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Identification of a POU factor involved in regulating the neuron-specific expression of the gene encoding diapause hormone and pheromone biosynthesis-activating neuropeptide in *Bombyx mori*

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Diapause hormone (DH) and PBAN (pheromone biosynthesis-activating neuropeptide) are two important insect neuropeptides regulating development and reproduction respectively. In the present study, we report two *Bombyx mori* transcription factors interacting specifically with the promoter of Bom-DH-PBAN (where Bom-DH stands for *B. mori* DH); we named them DHMBP-1 and -2 (DH-modulator-binding proteins 1 and 2). The developmental changes of DHMBP-1/-2 are closely correlated with that of Bom-DH-PBAN mRNA throughout the pupal stage. Competition assays indicate that DHMBP-1 from Chinese *B. mori* possesses binding characteristics similar to those of the POU-M1 protein from Japanese *B. mori*. POU-M1 cDNAs were cloned from various tissues of Chinese *B. mori* and were found to be distinct from the previously published POU-M1 in amino acid residues 108–136 because of insertion mutations. Owing to this difference in amino acid residues, we named this cDNA POU-M2. Even though POU-M2 differs from POU-M1 at the N-terminal, the POU domain and the binding properties of both POU-M1 and -M2 are the same. Functional analysis showed that overexpression of POU-M2 in the *Bombyx* cell line BmN activated the promoter of Bom-DH-PBAN, but failed to activate a promoter in which the POU-binding element was mutated. The transcriptional activity of POU-M2 is probably regulated by other factors binding to the upstream of the promoter sequence. We show that the POU-M2-binding site was able to activate the transcription of a heterologous promoter of the gene encoding *B. mori* larval serum protein. POU-M1 was found to exhibit the same transcriptional activities as POU-M2. Taken together, these results demonstrate that POU-M2 plays an important role in the transcriptional regulation of the Bom-DH-PBAN gene.

Key words: *Bombyx mori*, diapause hormone-modulator-binding protein, diapause hormone, pheromone biosynthesis-activating neuropeptide, POU, transcription factor.

INTRODUCTION

Insect diapause occurs as an alternative developmental programme in the life cycle, which ensures survival under unfavourable environments and increases mating chances by synchronizing the growth rate of the population [1,2]. *Bombyx mori* enters diapause at embryonic stage. Diapause is induced by DH (diapause hormone), which acts on developing ovaries of pharate adults to induce the laying of diapause eggs in the next generation. DH is a 24-amino-acid amidated peptide belonging to the FXPRL-NH_2 peptide family, which includes PBAN (pheromone biosynthesis-activating neuropeptide), melanization and reddish coloration hormone, myotropin and pyrokinin [2]. The DH cDNA from SG (suboesophageal ganglion) encodes a polypeptide precursor, which produces five peptides with the same FXPRL-NH_2 at the C-terminal by post-translational processing. The five peptides are DH, PBAN and α-, β- and γ-SG neuropeptides [3,4]. PBAN is a 33-amino-acid neuropeptide; in Lepidoptera, it can stimulate the pheromone gland of female adults to secrete sex pheromone to attract male adults for mating [5,6]. Similar cDNA structures were also found in many other Lepidopteran species, including *Helicoverpa zea* [7], *Helicoverpa assulta* [8], *Agrotis ipsilon* [9], *B. mandarina* [10], *Spodoptera littoralis* [11], *Helicoverpa armigera* [12], *Heliothis virescens* [13], *Manduca sexta* (GenBank® no. AY172672) and *Adoxophyes* sp. (GenBank® no. AF395670).

The Bom-DH-PBAN gene is expressed only in the SG of *B. mori* [14]. Expression of the Bom-DH-PBAN gene at early-middle and late stages of pupae is coincident with the occurrence of diapause and pheromone syntheses, suggesting that Bom-DH-PBAN gene expression is the initial event leading to diapause induction and pheromone production [15]. Thus the Bom-DH-PBAN gene is spatially and temporally expressed during different developmental stages. A genomic DNA analysis of Bom-DH-PBAN showed a canonical TATA box in its promoter region, and primer extension revealed two transcription-initiation sites [4]. However, the mechanism for regulating the expression of the Bom-DH-PBAN gene is still not clear.

In an effort to elucidate the regulatory mechanism of Bom-DH-PBAN gene expression, we found that two transcription factors, which we named DHMBP-1 and -2 (DH-modulator-binding proteins 1 and 2), interact with an octamer-like sequence near the TATA box. We also determined DHMBP-1 to be a POU

Abbreviations used: BmLSP, *Bombyx mori* larval serum protein; DH, diapause hormone; DHMBP, DH-modulator-binding protein; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; PBAN, pheromone biosynthesis-activating neuropeptide; RG, rabbit globin; RT, reverse transcriptase; SG, suboesophageal ganglion.

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The nucleotide sequence data reported have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY334012.

factor, which we named POU-M2, and found that overexpression of POU-M2 activated the promoter of Bom-DH-PBAN.

MATERIALS AND METHODS

Insects and nuclear protein extracts

A commercially available hybrid race (Jingsong × Haoyue, a bivoltine strain) of the silkworm, *B. mori*, was used in all experiments of the present study. The eggs were incubated at 25 °C, and larvae were reared on fresh mulberry leaves at 25–27 °C. The SGs were dissected out in an insect saline from day-1, day-5 and day-10 pupae; muscle was collected from day-10 pupae; embryos were collected on day 5 after laid; and silk glands were collected from day-2 fifth instar larvae.

Tissues were lysed in 4× tissue volume of buffer A [10 mM Hepes-K^+, pH 7.9/1 M sucrose/4 mM $MgCl_2$/1 mM KCl/1 mM DTT (dithiothreitol)/0.3 mM PMSF] using a glass Dounce homogenizer. The lysates were centrifuged for 10 min at 310 ***g*** in an SLA-1500 rotor. The supernatant was poured into a clean test tube and centrifuged for 20 min at 5000 ***g*** to pellet the nuclei. The pellets were suspended by 4× pellet vol. of buffer A and mixed with 1.2× buffer A volume of high sucrose solution (10 mM Hepes-K^+, pH 7.9/1.75 M sucrose/2 mM $MgCl_2$/1 mM KCl/0.1 mM EDTA/1 mM DTT/0.3 mM PMSF) and then centrifuged for 30 min at 23 000 ***g*** to pellet the nuclei. The nuclear pellets were suspended with 4× pellet vol. of nuclear extraction buffer (10 mM Hepes-K^+, pH 7.9/0.3 M sucrose/2 mM $MgCl_2$/350 mM KCl/100 mM NaCl/1 mM DTT/0.3 mM PMSF), and shaken gently for 30 min on ice. The nuclear extracts were centrifuged at 100 000 ***g*** for 1 h at 4 °C. Then, 0.33 g of solid $(NH_4)_2SO_4$ per ml of extract was added slowly, and the mixture was stirred gently for 30 min on ice. The protein was then precipitated by centrifugation at 12 500 ***g*** for 20 min, resuspended with 0.5 ml of dialysing solution [20 mM Hepes-K^+, pH 7.9/20 % (v/v) glycerol/100 mM KCl/0.1 mM EDTA/0.5 mM DTT/0.3 mM PMSF], and centrifuged at 12 000 ***g*** for 20 min to remove insoluble material. The supernatant was dialysed three times against 100 volumes of dialysis buffer at 4 °C. These samples were finally centrifuged for 20 min at 12 000 ***g***, and the supernatants were stored at − 70 °C.

Probes and probe labelling

The probes used in the experiments are shown in Figure 1. Probe P1, corresponding to − 282 to − 98 bp of the promoter of the Bom-DH-PBAN gene, was produced by PCR using primers P1F (5′-GAATTCCAAAGTCATCAG-3′) and P1R (5′-GGAATTCG-TACCATACGTTAAA-3′); probe P2, corresponding to − 114 to − 29 bp, was produced using P2F (5′-GGAATTCATTTTAA-CGTATG-3′) and P2R (5′-CGGATCCAGACCTTTATATAG-3′); probe P3, corresponding to − 79 to − 29 bp, was produced using P3F (5′-GGAATTCCCCTCATTTACATACATC-3′) and P2R. The *Eco*RI restriction enzyme site was designed in primers P1R, P2F and P3F. Probe S2 was prepared by annealing two oligonucleotides, namely 5′-TCCGACTTTTCCTATATAAAGG-3′ and P2R. Probes S1 and S3–S8 were prepared by annealing the forward sequences shown in Figure 1 with the common reversed sequence 5′-GTCGGACGGGG-3′. FIB, SC and SA were also prepared by annealing two partially complete oligonucleotides according to the known sequence [16,17]. All of the gaps in the probes produced by *Eco*RI digestion or by annealing oligonucleotides with partial overlap were filled using a Klenow fragment (Takara, Osaka, Japan) in the presence of [α-^{32}P]dATP, dCTP, dGTP and dTTP for labelling probes. Competitive probes

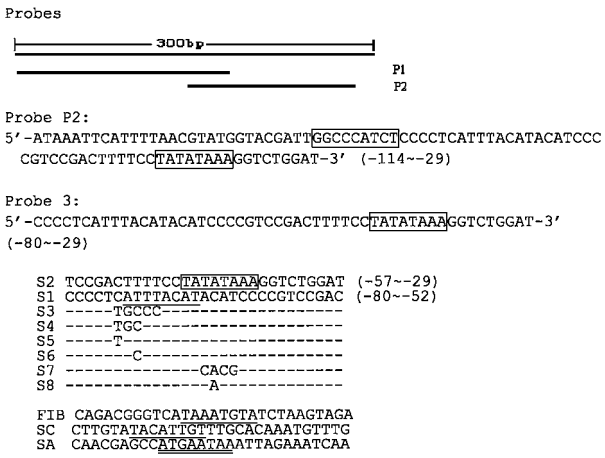

Figure 1 Probes used in EMSA

Overlapping probes P1 and P2 cover − 282 to − 29 bp of Bom-DH-PBAN promoter. Probes P1–P3 were produced by PCR using synthetic primers based on the sequence of Bom-DH-PBAN promoter, and other probes (S1–S8, FIB, SC and SA) were synthesized by annealing double-stranded oligonucleotides. The probes were labelled with [α-^{32}P]dATP by filling in the sticky ends produced by enzyme digestion or by partially annealing. FIB, SC and SA are three binding sites for POU and forkhead proteins in *Bombyx*. The TATA and CAAT boxes are indicated by rectangles, binding sites of POU in S1, FIB and SC probes are underlined and forkheads in SA are double underlined.

were produced by the same method, except that dATP was used instead of [α-^{32}P]dATP.

EMSA (electrophoretic mobility-shift assay)

In general, 10 fmol of a ^{32}P-labelled probe was incubated with nuclear protein extracts (5–10 μg) for 30 min at 27 °C in 20 μl of a reaction mixture containing 10 mM Hepes-K^+ (pH 7.9), 10 % glycerol, 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml BSA, 0.1 mM PMSF and 1 μg of poly(dI/dC) (Amersham Biosciences). The reaction mixtures were loaded on to a 5 % (w/v) native polyacrylamide gel and electrophoresed in 1 × TBE buffer (45 mM Tris/borate/1 mM EDTA) at 150 V. After electrophoresis, the gel was dried and subjected to autoradiography in the presence of an intensifying screen at − 70 °C for 16 h. Competition assays were performed by preincubating the reaction mixtures with the specified amount of excess unlabelled probes for 10 min before the addition of labelled probes.

RT (reverse transcriptase)–PCR amplification and quantification

Total RNA was extracted from the SG, silk gland, midgut, ovary, fat body, epidermis, muscles and cell lines (BmN, Bm5 and sf-21) by the single-step method of acid guanidinium thiocyanate–phenol–chloroform extraction [18], and quantified by measuring the UV absorbance at 260 or 280 nm. Total RNA (1 μg) containing 0.01 ng of RG (rabbit globin) mRNA (Gibco BRL, Gaithersburg, MD, U.S.A.) as an internal standard was reverse-transcribed at 42 °C for 1 h in a 1 × buffer (50 mM Tris/HCl, pH 8.3/75 mM KCl/3 mM $MgCl_2$), 10 mM DTT, 0.5 mM dNTP, 0.5 μg of oligo-$dT_{15\text{-}18}$ and RT AMV (avian myeloblastosis virus; Promega) to a final volume of 10 μl. The reaction was terminated by heating at 75 °C for 10 min.

PCR amplification of the Bom-DH-PBAN cDNA fragment was performed using primers PX1 (5′-TGGTTCGGCCCCA-GACTC-3′) and PX2 (5′-CAGTCTGGCGAAAAGCTCAT-3′),

corresponding to positions 134–151 and 468–485 of the Bom-DH-PBAN cDNA sequence under the following conditions: 30 cycles of 60 s at 93 °C, 60 s at 55 °C, 50 s at 72 °C, followed by 5 min at 72 °C. PCR amplification for the full reading frame of POU-M1/-M2 was performed using the primers PO1 (5′-CGGGATCCATGGCGGCGACCACGTACATG-3′) and PO2 (5′-CCCAAGCTTAGTGCGCTGCCAGTGTGTG-3′), corresponding to positions 179–201 and 1216–1236 of the reported POU-M1 cDNA sequence, under the following conditions: 30 cycles of 60 s at 93 °C, 60 s at 55 °C, 70 s at 72 °C, followed by 5 min at 72 °C. RG cDNA was amplified with the primers RP1 (5′-CACTTCGACTTCACCCACGG-3′) and RP2 (5′-TCAGCACGGTGCTCACGTTG-3′), corresponding to 372–391 and 742–761 bp of the RG cDNA sequence. Takara *Taq* (Takara, Osaka, Japan) was used in the amplification of Bom-DH-PBAN and RG, and LA *Taq* with high GC buffer (Takara) was used in the amplification of POU-M2.

For semi-quantitative measurements of RNA levels, 20 cycles of PCR were performed. The PCR products were electrophoresed on a 1.2 % (w/v) agarose gel, transferred on to a Hybond N^+ membrane (Amersham Biosciences) and hybridized with the corresponding probes labelled with [α-^{32}P]dCTP using a random-primed DNA-labelling kit (Takara).

Cloning and sequencing of POU-M2

The PCR product was ligated into pBluescript KS(+) vector, and sequenced by Takara (Dalian, China) on an automatic DNA sequencer using the dye terminator methodology. The correct POU-M2 sequence was digested by *Bam*HI and *Hin*dIII, and cloned into pBluescript KS(+) or pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.) plasmid. The recombinant plasmids were named T7-PM2 and EGFP-PM2 respectively. Positive clones were sequenced using the dideoxynucleotide chain-termination method (Takara, Dalian, China). POU-M1 cloned in pBluescript KS(+) (T7-PM1) was kindly provided by RIKEN DNA Bank, Japan (RDB 1829). The POU-M1 open reading frame was amplified using T7-PM1 as the template and PO1 and PO2 as primers. The PCR product was then cloned into pEGFP-C1 at the *Bam*HI–*Hin*dIII site, and the recombinant plasmid was named EGFP-PM1.

In vitro translation

The T7-PM1 and T7-PM2 plasmid DNAs (1 μg) were used as a template for *in vitro* translation in the TNT quick coupled transcription/translation system (Promega) containing 40 μl of TNT T7 Quick Master Mix, 1 μl of methionine (1 mM) and 8 μl of distilled water. The reaction was performed at 30 °C for 1.5 h. We used 2 μl of the translation product for EMSA.

Reporter plasmids

The genomic DNA of *B. mori* was extracted from silk gland as described previously [19], and 100 ng of DNA was used in each PCR. The upstream regions of the Bom-DH-PBAN gene starting at position + 18 and extending to − 4912 (DH 5k), − 3372 (DH 3.5k), − 2050 (DH 2k) or − 270 (DH 0.3k) were produced by PCR using the forward primers DHP1 (5′-CATAAGTGAATACGGCCCATTG-3′), DHPF2 (5′-CGGCACAGACGCGTCGCTCA-3′), DHPF3 (5′-CCTTACCAGCTATTTTTACTAGG-3′) and DHPF4 (5′-CATCAGGGTTAAAGCTTAAAAC-3′) and the reverse primer DHPR (5′-GTTGGTCCCTTCGGTGGT-3′). The PCR products were purified and cloned into the pGL2-basic plasmid containing luciferase gene (Promega) at *Sma*I site, and we named them 5kL, 3.5kL, 2kL and 0.3kL respectively. The POU-binding site mutations of 5kL and 2kL were produced by inverse PCR with the primers MR (5′-AGGGGAGATGGGCCAATCGTAC-3′) and MF (5′-TGCTTACATACATCCCCGTCCG-3′), corresponding to − 96 to − 75 bp and − 74 to − 53 bp of Bom-DH-PBAN promoter, using a MutanBEST kit (Takara), and we named them 5kM and 2kM. The underlined TGC is the mutated site.

To insert the S1 sequence (POU-binding element) to the proximal promoter of the BmLSP (*B. mori* larval serum protein) gene, inverse PCR was performed with the primers LSPF (5′-AAAAATTCGAATTGTATAAAAGGCGATGTG-3′) and LSPR (5′-TTTCGGAACATAAAAAACTTAGTTTATCAG-3′), corresponding to − 44 to − 15 bp and − 74 to − 45 bp of BmLSP promoter [20,21]. The inverse PCR product was blunted, phosphorylated and then ligated with the blunt-ended S1 sequence. Sequence and orientation were verified using LSPR and the forward strand of S1. The correct clone was named BmLSPn (BmLSP near). The insertion of S1 sequence into the 5′-end of BmLSP promoter was constructed by ligating the blunt-ended S1 sequence into the end-filled *Nhe*I restriction site at − 899 bp of BmLSP promoter.

Cell culture and transfection

Two *B. mori* cell lines (BmN, Bm5) and a *Spodoptera frugiperda* cell line (sf-21) were cultured in TC-100 medium with 10 % foetal calf serum at 27 °C.

For transient transfections, the cells were split in the ratio 1:3, cultured for 24 h and then transfected with 60 μl of transfection solution containing 5 μl of lipofectin, 1 μg of reporter plasmid DNA and 0.5 μg of internal control plasmid (pSV-β-galactosidase Control Vector; Promega) in 1 ml of serum-free medium for 4–6 h. For co-transfection, 9 μl of lipofectin, 1 μg of reporter plasmid DNA, 1 μg of EGFP-M2 plasmid and 0.5 μg of internal control plasmid were used. The serum-free medium was then replaced by the medium with 10 % foetal serum. The cells were incubated for another 48 h and harvested. Each treatment was repeated three times.

Measurement of the luciferase activity

Luciferase and β-galactosidase assays were performed using commercially available kits (Promega) according to the manufacturer's instructions. Luciferase activity in three separate experiments were determined in triplicate using a liquid-scintillation spectrometer (Beckman LS6000 series; Beckman, Fullerton, CA, U.S.A.). β-Galactosidase activity was measured using a spectrophotometer. The luciferase values were divided by the β-galactosidase values to control the transfection efficiency.

RESULTS

Two proteins bind to a 29 bp sequence at the proximal promoter of Bom-DH-PBAN

We first analysed the protein-binding elements at the proximal promoter, which is essential for transcription. Probes P1 and P2 overlap from − 282 to − 29 bp of the Bom-DH-PBAN promoter. P1 (− 282 to − 98 bp) contains no obvious binding sites of known transcription factors, and P2 (− 114 to − 29 bp) contains a canonical TATA box and a non-canonical CAAT box, whose core sequence GGCCCATCT has one base mismatch compared with the conserved GGCCAATCT motif (20 Bi; Figure 1). Incubation of the labelled probe P1 and nuclear protein

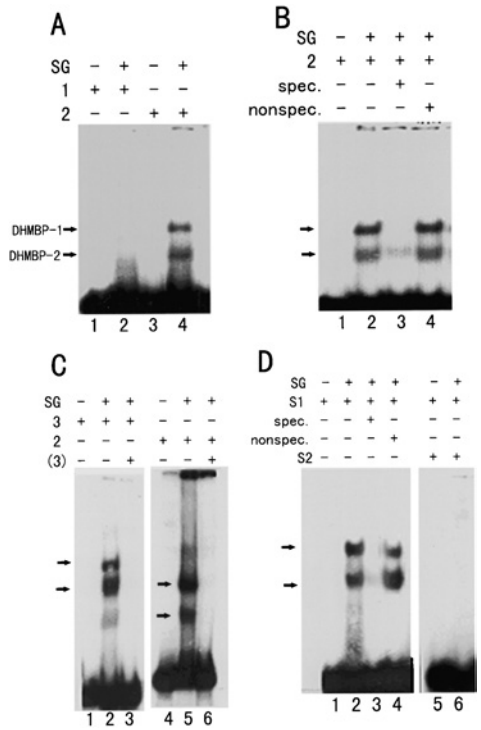

Figure 2 Detection of two transcription factors DHMBP-1 and -2 by EMSA

Different probes were incubated with 5–10 μg of nuclear protein extracts from SGs of day-1 pupae. (**A**) Probes P1 and P2 were incubated with nuclear protein extracts (lanes 2 and 4). Lanes 1 and 3, free probes; row 1, P1; row 2, P2. (**B**) P2 was incubated with nuclear protein extract without competitor (lane 2), 80-fold unlabelled P2 (lane 3) or 80-fold unrelated oligonucleotides (+30 to +100 bp of the PTTH gene of *B. mori*; lane 4). spec., specific competitor; non-spec., non-specific competitor. (**C**) P3 was incubated with nuclear extracts without competitor (lane 2) or with 80-fold unlabelled P3 (lane 3). P2 was incubated with nuclear extracts without competitor (lane 5) or with 80-fold unlabelled P3 (lane 6). Row (3), P3 as a specific competitor. (**D**) Probe S1 was incubated with nuclear extracts without competitor (lane 2), with 80-fold unlabelled probe S1 (lane 3) or 80-fold unrelated oligonucleotides (lane 4). Probe S2 was incubated with nuclear extracts (lane 6).

extract of SG produced no binding complex (Figure 2A, lane 2), whereas incubation of P2 generated two distinct binding complexes DHMBP-1 and -2 (Figure 2A, lane 4). A 50-fold excess of P2 competed with DHMBPs, but the intensity of the two bands remained unchanged when an unrelated DNA fragment was used as a competitor (Figure 2B, lanes 3 and 4). These results indicate that DHMBPs bind specifically to the promoter region of -114 to -29 bp. To narrow down the DNA region responsible for the specific binding, we designed a shorter probe P3 (-79 to -29 bp), which did not include the CAAT box, to test whether the DHMBPs belong to CAAT-box-binding protein. The DHMBPs were still detected with P3 (Figure 2C, lane 2), and unlabelled P3 could effectively compete with the interaction of DHMBPs with P2 (Figure 2C, lane 6). Thus DHMBPs bind to a region within P3. Probably, DHMBP is not TFIID, which is known to bind to the TATA box, since TFIID is a high-molecular-mass complex and its migration would be expected to be much slower compared with DHMBP. To test this hypothesis, two overlapping oligonucleotides, S1 (-80 to -52 bp) and S2 (-57 to -29 bp), were synthesized, and only S2 contained TATA box. We found that S1 interacted specifically with the two protein factors DHMBP-1 and -2 (Figure 2D, lane 2), whereas no shift band was observed when probe S2 was used (Figure 2D, lane 6). Our results suggest that the binding sites of DHMBP-1 and -2 are probably within the 29-bp S1 oligonucleotide.

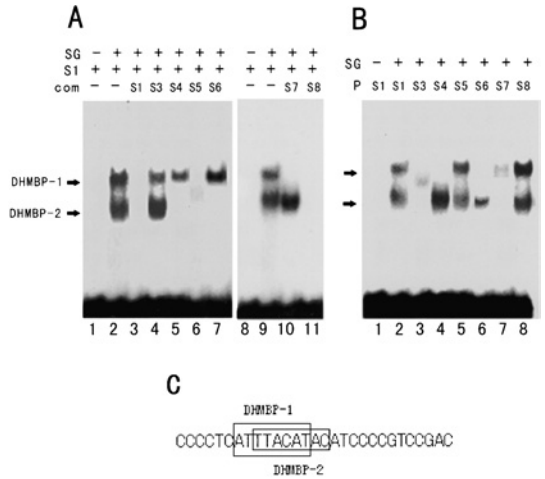

Figure 3 Determination of the accurate binding sites to DHMBP-1 and -2

Different probes were incubated with 5 μg of nuclear protein extracts from SG of day-1 pupae. (**A**) Probe S1 was incubated without competitor (lane 2) or with 80-fold unlabelled mutants S1 and S3–S8 (lanes 3–7, 10 and 11). com., competitor. (**B**) Binding of DHMBP-1 and -2 to S1 (lane 2) and its mutants S3–S8 (lanes 3–8). (**C**) The deduced binding sites of DHMBP-1 are in the large rectangle and DHMBP-2 in the small rectangle.

DHMBP-1 and -2 bind to an overlapping site

The 29-bp probe S1 consists of an octamer-like AT-rich sequence (ATTTACAT) flanked by GC-rich sequences (Figure 1). To determine the exact binding site of DHMBP-1 and -2, we designed a series of mutations in the nucleotides (S3–S7) at or around the octamer. The unlabelled mutants were used in competition assays (Figure 3A), and the labelled mutants were used in EMSAs (Figure 3B). Unlabelled S3 with the mutation of CATTTACAT to TGCCCACAT was unable to compete with the DHMBPs (Figure 3A, lane 4). We observed only a faint band when the labelled probe S3 was incubated with the nuclear protein extract (Figure 3B, lane 3), but the unlabelled S3 was unable to compete with this complex (results not shown). Therefore we conclude that the weak band was due to non-specific binding. The CATTT sequence is thus critical for the binding of both the DHMBPs. Unlabelled S4 and S6 oligonucleotides with changes in the octamer (Figure 1) competed only with the complex of DHMBP-2 (Figure 3A, lanes 5 and 7), and labelled probes S4 and S6 interacted only with DHMBP-2 (Figure 3B, lanes 4 and 6). Unlabelled S5 oligonucleotide with changes at the 5′-side of the octamer competed with both complexes (Figure 3A, lane 6), whereas the labelled probe S5 interacted with both DHMBP-1 and -2 (Figure 3B, lane 5). Unlabelled S7 oligonucleotide with a mutation adjacent to the 3′-side of the octamer competed only with the complex of DHMBP-1 (Figure 3A, lane 10), whereas labelled S7 probe interacted with DHMBP-1, although the complex was weaker compared with that of S1 (Figure 3B, lane 7). S8 with a single mutation had no effect on the binding of both the DHMBPs (Figure 3B, lane 8), which implies that the AT-rich sequence is also an important part of the binding site of DHMBP-2. DHMBP-1 and -2 have overlapping binding sites, which are summarized in Figure 3(C).

A spatiotemporal pattern of expression of DHMBPs during pupal development

We focused on the relationship between DHMBPs and Bom-DH-PBAN transcriptions. The same amount of nuclear extracts

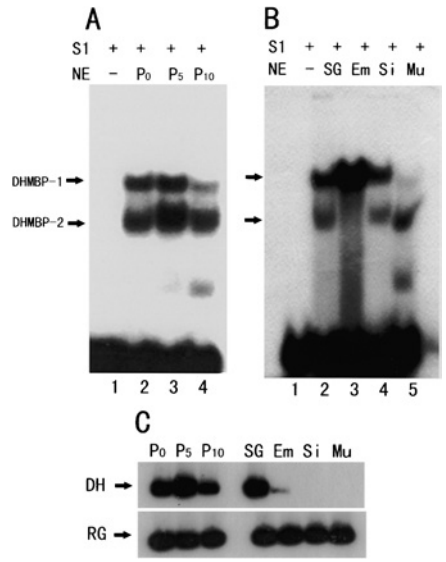

Figure 4 Developmental changes and tissue distribution of DHMBP-1 and -2

(**A**) Probe S1 was incubated with 5 μg of nuclear protein extracts (NE) from SG of day-1 (P0), day-5 (P5) and day-10 (P10) pupae (lanes 2–4). (**B**) Probe S1 was incubated with 10 μg of nuclear protein extracts from SG of day-1 pupae (lane 2), day-5 embryo (Em, lane 3), silk gland from day-2 fifth instar larva (Si, lane 4) and muscles of pharate adults (Mu, lane 5). (**C**) Changes of Bom-DH-PBAN mRNA in SG of pupae at different stages and its distribution in different tissues. Total RNA extracted from SGs was subjected to RT–PCR amplification with 18 cycles. PCR products (DH) were electrophoresed on a 1.2 % agarose gel and hybridized using labelled oligonucleotide as a probe. RG mRNA was added as an internal standard.

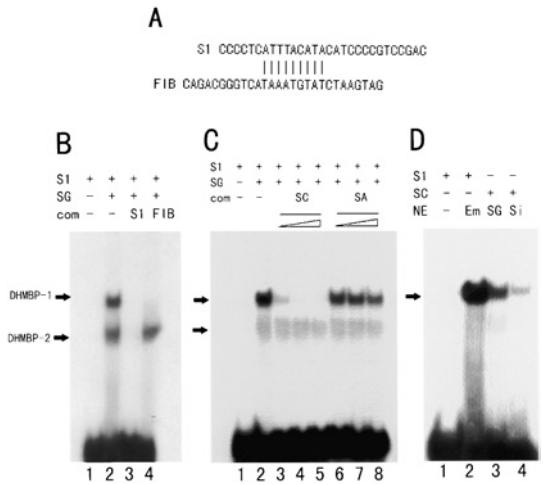

Figure 5 Competition assay of DHMBP-1

Different probes were incubated with 5 μg of nuclear protein extracts from SG of day-1 pupae. com., competitor. (**A**) Comparison of the sequences of S1 and FIB from the first intron of fibroin gene of *B. mori*. (**B**) Probe S1 was incubated without competitor (lane 2) or with 80-fold unlabelled probe S1 or FIB (lanes 3 and 4). (**C**) Probe S1 was incubated without competitor (land 2), with unlabelled probe SC (lanes 3–5) and SA (lanes 6–8). Triangles on the top of the panels show increasing amounts of competitors (40-, 80- and 160-fold). (**D**) Probe S1 was incubated with nuclear protein extract of embryo (lane 2), and probe SC was incubated with 10 μg of nuclear protein extract of SG (lane 3) and silk gland from day-2 fifth instar larva (lane 4). NE, nuclear protein extract.

from SGs at different pupal stages was incubated with probe S1. DHMBP-1 content increased from day 1 to day 5 and then decreased on day 10, a day before adult eclosion (Figure 4A). DHMBP-2 exhibited the same pattern, although the magnitude of the change was not as great as that of DHMBP-1. We also monitored the changes in Bom-DH-PBAN mRNA. The amount of Bom-DH-PBAN mRNA also increased progressively from day 1 to day 5, and decreased on day 10 (Figure 4C). Thus the changes in transcription factors DHMBP-1/-2 at pupal stage are closely correlated with those shown by Bom-DH-PBAN mRNA.

We examined the tissue distribution of DHMBPs in the nuclear extracts from SG, muscle, silk gland and day-5 embryo. We detected both DHMBP-1 and -2 in the nuclear extracts of SG and silk gland (Figure 4B). DHMBP-1 was abundant in embryos, whereas DHMBP-2 was absent from them. On the other hand, only DHMBP-2 was expressed in muscles. In previous studies, the Bom-DH-PBAN gene is transcribed only in the SG, and the transcription begins at the embryonic stage [14,15]. The results of semi-quantitative RT–PCR also showed that Bom-DH-PBAN mRNA only occurs in the SG and embryo (Figure 4C).

DHMBP-1 is probably a member of the POU protein family

The ATTTACAT sequence in S1 is similar to the octamer at position +290 in the intron of the fibroin gene (FIB; Figure 5A), which has been reported to interact with the POU-M1, forkhead and an unknown FMBP-1 [16]. Unlabelled FIB efficiently competed with DHMBP-1, but not with DHMBP-2 (Figure 5B, lane 4). Two probes, SC and SA, recognized specifically by POU-M1 and forkhead respectively, were also synthesized (Figure 1) and used in further competition assays. We found that a 40-fold excess of unlabelled SC competed with DHMBP-1 efficiently, but even unlabelled 160-fold excess of SA could not compete with it (Figure 5C). The SC could form a POU-M1 complex with the nuclear protein extract of the silk gland from day-2 fifth instar larva (Figure 5D, lane 4), which is in agreement with the previous findings [16,17]. SC probe also formed a complex with the nuclear protein extract of SG (Figure 5D, lane 3). The mobility of the two complexes described above was the same as that of DHMBP-1 from embryos binding to S1 probe (Figure 5D, lane 2). Since the probes of both SC and S1 are 29 bp, the molecular masses of DHMBP-1 and POU-M1 may be identical. To confirm the molecular mass of DHMBP-1, we partially purified DHMBP-1 from the nuclear extract of embryos using an affinity column, and a band of approx. 40 kDa that might correspond to POU-M1 was detected (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/380/bj3800255add.htm). On the basis of all these results, we suggest that DHMBP-1 is quite probably POU-M1.

Cloning and characterization of POU-M2 from Chinese *B. mori* similar to POU-M1 from Japanese *B. mori*

POU-M1 cDNA was first cloned from the silk gland of a Japanese *B. mori*. Its expression was also present in tissues other than silk gland [22,23]. We cloned a PCR fragment from the SG of the Chinese *B. mori*, Jingsong × Haoyue, using the primers designed according to the sequence of POU-M1 cDNA. The sequence includes a complete open reading frame of 1056 bp, which is identical with the known POU-M1, except for an insertion of a guanine at position 325 and two insertions of guanine and cytosine at position 404. The mutations cause a frameshift in the 28 amino acids at positions 109–136, but other parts of the sequence, including the POU-specific domain and POU homeodomain, remain the same as that of *B. mori* POU-M1 (Figure 6A). Fragments with the predicted size could be amplified from SG, embryo, midgut and fat body other than silk gland, but

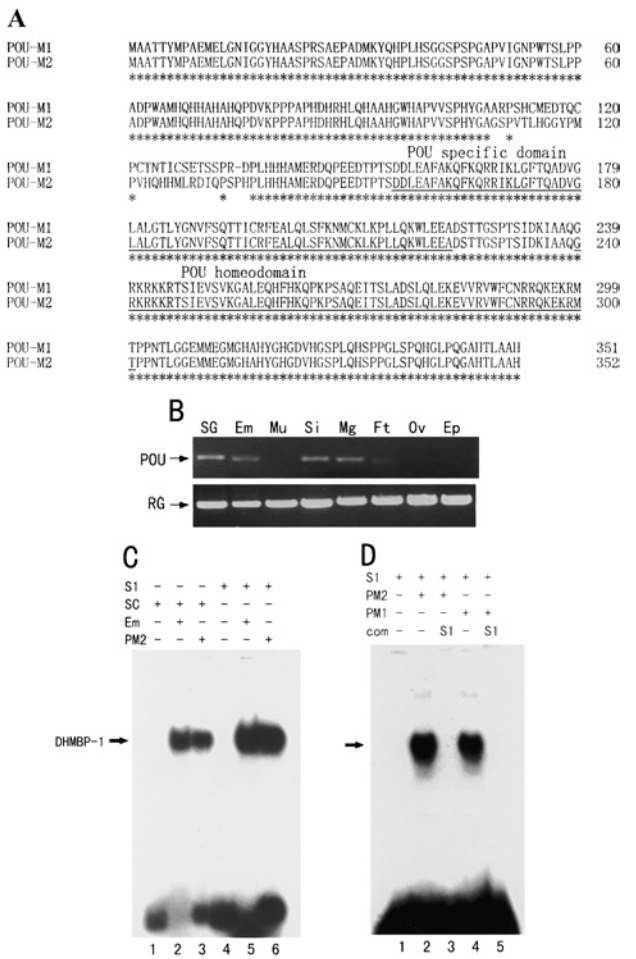

Figure 6 Cloning and characterization of POU-M2

(**A**) Comparison of the deduced amino acid sequence of POU-M2 cloned from SG of Chinese *B. mori* and the known POU-M1 cloned from silk gland of Japanese *B. mori*. Asterisks show identical amino acids. POU-specific domains and homeodomains are underlined. (**B**) The tissue distribution of the POU-M2 (PM2) transcript in different tissues of Chinese *B. mori*: embryo (Em), muscle (Mu), silk gland (Si), midgut (Mg), fat body (Ft), ovary (Ov) and epidermis (Ep). Total RNA (1 μg) from different tissues was reverse-transcribed and PCR amplification was performed with 30 cycles, and the expected band was 1 kb. RG mRNA was used as an internal standard for the RT–PCR. (**C**) EMSA of expressed POU-M2 and DHMBP-1 in the nuclear extract of embryo. We applied 2 μl of expressed POU-M2 (PM2) and 10 μg of nuclear extracts of embryo (Em) to the EMSA with S1 and SC. (**D**) EMSA of expressed POU-M1 (PM1) and POU-M2 (PM2). We applied 2 μl of expressed PM1 or PM2 to the EMSA with S1.

were undetectable in muscles and epidermis (Figure 6B). The sequences of the PCR fragments amplified from silk gland, embryo and midgut are the same as that from SG. These results suggest that the new POU member we have cloned is quite probably related to POU-M1 in the Japanese *B. mori* and, hence, we named it POU-M2 (GenBank® no. AY334012). The expressed POU-M2 could interact with S1 and SC probes and had the same mobility as DHMBP-1 in the nuclear extracts of the embryo (Figure 6C). To compare POU-M1 and POU-M2, we obtained POU-M1 cDNA, which was deposited in RIKEN DNA Bank by the laboratory of Dr Y. Suzuki (RDB 1829). Although the N-terminals of POU-M1 and POU-M2 are different, the binding characteristics of POU-M1 and POU-M2 with S1 probe were the same (Figure 6D). From these results, we have shown that DHMBP-1 and POU-M2 have similar DNA sequence-binding specificities, supporting the conclusion that DHMBP-1 is probably POU-M2.

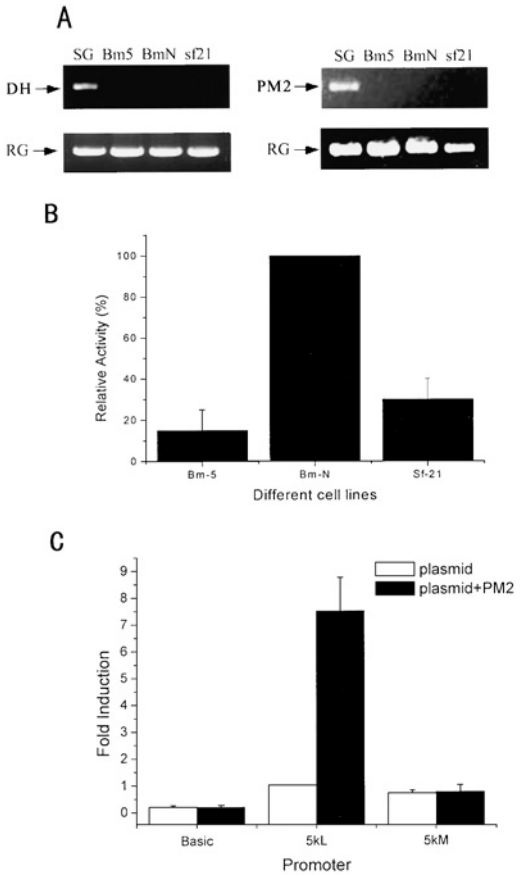

Figure 7 Overexpression of POU-M2 in cell lines activates the Bom-DH-PBAN promoter

(**A**) Expression of Bom-DH-PBAN (DH) and POU-M2 (PM2) genes in SG and insect cell lines. We reverse-transcribed 1 μg of total RNA from SGs or cell lines, followed by PCR amplification with 30 cycles. RG mRNA was used as an internal standard for the RT–PCR. (**B**) Luciferase activity of Bom-DH-PBAN promoter in insect cell lines Bm5, BmN and sf-21. We transfected 1 μg of 5kL DNA into different cell lines. Cells were harvested for luciferase assays at 48 h after transfection. Results are normalized relative to the luciferase activity in BmN cell line, which was set at 100 %. (**C**) Bom-DH-PBAN is activated specifically by POU-M2. We co-transfected 1 μg of 5kL or pGL2-basic without (open bar) or with (black bar) 1 μg of EGFP-PM2 into the BmN cell line. The content of lipofectin was adjusted based on the amount of plasmid DNA used. Cells were harvested, 48 h after transfection, for luciferase assays. Results are normalized relative to the luciferase activity of 5kL in BmN cell line, to which a value of 1.0 was assigned.

Overexpression of POU-M2 activates the Bom-DH-PBAN promoter

We detected the expression of the Bom-DH-PBAN gene in the insect cell lines BmN, Bm5 and sf-21 by RT–PCR. Bom-DH-PBAN mRNA could not be amplified in these cell lines, whereas the predicted 350 bp band was detected in the SG of pupae, which was used as a positive control (Figure 7A). Therefore we conclude that the Bom-DH-PBAN promoter in the genome may not be active in these cell lines. However, a low activity of luciferase controlled by the 4.8 kb Bom-DH-PBAN promoter (5kL) was detected in the BmN insect cell line (Figure 7B). Compared with the background, no luciferase activities were found in Bm5 and sf-21. Expression of POU-M2 was also not detected in the cell lines (Figure 7A). To determine whether POU-M2 could activate the Bom-DH-PBAN promoter, we co-transfected BmN cells with 5kL along with a POU-M2 expression plasmid EGFP-PM2. As shown in Figure 7(C), the forced expression of POU-M2 in BmN cells activated the Bom-DH-PBAN promoter significantly. In contrast, POU-M2 could not activate pGL2-basic without a promoter.

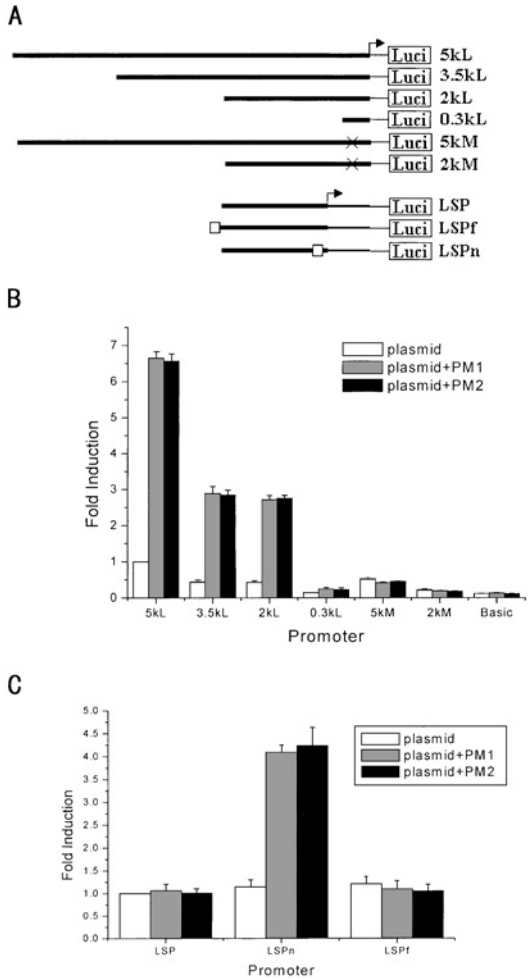

Figure 8 Deletion analysis of Bom-DH-PBAN promoter and functional analysis of POU-M2-binding site

(**A**) Diagram of luciferase reporter gene constructs. The bent arrow indicates the transcription-initiation site. The open bar represents the S1 sequence. The cross indicates mutation of the POU-binding site. (**B**) Luciferase activities of different Bom-DH-PBAN promoters. We transfected 1 μg of each reporter plasmid into BmN cell line without POU plasmid (open bars), with EGFP-PM1 (grey bars),or with EGFP-PM2 (black columns). Results are normalized relative to the luciferase activity of 5kL in BmN cell line, to which a value of 1.0 was assigned. (**C**) Luciferase activities of BmLSP, BmLSPn and BmLSPf. We transfected 1 μg of each reporter plasmid into BmN cell line without POU plasmid (open bars), with EGFP-PM1 (grey columns) or with EGFP-PM2 (black bars). Results are normalized relative to the luciferase activity of BmLSP in BmN cell line, to which a value of 1.0 was assigned.

Deletion and mutation analysis of the Bom-DH-PBAN promoter

Different deletions for the Bom-DH-PBAN promoter were performed to verify whether the *cis*-elements upstream are involved in the activation of the promoter (Figure 8A). POU-M1 and POU-M2 transfected in parallel with 5-, 3.5- or 2-kb-long promoters of the Bom-DH-PBAN gene had similar abilities to increase the activity (Figure 8B). The 3.5- and 2-kb-long promoters had a similar activity, which was nearly half of that of the 5-kb-long promoter. The 0.3-kb-long promoter had almost no activity whether it was co-transfected with POU-M1/-M2 or not (Figure 8B). These results suggest that the *cis*-elements upstream are essential for the Bom-DH-PBAN promoter activity. The 5- or 2-kb-long Bom-DH-PBAN promoter mutated at the proximal POU-binding site (5kM and 2kM) could not be activated by POU-M1 or POU-M2 (Figure 8B). Thus the defined POU-binding site is essential for Bom-DH-PBAN promoter activity. POU-M1 and POU-M2 showed similar activities in all the transfection assays.

Activation of a heterologous promoter induced by POU-M1/-M2

The BmLSP gene is highly expressed in the fat body of *B. mori* [20], and contains a canonical TATA box and several important *cis*-elements located at positions -593 to -303 and in the first intron [21]. The S1 sequence containing a POU-binding site was inserted into the upstream [BmLSPf (BmLSP far)] or the proximal site (BmLSPn) of the BmLSP promoter respectively. The insertion of the S1 sequence did not alter the activity of the BmLSP promoter (Figure 8C). Co-transfection of POU-M1 or POU-M2 increased the BmLSPn promoter activity approx. 4-fold, but had no effect on the BmLSP or BmLSPf promoter activity.

DISCUSSION

Several insect genes encoding neuropeptide hormones such as eclosion hormone [24], allatotropin [25], PTTH (prothoracicotropic hormone) [26], allatostatin [27] and DH [3,4] have been cloned over the years. However, regulation of these genes has not been reported so far. In the present study, we identified a POU transcription factor POU-M2 binding to the proximal promoter of the Bom-DH-PBAN gene. The co-transfection experiment demonstrated that POU-M2 directly activated the Bom-DH-PBAN promoter. These results indicate that POU-M2 is indeed involved in the regulation of Bom-DH-PBAN gene expression.

Using EMSA, the proteins DHMBP-1 and -2 bound to a 29 bp site were detected in the nuclear extracts. Our results suggest that DHMBP-1 is POU-M2, since (i) the interaction of DHMBP-1 and its specific probe S1 could be competed by the unlabelled SC specific to the *Bombyx* POU-M1 reported previously [22]; (ii) the complexes of SC with the nuclear protein extracts of SG and the silk gland had the same mobility as that of S1 with the nuclear protein extracts of embryo and SG; (iii) the characteristics of POU-M2 cloned from Chinese *B. mori* are identical with those of POU-M1, although their N-terminals are different; (iv) POU-M2 expressed *in vitro* could bind to S1 and had the same mobility as DHMBP-1; and (v) POU-M2 is effective in activating Bom-DH-PBAN promoter in co-transfection assays.

POU domain factors, which contain a POU-specific domain and a POU homeodomain binding to the octamer, play a key role in the development of the central nervous system, especially the neuroendocrine system [28]. *B. mori* POU-M1 was first cloned from the silk gland, and the expressed POU-M1 was found to bind to the SC element of *Bombyx* sericin-1 gene [22]. POU-M1 is similar to the *Drosophila* POU factor Cf1-a/drifter, and closely related to POU-III-type proteins in mammals, such as Brn-1, Brn-2 and Oct-6/Tst-1 [28]. Cf1-a/drifter was first found to be involved in the transcriptional regulation of the Dopa decarboxylase gene in selected dopaminergic neurons [29], and later shown to be critical for the differentiation and migration of certain tissues [30]. The function of these POU factors in regulating hormone expression is conserved across species. A POU family factor, *Pit-1*, is capable of transactivating the promoters of growth hormone, prolactin, and thyroid-stimulating hormone gene in mammals [31]. Although POU-M2 identified by us has a difference of 28 amino acids compared with POU-M1, the sequence of POU-specific domain and that of the POU homeodomain are the same, and their binding characteristics and *trans*-activities to Bom-DH-PBAN promoter are also identical. The POU-M2 from Chinese *B. mori* might be a gene allelic to POU-M1 from Japanese

B. mori. Recently, we cloned a POU-like cDNA from *Helicoverpa armigera* (Noctuidae, Lepidoptera) (GenBank® accession no. AY513764) and showed that Har-POU (*H. armigera* POU) has 94% identity with POU-M2 at the amino acid level, but only 87% identity with POU-M1. The 28-amino-acid sequence of POU-M2 at positions 109–136, which is not homologous with that of POU-M1, has 90% identity with that of Har-POU (see Supplementary Figure 2 at http://www.BiochemJ.org/bj/380/bj3800255add.htm). Thus the POU-M2-like transcription factor seems to be more widely distributed compared with POU-M1 in insects.

We investigated the developmental changes of DHMBPs and Bom-DH-PBAN during different pupal stages. The level of DHMBP expression increases from early to middle pupal stages (days 0 and 5), when the expression of the Bom-DH-PBAN gene also increases continuously and then decreases significantly on day 10, a day before adult eclosion. Thus the levels of transcription factors DHMBP-1/-2 at pupal stage are stage-dependent and parallel the changes in Bom-DH-PBAN mRNA content. Moreover, co-transfection of 5kL and EGFP-PM2 in insect cell lines also suggested that the POU-M2 could activate the Bom-DH-PBAN promoter directly. Although POU-M2 could promote the activity of the Bom-DH-PBAN promoter, probably other unknown transcription factors are also involved in controlling the transcription of Bom-DH-PBAN, since the spatial expression of POU-M2 and Bom-DH-PBAN do not strictly match. POU-M2 is widely expressed in several tissues, whereas Bom-DH-PBAN is only expressed in the SG [14]. It remains to be determined in future whether POU-M2 is co-expressed with Bom-DH-PBAN and regulates its transcription in the same cells.

Results of promoter deletions suggest that other transcription factors binding to the upstream of Bom-DH-PBAN promoter are also important for the activity of this promoter. Nevertheless, the activation induced by these factors is dependent on POU-M2 binding to the proximal promoter. The insertion of a POU-binding site into the proximal promoter of a heterologous gene BmLSP having several important upstream and downstream *cis*-elements also activates its transcription. The 0.3-kb-long Bom-DH-PBAN promoter without the upstream *cis*-elements fails to be activated by POU-M2. Probably, there are some interactions between POU-M2 and other activators binding to the upstream of Bom-DH-PBAN. One study has shown that Oct-1, a human POU gene, binds to the proximal promoter of the immunoglobulin gene and is essential for the function of its enhancer [32]. Its mechanism is such that the POU domain of Oct-1 directly facilitates recruitment of TBP (TATA-binding protein) to the promoter and then augments enhancer action [33]. It is intriguing to speculate that a similar mechanism may be involved in the transcriptional regulation of the Bom-DH-PBAN gene.

There is a canonical TATA box at -46 bp upstream of the transcription-initiation site of the Bom-DH-PBAN gene [4]. In the present study, we failed to find binding of TFIID to the S2 probe containing the TATA box in EMSA, even though a variety of binding conditions and different length probes containing the TATA box were tried. Additionally, the Bom-DH-PBAN gene has two transcription-initiation sites: a major one at $+1$ and an additional weaker one at -24 [4]. Nevertheless, both the sites, ACCACC ($+1$) and ACCGAT (-24), do not match well with the consensus sequence ATCAG/TTC/T at the cap site of insect mRNAs [34]. These results imply that the transcription-initiation site of this gene may not be determined by TFIID binding to the TATA box directly. POU domain proteins binding to the proximal promoter could recruit basal transcription factors and even played a crucial role in defining the site of transcription initiation instead of TFIID [35,36]. Therefore evidence from these experiments suggests that POU-M2 may take part in determining the transcription-initiation sites.

Preliminary results from our work on DHMBP-2 reveal that it has some interesting characteristics. We found that DHMBP-2 stimulated the binding of POU-M2, and DHMBP-2 was detected in both the nucleus and cytoplasm (see Supplementary Figure 3 at http://www.BiochemJ.org/bj/380/bj3800255add.htm). These results suggest that DHMBP-2 is quite probably a member of the HMG (high-mobility-group protein) family [37–39]. Therefore more information on the molecular structure of DHMBP-2 is needed for understanding the transcriptional regulation of this neuronal hormone gene.

We thank RIKEN DNA Bank for providing the POU-M1 clone (RDB 1829), deposited by the laboratory of Dr Y. Suzuki. We thank Dr John Ewer (Cornell University, Ithaca, NY, U.S.A.), Dr David Arnosti (Michigan State University, East Lansing, MI, U.S.A.) and Dr Rui-Ming Xu (Cold Spring Harbor Laboratory, NY, U.S.A.) for a critical reading of the manuscript. This work was partially supported by the Major State Basic Research Development Program (G20000162) of the Ministry of Science and Technology, People's Republic of China, a grant for Young Scientists from the Chinese Academy of Science and a grant from the National Natural Science Foundation of China (30070115).

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Received 29 September 2003/30 January 2004; accepted 9 February 2004
Published as BJ Immediate Publication 9 February 2004, DOI 10.1042/BJ20031482