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silk proteins. The molecular mechanisms involved in this short period are still not well-understood.

Multiprotein bridging factor 1 (MBF1) is a transcriptional coactivator. But very few researches in silkworm were reported. It is meaningful to realize its function of MBF1 gene because silkworm is a model insect and its unique silk synthesis character. This study describes expression patterns of MBF1 mRNA in the silk gland, epidermis, fat body, and midgut of the fifth instar larvae during the development of B. mori. We conducted the quantitative analysis of MBF1 mRNA with actin (A3) as the internal standard through SYBR Green I real-time RT-PCR method. The results showed MBF1 gene was expressed in all investigated organs but highly expressed in the silk gland. This suggests that MBF1 plays an important role in the development of the silkworm as a tissue-specific and stage-specific coactivator, showing its relation to biosynthesis of silk proteins.

Materials and methods

Experimental animals

The silkworm larvae of strain P50 were reared on mulberry leaves at 25°C under a 16 h light, 8 h dark photoperiod. The silk gland, epidermis, fat body and midgut of the fifth instar larvae (10 larvae as a sample) were dissected for analysis of developmental expression patterns.

Chemistry reagents

TRIZOL, pUCm-T vector, EZ spin column plasmid maxi-preps kit (Invitrogen, USA), fast ligation kit and hot start fluorescent PCR core reagent kits (SYBR Green I) were bought from Bio Basic Inc., Canada. MMLV first-strand cDNA synthesis kit was purchased from Sangon Company, Shanghai, China.

RNA extraction and cDNA synthesis

The total RNA was extracted from samples using TRIZOL reagent. The RNA was used as a template for first-strand cDNA synthesis using M-MLV reverse transcriptase with oligo dT_{18} primer under reaction conditions of 5 min at 70 °C, 10 min at 37°C, 60 min at 37°C, 10 min at 70°C. For gene fragment amplification, two primers were designed as MBF1's forward primer 5′-AAggatccATGTCTGACTGGG ATACAGT, and reverse primer 5′-ACAaagcttTTATTTCT GTCCGCCAGGAG; Actin's forward primer 5′-GCGCG GCTACTCGTTCACTACC, and reverse primer 5′-GGA TGTCCACGTCGCACTTCA.

The PCR was conducted in 20 μl system containing 13.1 μl double distilled water, 2 μl 10 × PCR buffer, 0.2 μl 25 mmol/l dNTP mix, 1 μl 10 mmol/l primer I, 1 μl 10 mmol/l primer II, 1.2 μl 25 mmol/l $MgCl_2$, 0.5 μl Taq DNA polymerase, 1 μl cDNA. The condition of reaction was set as 50 s at 94°C, 50 s at 60°C, 1 min at 72°C and final extension for 10 min at 72°C.

Cloning the PCR product and sequencing

The RT-PCR generated products were T/A cloned into pUCm 18-T vector and then sequenced.

Comparison of MBF1 transcription by semi-quantitative RT-PCR

The comparison of MBF1 transcription by semi-quantitative RT-PCR was performed according to a modified procedure of previously described protocol [14]. Actin gene, a house-keeping gene, was used as a reference gene to allow for normalization by visual inspection of mRNA levels. The quantity of products was screened in agarose gels. All experiments were repeated three times.

SYBR real-time quantitative PCR

PCR amplifications were performed using a Bio-RAD IQ™5 Multicolor real-time PCR detection system. The PCR was conducted in 25 μl system containing 9.5 μl ddH_2O, 12.5 μl Hotstart Fluo-PCR mix, 1 μl 10 mmol/l primer I, 1 μl 10 mmol/l Primer II, 1 μl cDNA. The PCR protocol was done by 30 s denaturation at 94°C, 30 s annealing at 60°C, and 30 s elongation at 72°C in 35 cycles. Fluorescence was detected at the end of every 72°C extension phase. To exclude the contamination of unspecific PCR products such as primer dimers, melting curve analysis was applied to all final PCR products after the cycling protocol.

Construction of the standard curve of the MBF1 and actin gene

To determine the copy number of the target transcript, the cloned plasmid DNA for MBF1 and actin were used to generate the standard curve. The plasmid DNA was purified using alkaline prep. Plasmids contained the cDNA inserts encoding the respective PCR products in a pUCm-T vector. The cloned plasmid DNA were consecutively diluted every tenfold at a range of 1×10^7–1×10^3 copies.

Comparison of MBF1 transcription by SYBR Green I RT-PCR

This was performed similar to the standard curve. Each cDNA from the silk gland, epidermis, fat body, and midgut of the first, fourth and eighth day of the fifth instar larvae

were run in triplicates. The threshold cycle (C_t) values were averaged from each reaction. Actin gene isolated in this study was used as a reference gene to normalize mRNA levels.

Data analysis

The data were expressed as mean±SD, and compared statistically by *t*-test, $P < 0.05$ being considered significant.

Results

Clone and sequence of the MBF1 gene

The total RNA was extracted from samples using the TRIZOL reagent. The RNA was used as the template for first-strand cDNA synthesis using M-MLV reverse transcriptase with oligo dT_{18} primer. The PCR was conducted using special primers for MBF1 and actin gene amplification. The electrophoresis bands of PCR product is shown in Fig. 1.

The RT-PCR generated products were T/A cloned into pUCm 18-T vector and then sequenced. The sequence of MBF1 cDNA is shown in Fig. 2. GenBank Blast search revealed that the resulting nucleotide sequences of MBF1 and actin gene shared the same identity with silkworm *B. mori*. It contained a single open reading frame of 146 amino acids. The deduced amino acid sequence of this factor predicts a basic protein (pI 10.7) with a molecular mass of 16.2 kDa.

Expression of MBF1 mRNA in organs with semi-quantitative RT-PCR

We used RT-PCR method to amplify MBF1 and act
from the silk gland, epidermis, fat body, and mi
PCR was carried out for 28 cycles for MBF

Fig. 1 The
a 458 bp
actin g

30 cycles for actin gene. The electrophoresis bands of PCR product is shown in Fig. 3.

As Fig. 3 shows, the highest expression level of MBF1 gene was detected in the silk gland. The accumulation of MBF1 mRNA in the silk gland reached its peak at the first day of the fifth instar larvae and then decreased significantly. The expressions of the MBF1 gene in the epidermis, fat body, and midgut were lower than that of the silk gland. In the midgut, the expression level of MBF1 gene has demonstrated an increasing trend with the development of larvae.

Standard curve analysis for SYBR Green I real-time quantitative PCR

Standard curves for each MBF1 and actin
obtained using standard plasmids that en
products. The quantitative nature is de
linear relationship between the log c
number and C_t value. We also con
titative relationship between the C_t
of the t concentrations of
samples. A range with a co
of 99 was described as
curve analysis revealed
product (Fig. 4c). W
gene samples, a
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Fig. 3 Semi-quantitative RT-PCR analysis of MBF1 transcripts in organs. Agarose gel analyses of MBF1 ampliÞcations in comparison with those of controls (actin). (1) Molting fourth instar; (2) the Þrst day of the Þfth instar larval; (3) the third day of the Þfth instar larval; (4) the Þfth day of the Þfth instar larval; (5) the seventh day of the Þfth instar larval

midgut to 8.57 in the silk gland of the Þrst day of the Þfth instar (Table 1 and Fig. 6). As Fig. 6 shows, the highest expression level of MBF1 gene was detected in the silk gland. The accumulation of MBF1 mRNA in the silk gland reached its peak at the Þrst day of the Þfth instar larvae and then decreased. The statistical signiÞcance is evidently different between different growths. Based on these results, we hypothesize that the silk protein synthesis is activated in the beginning of the Þfth instar. The MBF1 gene regulated the procedure in this stage.

Furthermore in the midgut, the expression of MBF1 mRNA has revealed the reverse phenomenon. The expression increased with the development of the Þfth instar, which was consistent with the results of semi-quantitative RT-PCR analysis. In the epidermis and fat body, the expression of MBF1 mRNA signiÞcantly decreased in the fourth day and reached its peak at the eighth day of the Þfth instar larvae (Fig. 7).

Discussion

MBF1 contains a nuclear export signal (NES)-like sequence in its C-terminal region, which is conserved among eukaryotes [15]. For example, amino acid residues 119–130 of *B. mori* MBF1, LGKIERAIGIKL, and the corresponding region of human MBF1, LGIERAIGLKL [16], are similar to the leucine-rich NES in HIV Rev protein, LPPLERLTL, and protein kinase inhibitor, LALKLAGLDI [17]. During larvae development, BmFTZ-F1, MBF1, and MBF2 form a nuclear complex at the molting stage D3. Transient expression of a reporter gene in S2 cells demonstrated that MBF1 and MBF2 enhance BmFTZ-F1-dependent transcription. These observations support that MBF1 and MBF2 serve as coactivators that mediate BmFTZ-F1-dependent transcriptional activation [18]. MBF1 can signiÞcantly stabilize BmFTZ-F1 binding to DNA [10, 19].

Takemaru et al. provides evidence acquired in vitro and in vivo that yeast MBF1 mediates GCN4-dependent transcriptional activation by bridging the DNA-binding region of GCN4 and TBP [8, 20]. These Þndings indicate that the coactivator MBF1 functions by recruiting TBP to promoters where DNA-binding regulators are bound [21]. Through immuno-precipitation, MBF1 was found to form a ternary complex including MBF1, TBP, and the bZIP protein tracheae defective (TDF)/Apontic. Many data demonstrate a crucial role of MBF1 in the development of the tracheae and central nervous system [22] via a direct interaction with the basic region of Drosophila Jun (D-Jun), MBF1 prevents an oxidative modiÞcation (S-cystenyl cystenylation) of the critical cysteine and stimulates AP-1 binding to DNA by preserving the redox-sensitive AP-1 activity. It has been shown that human MBF1 stimulates the transcriptional activity of steroidogenic factor 1, a human homolog of fushi tarazu factor 1, which is implicated in steroidogenesis [23]. Plant MBF1 can be rapidly induced by several stresses whereas animal MBF1 was not induced. MBF1 function in plants is controlled on the level of transcriptional induction, but not by nuclear translocation, unlike the case of MBF1 in animals [24].

The sensitivity of SYBR Green I analysis is more than adequate for the majority of research applications, and its quantitative nature is demonstrated by the linear relationship between the log of template concentration and cycle number at which ßuorescence rises above baseline. The increased sensitivity and speciÞcity of SYBR Green I real-time assay obtained by increasing the ampliÞcation size coupled with the low cost of SYBR Green dye as opposed to TaqMan probes will help to extend the applicability of SYBR Green I real-time PCR in detecting other viral pathogens as well as in cellular gene expression studies.

In this study, we described the expression pattern of MBF1 mRNA using both the semi-quantitative RT-PCR and the SYBR Green I real-time quantitative RT-PCR method. During the larvae stage, MBF1 mRNA was

Fig. 4 Example of real-time PCR for quantiÞcation of MBF1 mRNA. (a) Example of real-time PCR ampliÞcation stand curves of MBF1; (b) example of real-time PCR ampliÞcation recursive equation obtained by plotting ßuorescence date against their cycl number; (c) sample of real-time PCR ampliÞcation melting curves. The T_m is 84 C, the production is speciÞc

Fig. 5 Example of real-time PCR for quantiÞcation of actin mRNA. (a) Example of real-time PCR ampliÞcation stand curves of actin; (b) example of real-time PCR ampliÞcation recursive equation obtained by plotting ßuorescence date against their cycle number; (c) sample of real-time PCR ampliÞcation melting curves. The T_m is 84 C, the production is speciÞc

constitutively expressed in all investigated organs. It can be considered MBF1 is a very important gene in the growth of B. mori. The highest expression level of MBF1 gene was detected in the silk gland, revealing the strong relation with biosynthesis of silk proteins. The accumulation of MBF1 mRNA in the silk gland reached its peak at the Þrst day of the Þfth instar larvae and then decreased signiÞcantly. This implies high MBF1 activity is necessary for silk protein gene transcription in the beginning of Þfth instar, and the MBF1 expression was reduced with the gradual deterioration of the silk gland and ultimately silk protein synthesis ceased at the eight day of the Þfth instar. On the contrary, the expression of MBF1 mRNA reached its peak in epidermis and fat body at the eighth day of the Þfth instar larvae, suggesting MBF1 regulated the genes of metamorphosis such as chitinase and fatty acid synthetase. Thus, on the basis of this present research, we conclude that multiprotein bridging factor 1 (MBF1) is required for transcriptional activation of genes during the silkworm development.

Table 1 Relative quantiÞcation of MBF1 mRNA in organs by comparison with those of controls (actin)

Stage	Silk gland		Epidermis		Fat body		Middle gut	
First day of the Þfth instar	8.5745	0.0306	0.1481	0.0290	0.3051	0.1005	0.0302	0.0061
Fourth day of the Þfth instar	5.4731	0.0493**	0.0338	0.0067**	0.0992	0.1056**	0.1124	0.0113*
Eighth day of the Þfth instar	1.4881	0.3842**	0.1787	0.0084*	0.4702	0.0691**	0.2116	0.1013**

Unit: MBF1 mRNA copy/actin mRNA copy. Values are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$

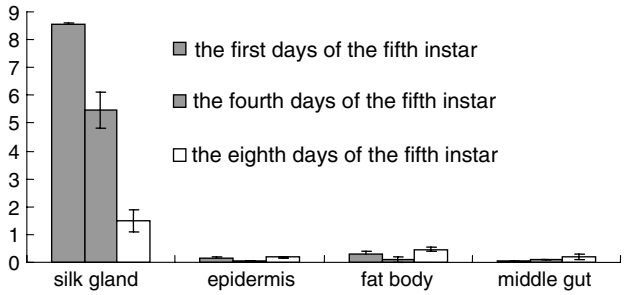

Fig. 6 MBF1 mRNA expression in all organs of Þfth instar. The levels of each MBF1 mRNA and actin were measured by SYBR Green I real-time RT-PCR. Silk glandÕs MBF1 mRNA expression was signiÞcantly higher than that of other organs ($P < 0.05$)

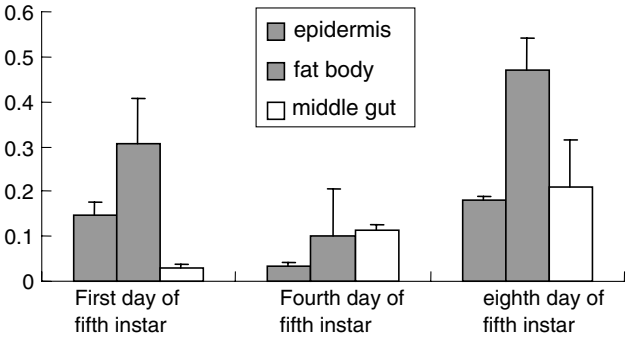

Fig. 7 MBF1 mRNA expression in epidermis, fat body and middle gut of Þfth instar. The levels of each MBF1 mRNA and actin were measured by SYBR Green I real-time RT-PCR

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