Spatiotemporal Expression Profile of the *Pumilio* Gene in the Embryonic Development of Silkworm

Liang Chen, Zaizhi You, Hengchuan Xia, Qi Tang, Yang Zhou, Qin Yao, and Keping Chen*

Institute of Life Sciences, Jiangsu University, No. 301 Xuefu Road, Zhenjiang 212013, P. R. China. Fax: +86-511-88791923. E-mail: kpchen@ujs.edu.cn

* Author for correspondence and reprint requests

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We previously identified a *pumilio* gene in silkworm (*Bombyx mori* L.), designated *BmPUM*, which was specifically expressed in the ovary and testis. To further characterize this gene's involvement in silkworm development, we have determined the spatiotemporal expression pattern of *BmPUM* during all embryonic stages. Real-time polymerase chain reaction (RT-PCR) analysis revealed that *BmPUM* was expressed in all stages of silkworm embryos and that its transcript levels displayed two distinct peaks. The first was observed at the germ-band formation stage (1 d after oviposition) and dropped to a low level at the gonad formation stage (5 d after oviposition). The second was detected at the stage of bristle follicle occurrence (6 d after oviposition), which was confirmed by Western blot analysis and immunohistochemistry. Nanos (Nos), functioning together with Pum in abdomen formation of *Drosophila* embryos, was also highly expressed at the beginning (0 h to 1 d after oviposition) of embryogenesis, but its transcript levels were very low after the stage of germ-band formation. These results suggest that BmPUM functions with *Bombyx mori* nanos (Bm-nanos) at the early stages of silkworm embryonic development, and may then play a role in gonad formation and the occurrence of bristle follicles. Our data thus provide a foundation to uncover the role of BmPUM during silkworm development.

Key words: Pumilio, Expression Pattern, RT-PCR

Introduction

The *pumilio* gene has originally been identified as a maternal effect gene in *Drosophila melanogaster*, where it plays a role in the differentiation of the anterior-posterior body axis of the embryo. It encodes an evolutionarily conserved RNA-binding protein, which participates in transcriptional regulation (Murata and Wharton, 1995; Zamore *et al.*, 1997). Its homologous genes have been characterized in many animals, such as nematodes, amphibians, and mammalians. Pumilio proteins, commonly contain the PUF domain, which is also known as the Pumilio homology domain (PUM-HD) (Wickens *et al.*, 2002). They are also called PUF protein family for their founder members PUM in *Drosophila melanogaster* and FBF in *Caenorhabditis elegans* (Zhang *et al.*, 1997). PUF proteins serve multiple functions. In coordination with their partner proteins, they are involved in the processes of differentiation and development, reproduction, cell cycle, memory, and mitochondrial biogenesis by inhibiting, activating, and positioning their respective target mRNA (Quenault *et al.*, 2011).

In *Drosophila melanogaster,* two *pum* transcripts are found (Macdonald, 1992). They are present at all developmental stages in *Drosophila* embryos, but are most abundant in early embryos (Macdonald, 1992). The Pum protein is situated in the cytoplasm and is concentrated in the subcortical region of the embryo (Macdonald, 1992). Recently, several functions of the Pum protein have been investigated. For example, the PUF domain of the Pum protein binds to related sequence motifs in the 3′UTR of the mRNA of the hunchback protein (*hb*) and recruits the transcription

factors Nanos (Nos) and Brain tumour (Brat) to repress the translation of *hb* mRNA and thus promotes the formation of the posterior embryo (Murata and Wharton, 1995; Sonoda and Wharton, 1999; Wreden *et al.*, 1997). In the embryo of *pumilio* mutants, the abdomen cannot be formed (Barker *et al.*, 1992). In addition, the Pum protein can repress the translation of the *cycB* mRNA which results in the repression of pole cell differentiation (Asaoka-Taguchi *et al.*, 1999). In a *pumilio* mutant, the pole cells exhibited features of apoptosis due to their premature differentiation and failure to migrate to the gonad (Asaoka-Taguchi *et al.*, 1999). It is difficult to distinguish between phenotypes of *pumilio* and *nanos* mutants, respectively. Studies have shown that *pumilio*, as well as *nanos*, are involved in the development of germ cells (Asaoka-Taguchi *et al.*, 1999). It has also been reported that the Pum protein can regulate the synaptic growth and plasticity in neuromuscular junctions of *Drosophila* by repressing the expression of its target mRNA, elF-4E, and furthermore, that it can regulate the motor neuron sodium channel current by binding to para-mRNA (Menon *et al.*, 2004; Muraro *et al.*, 2008). Pum proteins are engaged in various biological processes by binding to different target mRNAs. Transcriptome analysis has revealed that there are 1090 potential target mRNAs of the Pum protein in *Drosophila melanogaster* (Gerber *et al.*, 2006).

Distinct from the fruit fly, vertebrates like zebrafish, *Xenopu*s, rainbow trout, mouse, and human have two PUF genes, *pumilio-1 (Pum1)* and *pumilio-2 (Pum2)* (Spassov and Jurecic, 2003). Although the Pum proteins are evolutionarily conserved, their expression patterns differ between species. Rainbow trout *Pum1* and *Pum2A* are expressed in the brain and ovary at high levels (Kurisaki *et al.*, 2007). Mouse *Pum1* and *Pum2* are expressed in a variety of tissues but with preferential expression in fetal and adult hematopoietic stem cells (Spassov and Jurecic, 2003). Human *Pum1* and *Pum2* are expressed ubiquitously, but *Pum2* is predominantly expressed in germ cells (Spassov and Jurecic, 2002). A low level of *Pum1* and *Pum2* expression was found in the gonads of hatched female chicks, while *Pum2* was expressed at high level in 12-d-old embryonic tissues (Lee *et al.*, 2008).

Tian *et al*. (2009), using 3′ and 5′ rapid-amplification of cDNA ends (RACE), identified a *pumilio* gene in silkworm (*Bombyx mori* L.) designated *BmPUM* in our laboratory. Among all silkworm larval tissues tested, the BmPUM protein accumulates predominantly in the testis and ovary. To further investigate its expression and relationship with development, in this study the expression pattern of *BmPUM* in silkworm embryos was examined using the real-time polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry.

Materials and Methods

Insect materials

The C108 strain of *Bombyx mori* L. (Lepidoptera: Bombycidae) is inbred in our laboratory. Insects were reared at 25 °C on fresh mulberry leaves in an insect rearing chamber under short-day conditions (12 h light/12 h dark). The silkworm embryos were collected immediately after oviposition. Diapause-destined embryos were staged at the time of oviposition, and this time ±30 min was designated as 0 h. Then the eggs were maintained at 25 °C for a 12-h light/12-h dark photoperiod. After 0 h, 5 h, and 1 d to 10 d, embryos were collected and immediately used for RNA and protein extraction. BmN cells were purchased from Zhongshan Golden Bridge Biotechnology (ZSGB-bio) Co., Ltd., Beijing, China.

RT-PCR

Total RNA of different embryo stages was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. The quality and concentration of RNA samples were examined by ethidium bromide (EB)-stained agarose gel electrophoresis and absorbance measurement (Gene spec III; Naka, Hitachinaka, Japan). Two μg of total RNA were reverse-transcribed with oligo(dT)12-18 primer (500 μg/ml) and M-MLV reverse transcriptase (Takara, Otsu, Japan) in a total volume of 20 μl at 42 °C for 60 min; then the cDNA was diluted 10-fold for use. Quantitative PCR analysis of *BmPUM* and *Bm-nanos* transcript levels was performed using the MX 3000P™ real-time PCR system (Stratagene, La Jolla, CA, USA) and the Premix Ex Taq™ kit (Takara). PCR primers used in this experiment are listed in Table I. The housekeeping gene *Bm-actin* (GenBank Acc. No. NM_001126254) was used as an internal control. The expression of the vitellogenin gene *BmVLG* in the newly laid egg sample (0 h) served as the calibrator, and its relative expression level was set as 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level (Livak and Schmittgen, 2001).

Table I. Primers used in quantitative RT-PCR.

Target	Sequence of primer	Amplification length [bp]
BmPUM	5′-CTCGCCAACCACATCGTC-3′	313
	5′-TGTTGTTCAGGCGGGATAG-3′	
Bm-nanos	5′-AGGTCGCTAAAGTTCGCAGTG-3′	321
	5′-TGTTCGTCCCGCCCATAA-3′	
Bm-actin A3	5′-GCGCGGCTACTCGTTCACTACC-3′	284
	5′-GGATGTCCACGTCGCACTTCA-3′	

Western blot

Extracts were prepared by homogenizing eggs at various embryonic stages directly in ice-cold 1 × phosphate-buffered saline (PBS) (0.1 M, pH 7.6) containing four different protease inhibitors, *i. e.* phenylmethylsulfonyl fluoride (PMSF) (35 μg/ml), ethylenediaminetetraacetic acid (EDTA) (0.3 mg/ml), pepstatin (0.7 μg/ml), and leupeptin (0.5 μg/ml). An equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer was added to the homogenate, and the mixture was boiled for 10 min, then centrifuged at 12,000 × *g* for 10 min. The supernatant was saved for electrophoresis. Nuclear, cytoplasmic, and total proteins, respectively, were extracted from BmN cells using the Beyotime (Haimen, Jiangsu, China) nuclear and cytoplasmic protein extraction kit and RIPA lysis buffer (Beyotime) according to the manufacturer's instructions. Protein was quantified with the BCA protein assay reagent (Pierce, Rockford, IL, USA). Protein samples of 60 μg each were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% gel). Proteins were then blotted onto a polyvinylidene difluoride (PVDF) membrane, which was incubated for 3 h in PBS containing 0.5 μl/ml Tween (PBST) and 5% (w/v) defatted milk powder, at room temperature. Then the membrane was incubated with primary antibody against BmPUM (ZSGB-bio) (diluted 1:100), washed three times in PBST for 5 min each, and incubated at room temperature for 1.5 h with secondary antibody [diluted 1:1000, peroxidase-conjugated goat anti-rabbit IgG (ZSGB-bio)]. After washing as for the primary antibody, antigen bands were detected by incubation with 0.1% H_2O_2 and 3,3′-diaminobenzidine (DAB) as a chromogenic substrate. Anti-BmPUM polyclonal antibody was obtained from ZSGB-bio.

Immunohistochemistry

Embryos at different developmental stages were dissected from silkworm eggs after removal of egg shells, fixation, and removal of the vitelline membrane. The fixed embryos were transferred to 20% sucrose (dissolved in 0.1 M PBS) and kept for 24 h. Cryosectioning was carried out to obtain 6-μm sections of the fixed embryos. The sections were added to a 3% H_2O_2 solution to block endogenous peroxidase, and incubated with normal goat serum diluted 10 times in PBS containing 0.5 mg/ml bovine serum albumin (BSA) at 37 °C for 40 min. The sections were further incubated with anti-BmPUM rabbit antibody (ZSGB-bio) (diluted 1:200) as primary antibodies for 3 h at 37 °C and then washed 5 times in PBS. The negative control was prepared using only PBS. After incubation with goat anti-rabbit IgG conjugated fluorescein isothiocyanate (FITC) as secondary antibody for 1.5 h at 37 °C, the sections were treated with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min, and were then inspected by a confocal laser scanning microscope (Leica, Wetzlar, Germany).

Subcellular localization of BmPUM

Monolayers of BmN cells were seeded onto coverslips. They were washed in PBS and treated with 4% paraformaldehyde for 15 min. Then they were washed three times with cold PBS and permeabilized with 0.1% Triton X-100 solution for 15 min, followed by three washes in cold PBS. They were then processed as described above for immunohistochemistry.

Results

Developmental expression profiles of BmPUM in silkworm embryos

BmPUM mRNA transcript levels were determined by RT-PCR during the development of silkworm embryos. BmPUM was expressed in all embryonic stages examined (Fig. 1), with the highest expression level of the *BmPUM* gene at day 1 after oviposition, when the

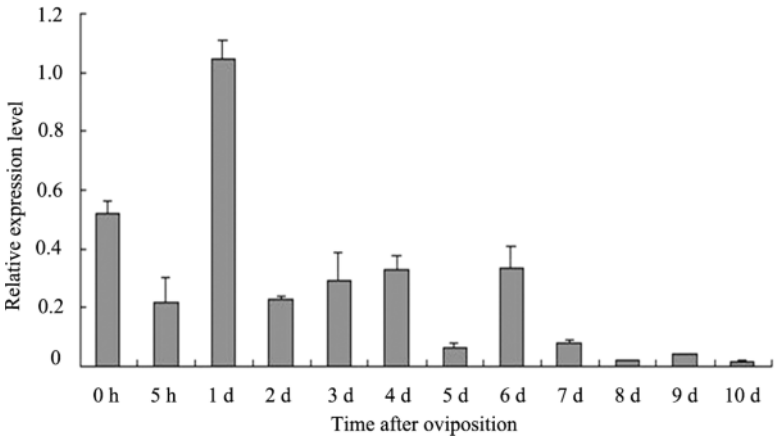

Fig. 1. RT-PCR analysis of *BmPUM* transcripts. RNA extracted from silkworm embryos at various times after oviposition was used as PCR template. The data show means ± standard errors for three replicates.

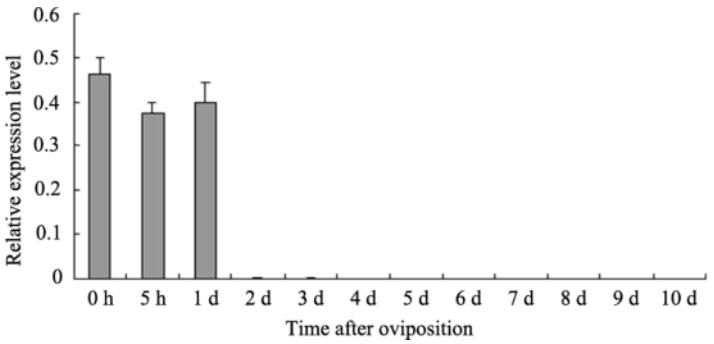

Fig. 2. RT-PCR analysis of *Bm-nanos* transcripts. See legend to Fig. 1 for further details.

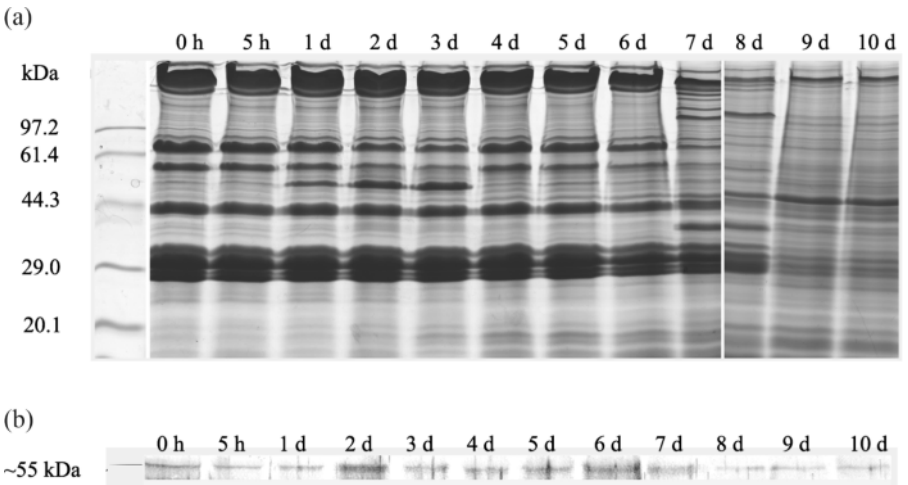

Fig. 3. Western blot analysis of BmPUM in total protein extracts from silkworm embryos at various times after oviposition. (a) Stained total proteins. (b) Western blot of BmPUM.

embryos were at the stage of germ-band formation, followed by a sharp decrease to a trough at day 5 after oviposition at the end of gonad formation. There was a second, lower peak at day 6 after oviposition (occurrence of bristle follicles) and a decrease again during days 7 to 10.

Developmental expression profiles of Bm-nanos in silkworm embryos

The *pumilio* and *nanos* genes jointly determine the abdominal segments during embryogenesis of *Drosophila melanogaster* (Murata and Wharton, 1995; Sonoda and Wharton, 1999; Wreden *et al.*, 1997). Therefore the expression profile of *Bm-nanos* at different developmental embryo stages was also examined. *Bm-nanos* was expressed at all stages examined (Fig. 2), its expression level being relatively high at 0 h, 5 h, and 1 d after oviposition, and thereafter decreasing rapidly from day 2 onward.

Western blot analysis of BmPUM in silkworm embryos

Western blot analysis was performed to examine the temporal distribution and content of BmPUM. Anti-BmPUM polyclonal antibody raised against the PUF domain gave a specific signal in all samples examined (Fig. 3) revealing peaks of BmPUM on days 2 and 6, respectively, with the higher one on day 2, thus indicating transcriptional control of the BmPUM level.

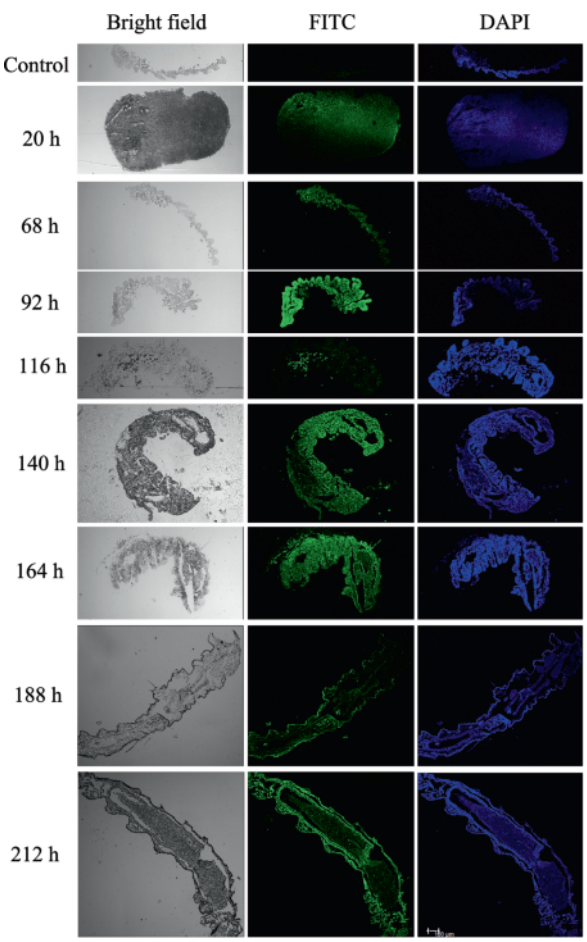

Fig. 4. Immunohistochemical analysis of BmPUM in silkworm embryos during development. Vertical rows represent bright field and green fluorescence for FITC-treated and DAPI-treated images. Horizontal rows represent negative control without primary antibody, and images taken at the indicated times; no sections of embryos of days 1 and 2 after oviposition were obtained. Because of their thinness, day 1 embryos were not sectioned and directly used for immunohistochemistry. Day 2 embryos disintegrated during embedding.

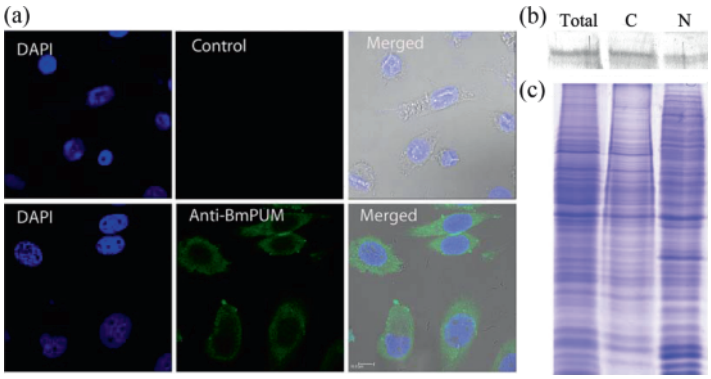

Fig. 5. Subcellular localization of BmPUM in BmN cells. (a) Immunofluorescence. Vertical rows represent visualization of nuclei, visualization of BmPUM, and the merged images, respectively. Horizontal rows represent the control and anti-BmPUM treatment, respectively. (b) Western blot analysis of BmPUM. Total, C, and N represent total proteins, cytoplasmic fraction, and nuclear fraction of BmN cells, respectively. (c) SDS gel electrophoresis of proteins in the fractions.

Immunohistochemical analysis of BmPUM in silkworm embryos

To further reveal the spatial distribution of BmPUM in various stages of silkworm embryos, immunohistochemistry was performed. Hybridization signals given by anti-BmPUM polyclonal antibody were observed in all developmental stages and tissues of the embryos (Fig. 4).

Subcellular localization of BmPUM in BmN cells

To study the subcelluar localization of BmPUM, BmN cells were examined by immunofluorescence analysis. BmPUM was largely located in the cytoplasm (Fig. 5a). To confirm this result, proteins extracted from cytoplasmic and nuclear fractions of BmN cells were analysed in Western blots. As shown in Fig. 5b, BmPUM protein was largely present in the cytoplasm, in accord with the immunofluorescence analysis.

Discussion

BmPUM was found to be expressed throughout all stages of silkworm embryonic development and in all tissues, both at the transcript and protein levels (Figs. 1, 3). The ubiquitous expression of *pumilio* has also been seen in other species. The transcripts of the two *pumilio* genes were detected in various stages of embryonic development in *Drosophila melanogaster* (Macdonald, 1992). The two *pumilio* genes of medaka (Japanese rice fish, *Oryzias latipes*), *Pum1* and *Pum2*, were expressed throughout embryonic development and in all tissues (Zhao *et al.*, 2012). Expression of *pumilio* may therefore be crucial in embryogenesis.

Based on the observed peaks in the levels of both *BmPUM* transcripts and BmPUM protein, the 5th day after oviposition (onset of gonad formation) seemed to be a turning point in embryo development. It is well known that primordial germ cells (PGCs) must undergo proliferation, invasion, and migration to form the gonad (Li *et al.*, 2003). In the embryonic development of *Drosophila*, the Pumilio protein plays a role in the migration of PGCs (Asaoka-Taguchi *et al.*, 1999), and the entire migration is complete by stage 14 ($\sim$ 11.5 h after oviposition), when the embryonic gonad is formed (Starz-Gaiano and Lehmann, 2001). Thus, day 5 after oviposition of silkworm embryo development is comparable to stage 14 of *Drosophila* embryos. In *pumilio* mutants, pole cells are unable to migrate to the germ ridge; as a result, the gonad fails to form (Asaoka-Taguchi *et al.*, 1999). FBF (a PUF protein in *Caenorhabditis elegans*) regulates the germline stem cell fate by binding to its target, gld-1 mRNA. In the FBF mutant, germ cells lose the capacity for self-renewal. In addition, FBF was also found to be responsible for germ cell survival, migration, and repression of premature differentiation (Suh *et al.*, 2009). The expression pattern of BmPUM suggests that it may be involved in proliferation of PGCs, their migration, and formation of gonads during embryonic development in *Bombyx mori*.

The RT-PCR analysis of the *Bm-nanos* transcripts showed that this gene, like *BmPUM*, was highly expressed at the beginning (0 h to 1 d after oviposition) of embryogenesis (Fig. 2). The FBF protein interacts with nanos and inhibits the translation of *fem-3* mRNA that controls the sperm-oocyte switch in *Caenorhabditis elegans* (Kraemer *et al.*, 1999). In *Drosophila,* Pumilio together with nanos regulates the abdomen formation by repressing the translation of *hb* mRNA (Murata and Wharton, 1995; Sonoda and Wharton, 1999; Wreden *et al.*, 1997) and suppresses the differentiation of pole cells by repressing the translation of *cycB* mRNA (Asaoka-Taguchi *et al.*, 1999). So we speculated that, in cooperation with nanos, BmPUM may also perform similar functions in early silkworm embryonic development. The expression of *Bm-nanos* was kept at a low level after day 2 of oviposition (Fig. 2), whereas that of *BmPUM* exhibited a second, lower peak on day 6 when the embryos were at the bristle follicles stage. Defect bristles were reported for the *pum* mutant in *Drosophila* (Lehmann and Nüsslein-Volhard, 1987). Likewise, BmPUM may carry out its functions with different partner proteins in the process of bristle follicle formation.

Subcelluar localization and Western blot analyses demonstrated that BmPUM was largely located in the cytoplasm, with a small amount in the nucleus (Fig. 5). In *Drosophila*, the Pum protein has been localized to the cytoplasm (Macdonald, 1992). However, a study of the crystal structure of the *Drosophila* Pum protein suggested that the PUF domain may function to guide RNA out of the nucleus, in addition to regulating the translation and degradation of mRNA (Edwards *et al.*, 2001). Our finding that a small amount of BmPUM protein was present in the nucleus, supports the above report. The human Pum2 protein was abundant in both cytoplasm and nucleus of spermatogonia, but was confined to the cytoplasm of spermatocytes and oocytes (Moore *et al.*, 2003). Obviously, BmPum protein had the same distribution as the Pum

proteins in these species, implying that the BmPUM protein may act at the level of both transcription and translation.

In conclusion, *BmPUM* was expressed throughout the embryonic development, and its transcript levels peaked at day 1 after oviposition (germ-band formation), sharply decreased to a low level at day 5 (gonad formation), and then rose again 6 d after oviposition (occurrence of bristle follicles). *Bm-nanos* was also highly expressed at the beginning (0 h to 1 d after oviposition) of embryogenesis. Taken together, the BmPUM protein may function with Bm-nanos at the early stages of silkworm embryonic development and may be involved in gonad and bristle follicle formation. The BmPUM protein was localized to both cytoplasm and nucleus, implying that the regulation of silkworm embryonic development by the BmPUM protein may occur at the level of both transcription and translation.

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