mori*, was used in all experiments of the present study. The larvae were reared on fresh mulberry leaves at 25±1°C under a photoperiod of L14:D10 (light: dark). Brains and other tissues were dissected in insect saline (0.75% NaCl) from larvae, pupae, and adults and stored at -70°C until use.

RNA isolation, cDNA synthesis, and polymerase chain reaction (PCR)

Total RNA was isolated from the brains of *B. mori* by using an acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The 20 brains were homogenized in Solution D, placed on ice for 5 min, and then sodium acetate and chloroform/ isoamylalcohol (49:1) were added. The mixture of homogenized brains was centrifuged at 10,000×g at 4°C for 20 min. The supernatant was transferred into a new tube, and then the isopropanol was added. After centrifugation, the RNA pellet was washed in 75% ethanol and then dissolved in the ddH_2O. One microgram of total RNA was reverse transcribed at 42 °C for 1 h in a 10 µl final volume reaction mixture containing reaction buffer, 10 mM DTT, 0.5 mM dNTP, 0.5 µg oligo-dT18, and reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Japan).

Two specific primers MF (5′-CAAGATTGCTGCCACCATG-3′) and MR (5′-CCGAAGGCCTCATAGCCTA-3′) were designed based on the known BMEF2A sequences (Shiomi et al., 2005). PCR reaction was performed with primers MF and MR under the following conditions: 30 cycles of 40 s at 94°C, 60 s at 55°C, and 90 s at 72°C. A DNA band corresponding to approximately 1.2 kb of the expected size was excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Japan). The purified PCR product was ligated into T-vector (Takara, Japan) and then transformed into XL-1 blue competent bacteria. The positive recombinant clone with an insert was sequenced using the dideoxy-nucleotide chain termination method (Takara, Dalian, China).

Sequence analysis and molecular modeling of BMEF2B construction

The DNA and amino acid sequence analysis were performed using the program of DNAstar of Lasergene 6 software (Zhao et al., 2007). Alignments of the protein-coding genes for each of the available MEF2s were made using the programs Clustal X (Thompson et al., 1997).

We used the SWISS-MODEL for modeling the 3D structure of BMEF2B. The SWISS-MODEL is a fully automated protein structure homology-modeling server via the ExPASy web server (http://swissmodel.expasy.org/) (Arnold et al., 2006).

Tissue distribution of BMEF2B mRNA

Total RNA was extracted from different tissues, and then subjected to thirty PCR cycles. One microgram of total RNA was reverse transcribed, and then subjected to thirty PCR cycles. PCR reaction was performed with primers MF and MR under the following conditions: 30 cycles of 40 s at 94°C, 60 s at 55°C, and 90 s at 72°C.

Developmental expression of BMEF2B mRNA

The developmental expression of BMEF2B mRNA was measured with the combined methods of quantitative RT-PCR and Southern blot analysis according to previously described procedures (Wei et al., 2005; 2008). Total RNA was extracted from 20 brains in different stages. The first strand cDNA was synthesized from 1 µg of total RNA at 42°C for 1 h, prepared with an AMV reverse transcript system kit (TaKaRa, Japan). The MEF2B cDNA fragment was amplified with the primers MF and MFR2 (Figure 1) for 20 cycles to ensure that the reaction was in the linear range, based on our preliminary experiment (data not shown). The PCR products were electrophoresed on a 1.2% agarose gel and transferred to a Hybond-N^+ membrane. The 500 bp fragment corresponding to nucleotide position 1-500 of the MEF2B cDNA was labeled with [α-^{32}P]-dCTP using a random primed DNA labeling kit (Takara, Japan). Southern hybridization with the labeled MEF2B cDNA as a probe and signal detection was the same as described by Wei et al. (2005, 2008).

RESULT

Cloning and characterization of BMEF2B cDNA

Based on the sequences of BMEF2 reported by Shiomi et al. (2005), two specific primers MF and MR were designed and used for PCR amplification. A 1218 bp fragment was amplified, cloned and sequenced (Figure 1). The sequence includes a complete open reading frame of 1206 bp, which is identical with the known BMEF2 (Shiomi et al., 2005), but shows an absence of 12 nucleotides (GCACTCACAAAG) and three nucleotides mutation (Figure 1). These results suggest that the new MEF2 isoform we have cloned from the strain "Liangguang 2" is quite probably related to BMEF2 in the strain "N4" of *B. mori* (GenBank no. AB121093), hence, we named it BMEF2B (GenBank no. EF100967). The isoform from the strain “N4” was termed as BMEF2A.

The open reading frame (ORF) of BMEF2B started with ATG, and terminated by a TAA stop codon, which encodes a predictive precursor protein containing 401 amino acids (Figure 1). A MADS box and an adjacent MEF2 domain are encoded within an 86-amino acid N- terminal sequence. These two regions are highly conserved in MEF2s from various organisms (Figure 2). By compari-

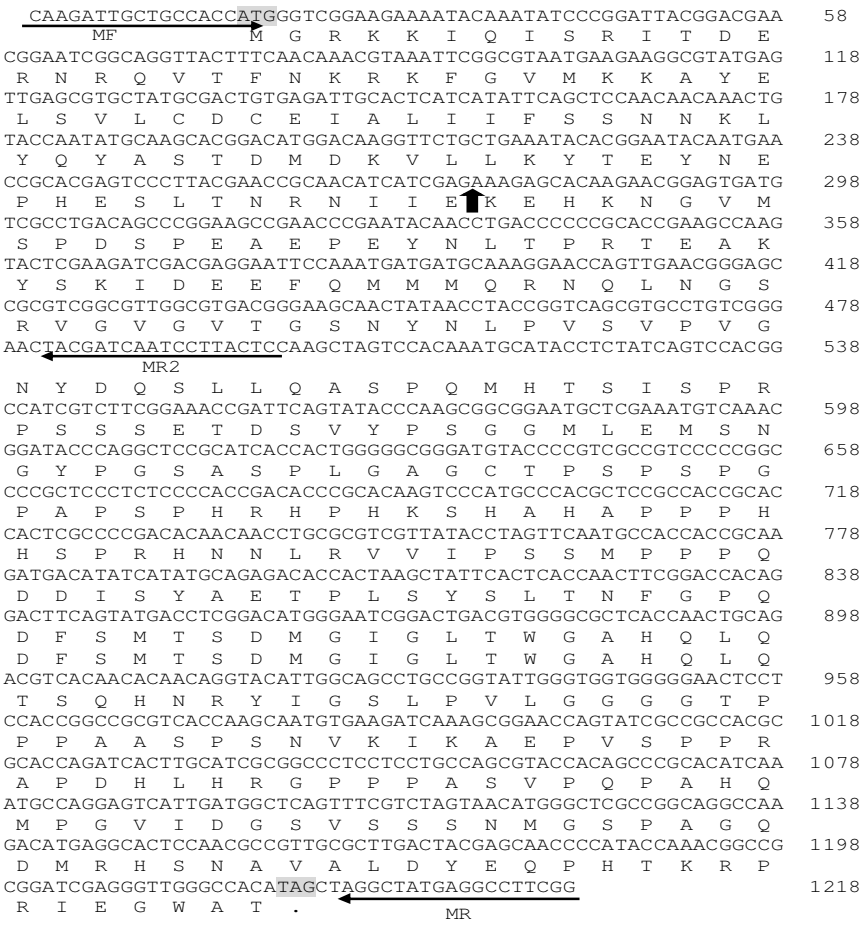

Figure 1. Nucleotide and deduced amino acid sequences of MEF2B cDNA in B. mori. The suggested start codon (ATG) and stop codon (TAG) are shaded. The vertical arrow shows the site that thare are 12 bp absence in compared to the MEF2A cDNA sequence. Arrows below the nucleotide sequence represent the position of the different synthetic primers used in PCR. Specific primers for amplification are MF (5' CAAGATTGCTGCCACCATG-3') and MR (5'-CCGAAGGCCTCATAGCCTA-3') .

son with BMEF2A, there are four amino acids absence (ALTK) in the downstream of MEF2 domain of BMEF2B, which is similar to the six isoforms of *D. melanogaster* (Lilly et al., 1994; Hoskins et al., 2007).

Model building of BMEF2B 3-D structure

We used the SWISS-MODEL (http://swissmodel.expasy.org) to model the 3-D structure of BMEF2B. The precursor protein that containing 401 amino acids could not be modeled, and only the model of partial fragment of the N terminal 93 aa including MADS box and an adjacent MEF2 domain could be obtained. For the N terminal 93 aa of BMEF2B, the Ala44-Phe48 and Leu54-Ala58 are suggested as two anti-parallel β-turns. The Glu14-Leu38, Met62-Thr70 and Asn81-Gly92 form two α-helixes. The topology structure of the BMEF2B is α-β-β-α-α (Figure 3), which is according to the crystal structure of the MADS-box/MEF2S domain of human MEF2B (Han et al., 2003). For the BMEF2A, only the N terminal 91 aa could be modeled, and the structure is similar to that of BMEF2B (data not show).

Tissue expression of Osf-EH mRNA

The tissue specificity of EH mRNA was also examined using RT-PCR (Figure 4). The BMEF2B mRNA was detected not only in the central nervous system (CNS), including brain, suboesophageal ganglion (SG), thoracic ganglion (TG), and abdominal ganglion (AG), but in the non-neural tissues, such as the midgut (Mg), fat body (FB), and epidermis (Ep) from larvae of the 5th instar, and muscle (Ms) and ovary (Ov) from adults. The tissue distribution of BMEF2B is similar to that of BMEF2A (Shiomi et al., 2005).

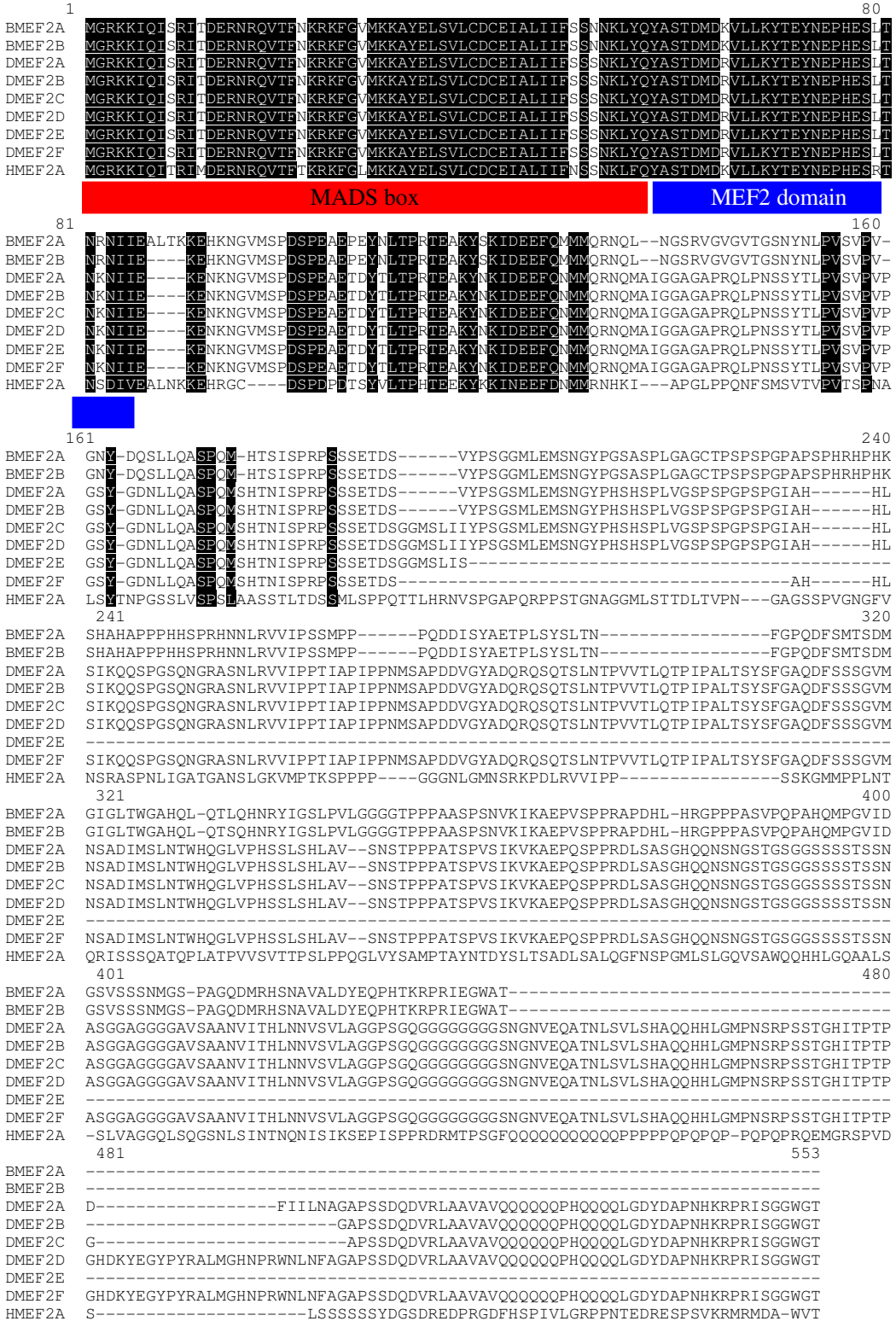

Figure 2. Alignment of deduced amino acid sequences of MEF2s. BMEF2A, MEF2A of *B. mori* (AB121093); BMEF2B, MEF2B of *B. mori* (EF100967); DMEF2A, MEF2A of *D. melanogater* (NM_057673); DMEF2B, MEF2B of *D. melanogater* (NM_057671); DMEF2C, MEF2C of *D. melanogater* (NM_057670); DMEF2D, MEF2D of *D. melanogater* (NM_057672); DMEF2E, MEF2E of *D. melanogater* (NM_165722); DMEF2F, MEF2F of *D. melanogater* (NM_206067); HMEF2A, MEF2A of *H. sapiens* (BC013437). The conserved residues are shaded. The MADS box and the MEF2 domain are shown in red and blue, respectively.

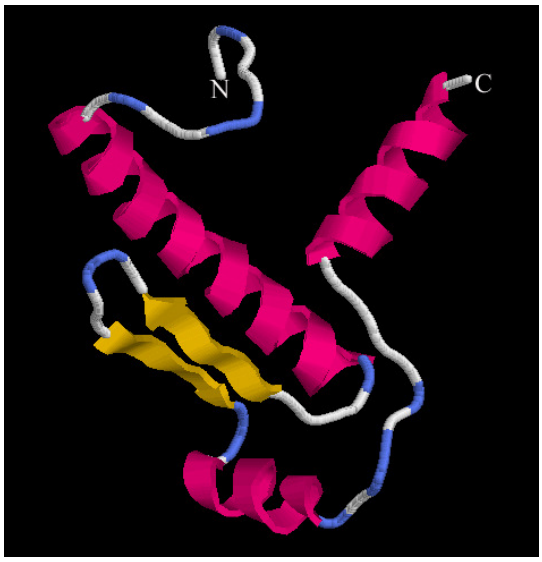

Figure 3. The predicted 3 D structure of BMEF2B of *B. mori*. The model was model using SWISS-MODEL, a fully automated protein structure homology-modeling server via the ExPASy web server.

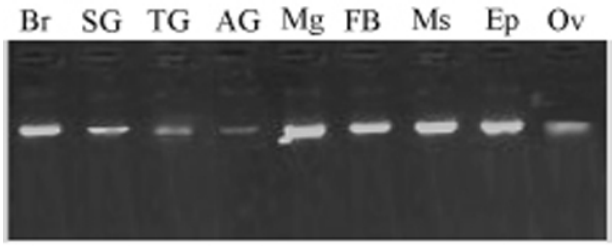

Figure 4. RT-PCR analysis of the tissue expression of BMEF2B mRNA in *B. mori*. Total RNA extracted from the tissues was reverse transcribed and then subjected to 30 PCR cycles using MF and MR. Brain (Br), suboesophageal ganglion (SG), thoracic ganglion (TG), abdomen ganglion (AG), midgut (Mg), fat body (FB), and epidermis (Ep) were dissected from larvae of the 5th instar, and muscle (Ms) and ovary (Ov) were dissected from adults, respectively.

Developmental expression of BMEF2B mRNA

The developmental changes of BMEF2B mRNA in the brain were measured using RT-PCR combined with Southern blots. The expression levels of BMEF2B mRNA were consistently high from larvae to adults. After the pupal ecdysis, the BMEF2B mRNA decreased gently, but increased again, and kept a higher level after eclosion (Figure 5).

DISCUSSION

In vertebrates, four isoforms of MEF2 gene, designated MEF2A, -B, -C and -D, had been cloned and sequenced. After whole genome sequence, seven MEF2 factors,

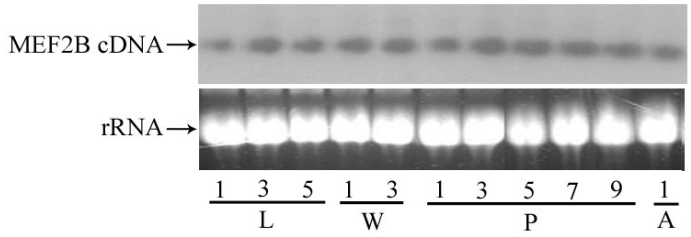

Figure 5. Developmental changes of BMEF2B mRNA in the brain. RNA was extracted from 20 brains of larvae, pupae, and adults. One microgram of total RNA was subjected to reverse transcription and PCR amplification (20 cycles), then PCR products were electrophoresed on a 1.2% agarose gel and hybridized using labeled oligonucleotide as a probe. L, P, and A represent 5th instar of larvae, pupae, and adults, respectively. The Arabic numerals indicate the day of larval, pupal, and adult stages.

MEF2A-F, were identified in *D. melanogaster*. These factors all show homology within a conserved MADS domain as well as an adjacent domain known as the MEF2 domain (McKinsey et al., 2002). One isoform of MEF2 gene, termed BMEF2A, had been cloned from the stain "N4" of *B. mori* (Shiomi et al, 2005). In the present study, a new isoform of MEF2 gene, named as BMEF2B, was cloned from the strain "Liangguang 2" of *B. mori*. By comparison with BMEF2A, there are 12 nucleotides absence (GCACTCACAAAG) and three nucleotides mutation in BMEF2B. The ORF of BMEF2B encodes a 401 amino acids precursor. The N-terminal 86 amino acids of BmMEF2B are highly conserved and include a MADS box and a MEF2 domain. The MADS box is essential for DNA binding and dimerization, and the MEF2 domain plays an important role in DNA binding affinity as well as an indirect role in dimerization. The C-terminal portion of BMEF2B may be required for its transcriptional activation (Molkentin et al., 1996). Using the SWISS-MODEL, the structure of the BMEF2B was prected as α-β-β-α-α, which is according to the crystal structure of the MADS-box/MEF2S domain of human MEF2B (Han et al., 2003).

In the present study, BMEF2B mRNA could be detected in the central nervous system and other non-neural tissues, including midgut, fat body, epidermis, muscle and ovary. However, the BMEF2B mRNA in different tissues showed different pattern: the BMEF2B mRNA in midgut, fat body, epidermis and muscle are higher than those in other tissues; the BMEF2B mRNA in brain is higher than those in other neural tissues. The distribution and expression pattern in different tissues are similar to the expression of BMEF2A (Shiomi et al., 2005). BmMEF2A had been identified as a factor to enhance PTTH gene expression (Shiomi et al., 2005), but the expression of PTTH gene is different to that of BMEF2. PTTH mRNA in *B. mori* (Adachi-Yamada et al., 1994) and *Helicoverpa armigera* (Wei et al., 2005) mainly expressed in brain, but low level in other tissues, such as midgut, epidermis and silk gland. The above different

pattern may relate to the other functions of MEF2 factors. In mammals, MEF2 can bind directly to the promoters or enhancers of the majority of musclespecific genes and interacts with members of the MyoD family of basic helix–loop–helix (bHLH) proteins to activate the skeletal muscle differentiation program. MEF2 factors have also been implicated in myocyte hypertrophy (Kolodziejczyk et al., 1999), in the formation of slow twitch skeletal muscle fibers (Wu et al., 2000), and in vascular smooth muscle cell proliferation, differentiation and patterning (Lin et al., 1998).

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