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Expression profiles and localization of *vitellogenin* mRNA and protein during ovarian development of the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

Ovarian maturation of the giant tiger shrimp (*Penaeus monodon*) results from rapid synthesis and accumulation of a major yolk protein, vitellin which is derived from its precursor, vitellogenin. The expression profiles and localization of *P. monodon vitellogenin 1* (*PmVtg1*) were examined. *PmVtg1* mRNA was expressed only in ovaries and hepatopancreas but not in other tissues of female and testes of male broodstock. In wild intact broodstock, *PmVtg1* mRNA was expressed at a low level in stage I ovaries and up-regulated in stages II and III being significantly reduced in stage IV ovaries ($P < 0.05$). In eyestalk-ablated broodstock, the expression level of *PmVtg1* mRNA was increased to nearly a peak level in vitellogenic (stage II) ovaries and peaked in cortical rod (stage III) ovaries ($P < 0.05$). The level of *PmVtg1* mRNA in hepatopancreas of intact shrimp with stages II and III ovaries was significantly greater than those with stages IV and V (post-spawning) ovaries. Interestingly, *PmVtg1* mRNA in hepatopancreas was 25–40 times higher than that in ovaries of intact shrimp with stages I–III of ovarian development. *In situ* hybridization revealed that *PmVtg1* mRNA was clearly localized in the cytoplasm of follicular cells surrounding late previtellogenic, vitellogenic and cortical rod oocytes. Quantitative real-time PCR against different periods of spawned eggs suggested that *PmVtg1* mRNA was also synthesized in oocytes. Western blot analysis revealed a similar profile of ovarian *P. monodon* vitellin and *PmVtg1* mRNA during ovarian development and also suggested that the Vtg protein in hepatopancreas and Pm-vitellin in ovaries should have encoded from different *Vtg* genes. Immunohistochemistry confirmed that Pm-vitellin was localized in developing, vitellogenic and mature oocytes but not in the follicular cells.

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1. Introduction

The giant tiger shrimp (*Penaeus monodon*) is one of the world's most economically important culture decapods. Farming of this species still relies almost entirely on ocean-caught females due to the constraint that breeding of pond-reared *P. monodon* is rather difficult and rarely produces the sufficient amount of high quality larvae desired by the industry (Klinbunga et al., 2009; Limsuwan, 2004; Withyachumnarnkul et al., 1998). These problems impede the application of domestication and selective breeding programs for genetic improvement of commercially desired traits in this species (Benzie, 1998; Sittikankaew et al., 2010; Withyachumnarnkul et al., 1998).

Knowledge on molecular mechanisms and functional involvement of reproduction-related genes and proteins in ovarian development are necessary for better understanding the reproductive maturation of *P. monodon* in captivity. This basic knowledge may resolve the major constraint on reduced degrees of reproductive maturation and also provide the possible way to induce maturation without the use of eyestalk ablation in this economically important species (Klinbunga et al., 2009; Preechaphol et al., 2007).

Gonad development and maturation require precisely coordinated expression of specific gene classes (Grimes, 2004; Qiu and Yamano, 2005; Qiu et al., 2005). Ovarian maturation of *P. monodon* results from rapid synthesis and accumulation of a major yolk protein, vitellin which is derived from its precursor, vitellogenin (Vtg) and cortical rod (Cr) formation (Charniaux-Cotton, 1985; Harrison, 1990; Lubzens et al., 1997; Quackenbush, 2001). Ovaries and hepatopancreas are sources of vitellogenin synthesis in penaeid shrimp, (Avarre et al., 2003; Phiriyangkul

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and Utarabhand, 2006; Phiriyangkul et al., 2007; Raviv et al., 2006; Tiu et al., 2006; Tsang et al., 2003; Tsutsui et al., 2005). Although Tiu et al. (2008) reported that the vitellogenin protein (called Pm-vitellin in this study) was evenly distributed in the ooplasm of developed and mature oocytes, no information has been provided about the localization of *Vtg* mRNA in ovaries of *P. monodon*.

In this study, tissue distribution analysis and the expression profiles of *vitellogenin 1* (*PmVtg1*) mRNA in ovaries and hepatopancreas in different ovarian stages and in spawned eggs of *P. monodon* were examined by quantitative real-time PCR. Localization of ovarian *PmVtg1* mRNA was examined using *in situ* hybridization. In addition, localization of vitellin (Pm-vitellin) in different stages of ovarian development of *P. monodon* was also determined using immunohistochemistry. The presence of *PmVtg1* mRNA in hepatopancreas, follicular cells and spawned eggs are reported and the absence of Pm-vitellin protein in follicular cells is confirmed.

2. Materials and methods

2.1. Experimental animals

Juvenile *P. monodon* (4-month-old) were purchased from a commercial farm in Chachoengsao (eastern Thailand, $N = 5$). Female broodstock were wild-caught from the Andaman Sea (west of peninsular Thailand) and acclimated under the farm conditions for 2–3 days. Ovaries of post-spawning shrimp were collected immediately after spawning (stage V, $N = 6$). Ovaries and hepatopancreas were dissected out from each intact shrimp and weighted. For the eyestalk ablation group, wild shrimp were acclimated for 7 days before unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after the ablation. The ovarian developmental stages of *P. monodon* were classified according to the gonadosomatic indices (GSI, ovarian weight/body weight × 100) to previtellogenetic (stage I, GSI < 1.5%, $N = 4$ and 7 for intact and eyestalk-ablated broodstock, respectively), vitellogenetic (stage II, GSI = 2–4%, $N = 11$ and 12), early cortical rod (stage III, GSI = 4–6%, $N = 5$ and 10) and mature (stage IV, GSI > 6%, $N = 6$ and 5) ovaries, respectively. The ovarian stage of each shrimp was further confirmed by a conventional histology (Qiu et al., 2005). For tissue distribution analysis, various tissues of female broodstock, ovaries of female juveniles and testes of male broodstock ($N = 4$ for each group) were collected. Shrimp tissues were immediately placed in liquid N_2 and kept at −70 °C until needed. Moreover, eggs of wild intact broodstock were collected at 0, 1, 2, 3, and 6 h post spawning ($N = 3$ for each time point). These specimens were used for expression analysis of *PmVtg1* mRNA for inferring its expression in oocytes during oogenesis.

2.2. Isolation of total RNA and first strand cDNA synthesis

Total RNA was extracted from shrimp tissues using TRI Reagent (Molecular Research Center). Ten micrograms of the extracted total RNA were treated with DNase I (0.2 U/μg of RNA, GE Healthcare) at 37 °C for 30 min and subjected to phenol–chloroform extraction. One and a half microgram of DNase I-treated total RNA was reverse-transcribed using an Improm-II™ Reverse Transcription System (Promega).

2.3. EST analysis

Total RNA was extracted from vitellogenic ovaries of wild *P. monodon* broodstock and mRNA was further purified from total RNA using a QuickPrep Micro mRNA Purification Kit (GE Healthcare). Expressed sequence tags (ESTs) from a conventional ovarian cDNA library previously constructed was further analyzed as described in Preechaphol et al. (2007).

2.4. Tissue distribution analysis

Expression of *vitellogenin 1* (*PmVtg1*) mRNA in various tissues of *P. monodon* was examined by RT-PCR. The amplification reaction was carried out in a 20 μl reaction volume containing 1× *Ex Taq*™ buffer, 0.2 mM each dNTP, 0.5 μM each of $PmVtg1_{134}$-F (5′-AGGCATCACAG TAACTGAGACCGAT-3′) and $PmVtg1_{134}$-R: 5′-CAGGTGTTGGGTAACG TTCTTGAC-3′, 25 ng the first strand cDNA template and 0.5 unit of *TaKaRa Ex Taq*™ (Takara, Japan). $EF\text{-}1\alpha_{500}$ (F: 5′-ATGGTTGTCAACTTTG CCCC-3′) and R: 5′-TTGACCTCCTTGATCACACC-3′ were included as the positive control. RT-PCR was initially performed by predenaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 7 min. The amplicon was electrophoretically analyzed using 1.5% of agarose gels and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

2.5. Quantitative real-time PCR

The expression level of $PmVtg1_{134}$ and the control ($EF\text{-}1\alpha_{214}$; F: 5′-TCCGTCTTCCCCTTCAGGACGTC-3′ and R: 5′-CTTTACAGACACG TTCTTCACGTTG-3′) transcripts in different shrimp groups were examined in a 10 μl reaction volume containing 5 μl of 2× LightCycler 480 SYBRGreen I Master (Roche), 50 ($PmVtg1_{134}$) or 5 ($EF\text{-}1\alpha_{214}$) ng the first strand cDNA template, 0.2 μM each of gene-specific primers. The quantitative real-time PCR assay of each sample was run in duplicate using a Lightcycler 480. The thermal profiles were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 72 °C for 30 s. The average quantification of $PmVtg1_{134}$ and $EF\text{-}1\alpha_{214}$ mRNA of each sample was evaluated from the respective standard curves generated from 10^3 to 10^8 copies of recombinant plasmids of the target or reference sequences (in triplicate).

In addition, the expression level of *PmVtg1* mRNA in hepatopancreas of wild intact shrimp with different stages of ovarian development (stages I–V) was examined ($N = 6$ for each group). To examine whether *PmVtg1* mRNA is expressed in *P. monodon* oocytes, quantitative real-time PCR was also carried out against the cDNA template from eggs (500 ng) of three broodstock individuals each at 0, 1, 2, 3 and 6 h after spawning. RT-PCR of *nuclear autoantigenic sperm protein* (*PmNASP*, 100 ng template) which is abundantly expressed in all stages of ovaries of *P. monodon* (Karoonuthaisiri et al., 2009) was carried out from the same template for quality control of the synthesized cDNA template.

The relative expression level (copy number of $PmVtg1_{134}$ and that of $EF\text{-}1\alpha_{214}$) between different groups of broodstock possessing different stages of ovarian development were statistically tested using one way analysis of variance (ANOVA) followed by Duncan's new multiple range test. Significant results were considered when $P < 0.05$.

2.6. In situ hybridization

Pieces of ovaries were dissected out from wild intact and eyestalk-ablated *P. monodon* broodstock and fixed overnight at 4 °C in 4% paraformaldehyde prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.2) for *in situ* hybridization or Davidson's fixative for immunohistochemistry. The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at −20 °C until used. Tissues were histologically prepared and embedded in paraffin. Conventional paraffin sections (5 μm) were carried out. The sense and anti-sense cRNA probes: primers $PmVtg1_{927}$-T7/F, 5′-TAATACGACTCAC TATAGGGACCGACCCTTGTATTCTCTAATGC-3′ and $PmVtg1_{927}$-R/SP6, 5′-*ATTTAGGTGACACTATAGAA*AGCCGTCAACATTTAGGCTATGTAA-3′ containing T7 (underlined) and SP6 (italicized and underlined) promoter sequences, were synthesized by using DIG RNA labeling mix (Roche). Tissue sections were dewaxed with xylene and dehydrated in absolute ethanol. The sections were prehybridized with 2× SSC containing 50%

Table 1
ESTs significantly matched *vitellogenin* found in the ovarian cDNA library of *P. monodon*.

Clone No.[a]	Matched gene	Closest species	*E*-value
OV-N-N01-1287-W	*Vitellogenin*	*Fenneropenaeus chinensis*	1×10^{-119}
OV-N-N01-0265-W	*Vitellogenin 1*	*Penaeus monodon*	1×10^{-131}
OV-N-N01-0305-W	*Vitellogenin 1*	*Penaeus monodon*	1×10^{-132}
OV-N-N01-0670-W	*Vitellogenin 1*	*Penaeus monodon*	1×10^{-113}
OV-N-N01-1224-W	*Vitellogenin 1*	*Penaeus monodon*	1×10^{-133}
OV-N-N01-1300-W	*Vitellogenin 1*	*Penaeus monodon*	1×10^{-131}

[a] http://pmonodon.biotec.or.th.

deionized formamide, 1 μg/μl yeast tRNA, 1 μg/μl salmon sperm DNA, 1 μg/μl BSA, and 10% (w/v) dextran sulfate at 50 °C for 30 min, and were hybridized with either the antisense or sense probe in the prehybridization solution overnight at 50 °C. After hybridization, the tissue sections were washed twice with 4× SSC for 5 min each and once with 2× SSC containing 50% formamide for 20 min at 50 °C. The sections were immersed in prewarmed RNase A buffer (0.5 M NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA) at 37 °C for 5 min and treated with RNase A (10 μg/ml) at 37 °C for 30 min. Tissue sections were washed four times with RNase A buffer (37 °C, 10 min each) and 2× SSC (50 °C, 15 min each). High stringency washing was carried out twice in 0.2× SSC at 50 °C for 20 min each. The bound probes were detected with a DIG Wash and Block Buffer Kit (Roche) (Qui and Yamano, 2005).

2.7. Western blot analysis

Approximately 0.5 g of frozen ovaries or hepatopancreas of *P. monodon* was ground to the fine powder in the presence of liquid N_2 and homogenized in 1.2 ml of the TCA–acetone extraction buffer: 10% TCA in acetone containing 0.1% DTT and Complete protease inhibitor cocktail (Roche) and left at −20 °C for 1 h. The mixture was centrifuged at 10,000 *g* for 30 min at 4 °C and the supernatant was discarded. The pellet was washed three times with acetone before centrifuged at 10,000 *g* for 30 min at 4 °C. The resulting pellet was air-dried and dissolved in the lysis buffer (30 mM Tris–HCl, 2 M thiourea, 7 M urea, 4% CHAPS, w/v). Protein concentrations of the tissue extract were measured by a dye binding method (Bradford, 1976). Thirty micrograms of total proteins were heated at 100 °C for 5 min and immediately cooled on ice. Proteins were size-fractionated on a 12.5% SDS-PAGE (Laemmli, 1970). Electrophoretically separated proteins were transferred onto a PVDF membrane (Hybond P; GE Healthcare) (Towbin et al., 1979) in 25 mM Tris, 192 mM glycine (pH 8.3) buffer containing 10% methanol at 100 V for 90 min. The membrane was treated in the DIG blocking solution (Roche) for 1 h and incubated with anti-vitellin PAb (kindly provided by Prof. K. Aida, University of Tokyo, Japan; Kawazoe et al., 2000; 1:2500 in the blocking solution) for 1 h at room temperature. The membrane was washed 3 times with 1× Tris Buffer Saline-Tween20 (TBST; 50 mM Tris–HCl, 0.15 M NaCl, pH 7.5, 0.1% Tween20) and incubated with goat anti rabbit IgG (H + L) conjugated with alkaline phosphatase (Bio-Rad Laboratories) at 1:3000 for 1 h and washed 3 times with 1× TBST. Immunoreactive signals were visualized using NBT/BCIP (Roche) as the substrate.

2.8. Immunohistochemistry

Deparaffinized sections were incubated in the blocking solution I (3% H_2O_2 in methanol) for 15 min to remove endogenous peroxidase. The sections were then rinsed briefly with PBS, pH 7.2 (0.35 g NaH_2PO_4, 1.28 g Na_2HPO_4 and 8 g NaCl in 1000 ml of distilled H_2O). After treatment in the blocking solution II (Roche) for 1 h, the sections were incubated with purified anti-vitellin PAb (1:500) for 1 h in a humid chamber. The sections were rinsed three times for 5 min each with PBS, pH 7.2 and further incubated with goat anti-rabbit IgG-HRP conjugate (simple stain PO, Nichirei) for 30 min. The sections were again rinsed three times for 5 min each with PBS, pH 7.2. Localization of the antigen was visualized using diaminobenzidine (Wako) as the substrate. Tissue sections were also incubated with the blocking solution II to examine the specificity of the antiserum.

3. Results

3.1. Identification of PmVtg1 cDNA by EST analysis

EST analysis was extended from the information reported in Preechaphol et al. (2007). In total, 2330 clones were unidirectional sequenced (data not shown) and 5 ESTs that significantly matched *P. monodon vitellogenin 1* (*PmVtg1*) and 1 EST that matched *Fenneropenaeus chinensis vitellogenin* previously deposited in GenBank (*E*-value < 10^{-4}) were obtained. *PmVtg1* was not found in the libraries established from other tissues including testes, hepatopancreas, hemocytes, intestines, lymphoid organs and eyestalk (http://pmonodon.biotec.or.th) (Table 1).

3.2. Tissue distribution analysis of PmVtg1 mRNA

PmVtg1 mRNA was expressed in various ovarian stages of intact and eyestalk-ablated broodstock but the positive amplification product was not observed in juvenile ovaries. *PmVtg1* mRNA was also abundantly expressed in hepatopancreas but not in other tissues of female broodstock and testes of male broodstock (Fig. 1).

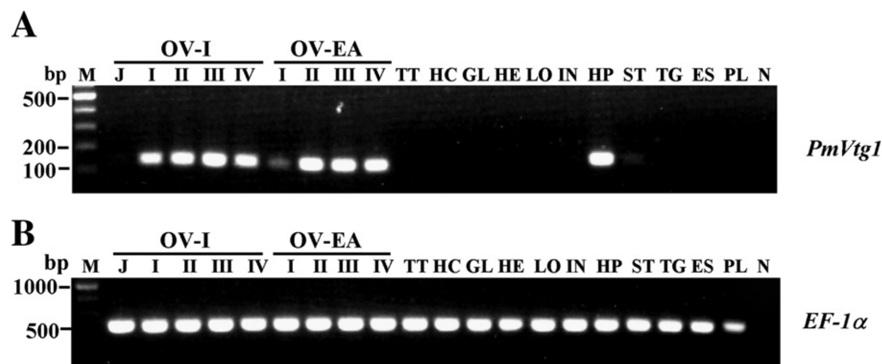

Fig. 1. Tissue distribution analysis of *PmVtg1* mRNA using the first strand cDNA of juvenile ovaries (J) and stages I, II, III and IV ovaries of wild intact and eyestalk-ablated female broodstock, testes (TT) of male broodstock and various tissues of female broostock of wild *P. monodon*: hemocytes (HC), gills (GL), heart (HE), lymphoid organs (LO), intestine (IN), hepatopancreas (HP), stomach (ST), thoracic ganglion (TG), eyestalks (ES), and pleopods (PL). *EF-1α* was successfully amplified from the same template (B). Lanes M and N are a 100 bp DNA marker and the negative control (without cDNA template), respectively.

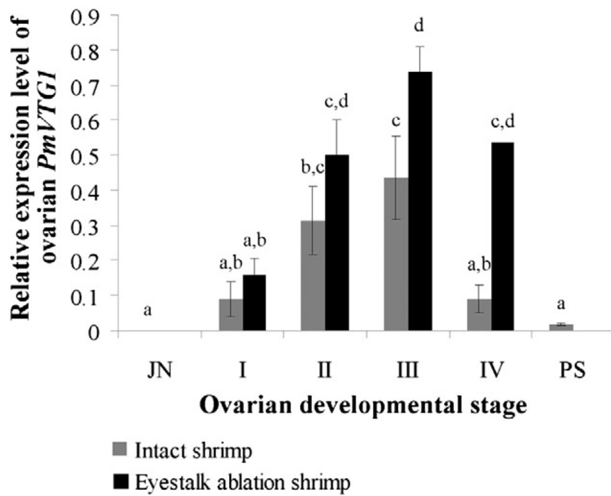

Fig. 2. Histograms showing the relative expression profiles of *PmVtg1* mRNA (50 ng cDNA tempate) during ovarian development of intact and unilateral eyestalk-ablated *P. monodon* broodstock. The expression level was measured as an absolute copy number of *PmVtg1* and normalized by that of *EF-1α* from the same shrimp. Each histogram corresponds to a particular ovarian stage. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$). JN = cultured juveniles; I–IV = stages I–IV ovaries of intact or eyestalk-ablated broodstock; PS = post-spawning intact broodstock.

3.3. Expression profiles of PmVtg1 mRNA during ovarian development of P. monodon

The average GSI of shrimp in this study was increased from 1.36 ± 0.36 (stage I) at the beginning of the experiment to 5.06 ± 1.21, 6.90 ± 0.03 and 8.79 ± 2.36 for shrimp with stages II, III and IV ovaries within 7 days after unilateral eyestalk ablation.

Quantitative real-time PCR was used for determination of gene expression in naturally maturing shrimp and unilateral eyestalk-ablated shrimp. The expression level of *PmVtg1* mRNA in juvenile (premature) ovaries was extremely low (relative expression level = 0.0000368). The expression level was slightly greater in stage I ovaries of intact broodstock but the result was not significantly different from the juvenile stage ($P > 0.05$, Fig. 2). Its expression level was increased in stage II ($P > 0.05$), significantly increased in stage III ($P < 0.05$) and decreased to the basal level (of stage I) in mature ovaries of intact broodstock ($P < 0.05$). In eyestalk-ablated broodstock, a low expression level of *PmVtg1* mRNA was also observed in stage I ovaries. Its expression was significantly increased in stage II ovaries ($P < 0.05$), peaked in stage III ovaries before being slightly reduced in mature ovaries. However, the expression levels in stages II, III and IV ovaries of eyestalk-ablated broodstock were not significantly different ($P > 0.05$, Fig. 2). The level of *PmVtg1* mRNA in stage III and IV ovaries of eyestalk-ablated broodstock was significantly greater than that in the same ovarian stages in intact broodstock ($P < 0.05$, Fig. 2).

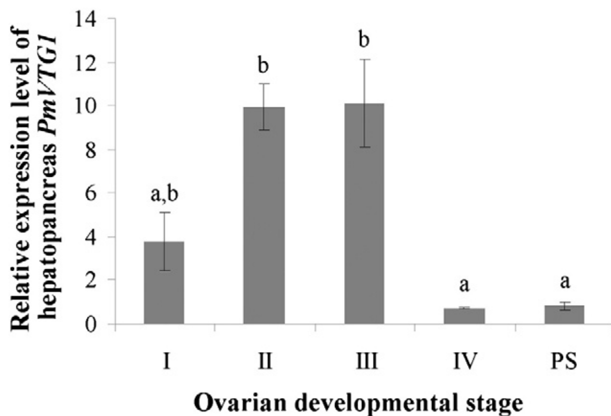

Fig. 3. The relative expression profiles of *PmVtg1* mRNA in hepatopancreas (50 ng cDNA tempate) of intact shrimp having stages I–IV and post-spawning ovaries (PS; recognized as stage V, $N = 6$ for each stage). Each histogram corresponds to a particular ovarian stage. The same letters above bar indicate that the expression levels were not significantly different ($P > 0.05$).

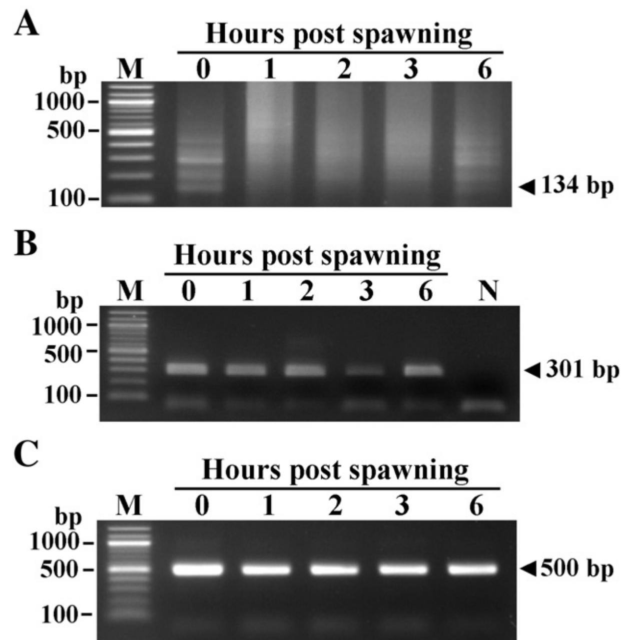

Fig. 4. RT-PCR for amplification of *PmVtg1* mRNA in spawned eggs (500 ng cDNA tempate, A) at 0. 1, 2, 3, and 6 h post spawning. *PmNASP* (100 ng template, positive control; B) and *EF-1α* (100 ng template, reference control; C) were successfully amplified from the same template.

3.4. Expression profiles of PmVtg1 mRNA in hepatopancreas of P. monodon having different ovarian developmental stages and in spawned eggs

In hepatopancreas, the relative expression level of *PmVtg1* mRNA peaked in broodstock shrimp possessing stages I and II ovaries. Its expression in hepatopancreas was significantly reduced following the progression of ovarian development in stages III and IV ovaries ($P < 0.05$, Fig. 3). The relative expression level of *PmVtg1* mRNA in hepatopancreas was 40, 30 and 25 fold greater than that in ovaries in non-ablated shrimp having stage I-III ovaries, respectively. At the mature stage, the level of *PmVtg1* mRNA in hepatopancreas and ovaries was comparable ($P > 0.05$).

The positive amplification results of abundantly expressed transcript, *nuclear autoantigenic sperm protein* (*PmNASP*) was observed suggesting the good quality of the cDNA template prepared from spawned eggs (Fig. 4). A relatively low expression level (0.0002–0.0003) of *PmVtg1* mRNA was also found in eggs at 0, 1, 2, 3 and 6 h after shrimp were spawned (Figs. 4 and 5). Interestingly, the relative expression level of egg *PmVtg1* mRNA was approximately 10,000 fold lower than that of stage IV ovaries of intact broodstock.

3.5. Localization of PmVtg1 mRNA in ovaries of P. monodon

The anti-sense cRNA probe gave the positive hybridization signals in follicular cells surrounding late previtellogenic, vitellogenic and cortical rods oocytes but not in follicular layer, oogonia or any stage of oocytes in both intact and eyestalk-ablated broodstock (Fig. 6C-F and I-L). No signal was found with the sense cRNA probe (Fig. 6B and H).

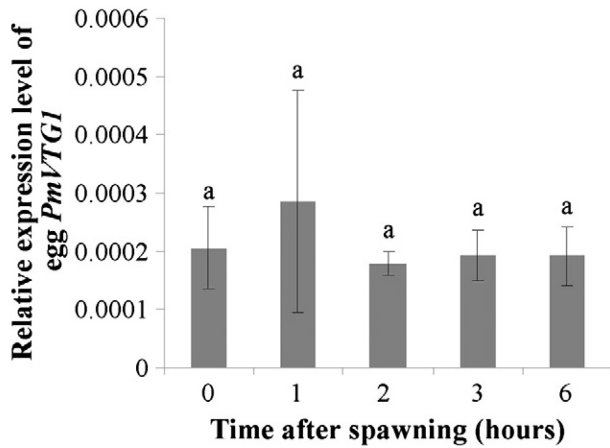

Fig. 5. The relative expression level of *PmVtg1* mRNA in spawned eggs at 0. 1, 2, 3, and 6 h after ovulation (500 ng cDNA tempate for *PmVtg1* and 100 ng template for *EF-1α*). Each histogram corresponds to a particular time point. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$).

3.6. Expression profiles of Pm-vitellin protein in ovaries and hepatopancreas of P. monodon having different stages of ovarian development

In wild intact broodstock, the ovarian Pm-vitellin protein was expressed at a low level in stage I ovaries. Its expression levels were greater during vitellogensis and mature stages. In contrast, the positive immunoreactive band was not observed against proteins from hepatopancreas (Fig. 7).

3.7. Localization of Pm-vitellin protein in ovaries of P. monodon

Immunohistochemistry revealed that Pm-vitellin was distributed in the ooplasm of late previtellogenic, vitellogenic, early cortical rod and mature oocytes in both intact and eyestalk-ablated broodstock of wild *P. monodon* females. Unlike results from *in situ* hybridization, the reactive signal was not observed in the cytoplasm of follicular cells surrounding oocytes and other locations of ovaries (Fig. 8C–F and I–L). Positive immunohistological signal was not observed with the blocking solution (Fig. 8B and H).

Fig. 6. Localization of *PmVtg1* mRNA (blue) during ovarian development of intact (B–F) and eyestalk-ablated (H–L) broodstock of wild *P. monodon* visualized by *in situ* hybridization using the sense (B and H) and antisense (C–F and I–L) *PmVtg1* cRNA probes. Oocyte stages were classified by a conventional hematoxylin/eosin staining (A and G). Arrowheads indicate the positive hybridization signals. EP = early previtellogenic oocytes; LP = late previtellogenic oocytes; EVG = early vitellogenic oocytes; LVG = late vitellogenic oocytes; ECR = early cortical rod oocytes; LCR = late cortical rod oocytes; Cr = cortical rods; N = nucleus; Cm = cell membrane of oocytes; Fc = follicular cells; Fl = follicular layers; Oop = ooplasm of oocytes.

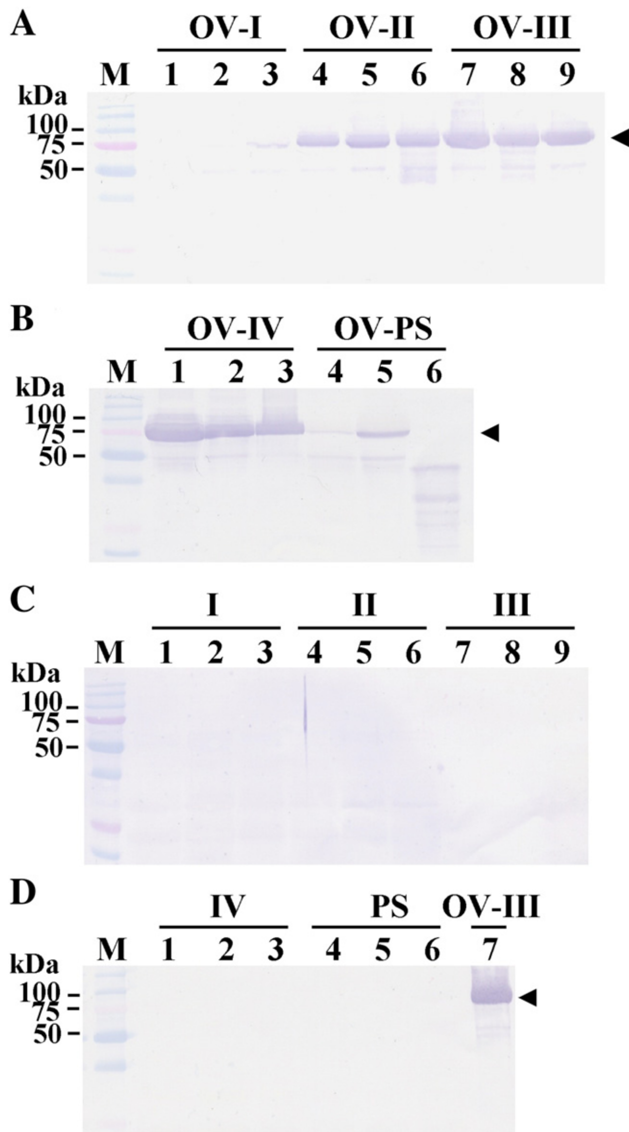

Fig. 7. Western blot analysis illustrating the expression profiles of the Pm-vitellin protein in ovaries (A and B) and PmVtg1 protein in hepatopancreas (C and D) in different stages (I–IV and post-spawning, stage V) of ovarian development of wild intact broodstock of *P. monodon*. OV-III (panel D) = total ovarian proteins of *P. monodon* broodstock having stage III ovaries used as the positive control of Western blot analysis.

4. Discussion

A typical ovarian cDNA library was established for isolation of genes involving ovarian (and oocyte) development in *P. monodon*. EST analysis was extended from 1051 clones in the previous study (Preechaphol et al., 2007) to 2330 clones in the present study and results indicated that *PmVtg1* was expressed only in the ovarian cDNA library but not in those established from other tissues of *P. monodon* (data not shown). Although *P. monodon* Vtg protein is reported to be present in hepatopancreas (Avarre et al., 2003; Raviv et al., 2006; Tiu et al., 2006; Tsang et al., 2003; Tsutsui et al., 2005). EST representing *PmVtg1* was not observed in the hepatopancreas library after 5419 clones were sequenced because the library was originally established from juvenile shrimp (Tassanakajon et al., 2006; http://pmonodon.biotec.or.th).

Five ESTs significantly matched *Vtg1* of *P. monodon* (*E*-value = 1e-113–1e-133). Interestingly, one EST showed significant similarity with *Vtg* of *F. chinensis* (*E*-value = 1e-119) suggesting that more than one *Vtg* genes may be involved in the accumulation of the vitellin protein in ovaries of *P. monodon* (Subramoniam, 2011; Tiu et al., 2006) as previously reported in *Metapenaeus ensis* (Kung et al., 2004) and *F. merguiensis* (Phiriyangkul et al., 2007).

In penaeid shrimp, vitellogenin is synthesized in both hepatopancreas and ovaries, transported to oocytes and accumulated in the ooplasm as vitellin for utilization as a nutritional source during embryogenesis (Fainzilber et al., 1992; Kawazoe et al., 2000; Tiu et al., 2006; Yano, 1995). The levels of hemolymph Vtg protein increase during ovarian development of penaeid shrimp (Meusy and Payen, 1988; Okumura et al., 2006; Tiu et al., 2006; Yano et al., 1996). In this study, tissue expression analysis based on conventional RT-PCR further confirmed that *PmVtg1* mRNA was transcribed in hepatopancreas and ovaries but not in other tissues of female broodstock. Several publications have reported that *Vtg* mRNA is exclusively expressed during vitellogenesis in broodstock shrimp (Avarre et al., 2003; Tsutsui et al., 2000; Xie et al., 2009). Using quantitative real-time PCR, we illustrated that *PmVtg1* mRNA was earlier expressed in premature ovaries of juveniles (approximately 4-month-old) of *P. monodon* with a very low level of expression (mean relative expression level = 0.0000368).

In the kuruma shrimp (*Marsupenaeus japonicus*), the *Vtg* mRNA levels were maintained at low levels during previtellogenesis for its natural maturation. The expression levels in hepatopancreas and ovaries were increased as vitellogenesis progressed, but decreased during the later stages of maturation. For eyestalk-ablated *M. japonicus*, changes in mRNA levels differed in both tissues where an obvious increment of the mRNA levels was found in the ovaries whereas negligible changes were observed in the hepatopancreas (Tsutsui et al., 2000). In *P. semisulcatus*, both hepatopancreas and ovaries are involved in the expression of *Vtg* mRNA but the expression level of *Vtg* mRNA levels in shrimp carrying the same diameter of ovaries differed significantly according to the molting stages (Avarre et al., 2003). The information suggested that the expression of ovarian *Vtg* mRNA is influenced by both ovarian developmental and molting stages.

In this study, the relative expression level of *PmVtg1* mRNA in hepatopancreas was much greater (25–40 fold) than that in ovaries in intact shrimp having stages I–III ovaries. However, the level of *PmVtg1* mRNA in these organs was not different in shrimp with stage IV ovaries. In contrast, the expression level of *Vtg* mRNA in hepatopancreas is much lower than that in ovaries at all stages of ovarian development in naturally maturing *F. merguiensis* (Phiriyangkul et al., 2007). Accordingly, contributions of ovaries and hepatopancreas to yolk production may differ among different shrimp species.

Okumura et al. (2006) examined the expression level of *Vtg* mRNA and protein in ovaries and hemolymph of *M. japonicus* over a 7-day period after bilateral eyestalk ablation. The ovarian weight and hemolymph Vtg protein levels increased in ablated females. The ovarian *Vtg* mRNA levels increased concomitantly with vitellin protein accumulation in ovaries after eyestalk ablation.

Similarly, the potential effect of unilateral eyestalk ablation on expression of ovarian *PmVtg1* mRNA was also observed. Different expression profiles of *PmVtg1* mRNA between intact and eyestalk-ablated broodstock were observed. In intact broodstock, *PmVtg1* mRNA was down-regulated from stage III in stage IV (mature) ovaries and after spawning. In contrast, the expression level of *PmVtg1* mRNA was high in stage II (vitellogenic) ovaries, peaked in stage III (early cortical rod) ovaries and slightly reduced to the previous level in stage IV (mature) ovaries in eyestalk-ablated broodstock. The information clearly suggested the effect of eyestalk ablation on inducing of ovarian development resulting in short multiple spawning in *P. monodon* (Okumura et al., 2006; Wongprasert et al., 2006).

Although eyestalk ablation causes a rapid increase of ovarian *PmVtg1* mRNA during vitellogenesis and final maturation of *P. monodon*, a lower number of oocyte resorption was observed in non-ablated compared to ablated shrimp (S. Klinbunga, unpublished data). Wongprasert et al. (2006) reported lower quality of spawns observed in eyestalk-ablated domesticated broodstock after multiple spawning as the hatching rate

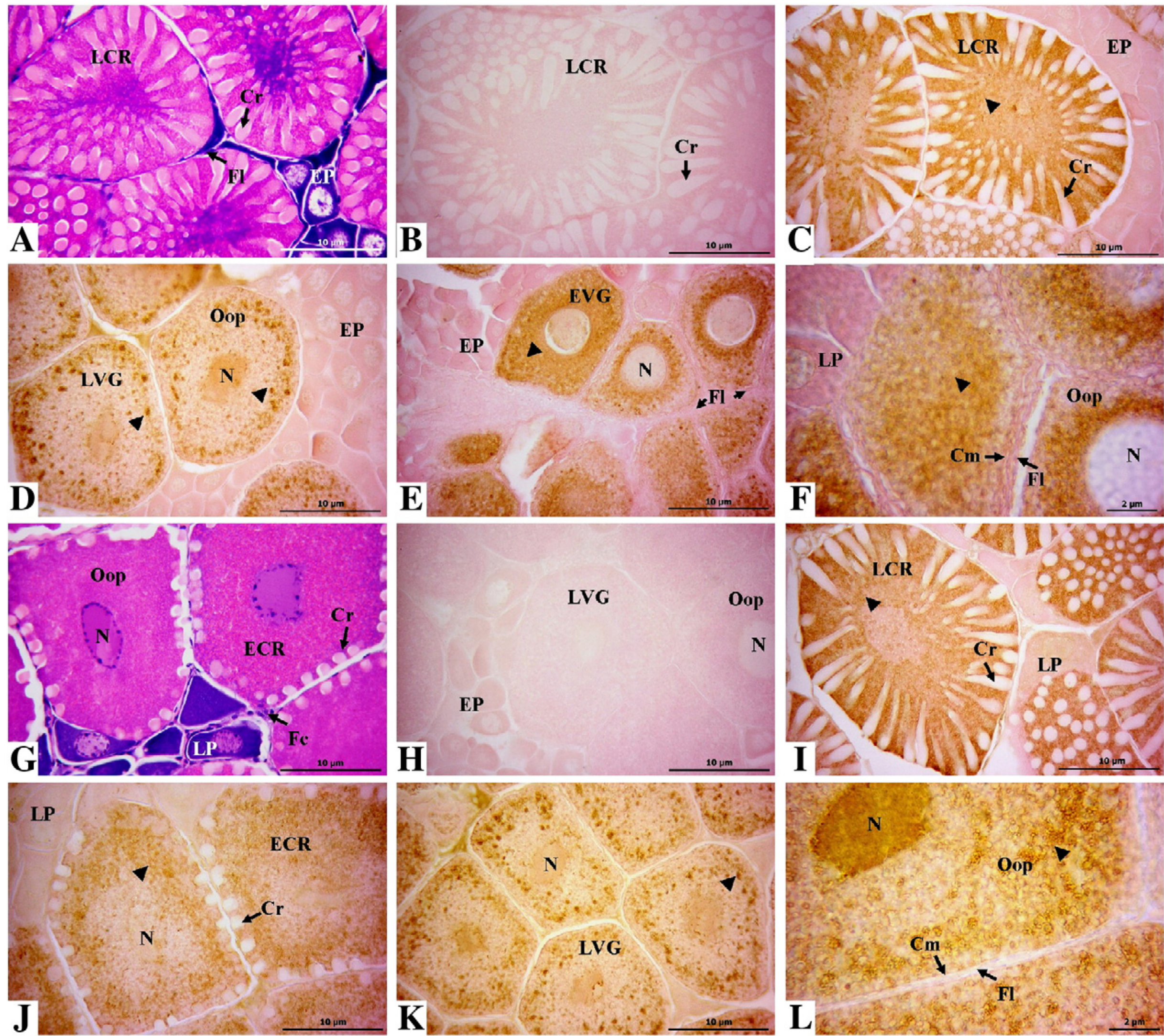

Fig. 8. Immunohistochemical localization of Pm-vitellin (brown) in ovaries of intact (B–F) and eyestalk-ablated (H–L) *P. monodon* broodstock using anti-vitellin PAb (1:500) (C–F and I–L). Hematoxylin and eosin staining (A and G) was carried out for classification of oocyte stages. The blocking solution (B and H) was included as the negative control. EP = early previtellogenic oocytes; LP = late previtellogenic oocytes; EVG = early vitellogenic oocytes; LVG = late vitellogenic oocytes; ECR = early cortical rod oocytes; LCR = late cortical rod oocytes; Cr = cortical rods; N = nucleus; Cm = cell membrane of oocytes; FC = follicular cells; Fl = follicular layers; Oop = ooplasm of oocytes.

was significantly reduced from 65.5 ± 14.5% in the first spawn to 21.5 ± 3.5 in the third spawn. Therefore, induction of ovarian development and maturation of *P. monodon* without the use of eyestalk ablation seems to be an appropriate approach to consistently obtain high quality spawners.

There has been no evidence on the autosynthesis of *Vtg* mRNA within oocytes of penaeid shrimp. In this study, spawned eggs without surrounded follicular cells were used for determination of *PmVtg1* gene expression using quantitative real-time PCR and extremely low expression levels were found (relative expression level of 0.0002–0.0003 from 500 ng template). These levels of *PmVtg1* expression could not be detected by *in situ* hybridization. In *M. japonicus*, it was suggestive that the yolk protein is synthesized by the follicular cells (and follicular epithelium) rather than by the oocytes (Tsutsui et al., 2000).

Unlike the crab (*Pachygrapsus crassipes*) where the Vtg protein was autosynthesized within the oocytes (Lui and O' Connor, 1977), *Vtg* mRNA was exclusively expressed in the ovarian follicle cells and hepatopancreas but not in oocytes in *M. japonicus* (Tsutsui et al., 2000). In this study, the spatial and cellular distributions of the *PmVtg1* transcript in ovaries of *P. monodon* broodstock were identified by *in situ* hybridization. The signals of *PmVtg1* mRNA were only detected in the cytoplasm of follicle cells surrounding developing and nearly mature oocytes but not in oocytes of *P. monodon*. The contradictory results on the finding of *PmVtg1* mRNA expression in oocytes (inferred from spawned eggs which were not surrounded by follicular cells) by quantitative real-time PCR but not *in situ* hybridization should be due to the sensitivity of the detection methods. In addition, the disappearance of *PmVtg1* hybridization from the ooplasm of oocytes may due to a significance increase in oocytes during vitellogenesis and final maturation of oocytes.

Previous studies have indicated the presence of multiple *Vtg* genes in *M. ensis* (Kung et al., 2004; Tsang et al., 2003), *Macrobrachium rosenbergii* (Yang et al., 2000) and *M. japonicus* (Tsutsui et al., 2000). In *M. ensis*, the *MeVg1* and *MeVg2* genes share only a 50% overall amino acid sequence identity (Kung et al., 2004; Tsang et al., 2003). Moreover, Tiu et al. (2006) characterized the complete gene sequence (14 introns and 15 exons) and full-length cDNA (7.8 kb encoding a protein of 2584 amino acids) of *P. monodon Vtg1* (*PmVtg1*). The existence of multiple *PmVtg* genes was revealed by RT-PCR and genomic cloning. In this study, the positive immunological signal was observed from Western blot analysis of total ovarian proteins from vitellogenic and mature (stages II-IV)

ovaries and post-spawning (stage V) ovaries but no signal was found against total proteins from hepatopancreas of the same shrimp individuals. This further confirms the presence of multiple *Vtg* genes in *P. monodon* and that the Vtg protein in hepatopancreas and Pm-vitellin in ovaries may have encoded from different *Vtg* genes.

Previously, Tiu et al. (2008) illustrate positive immunoreactive signals of the vitellin protein in ooplasm of oocytes but not in follicular cells in *P. monodon*. Using immunohistochemistry, similar results were confirmed in the present study. Yano et al. (1996) examined the route of vitellin uptake into the oocytes of *M. japonicus* using immunohistochemistry and electron microscopy. The positive signal was observed in the enlarged follicle cells surrounding oil globule stage oocytes of the early vitellogenic ovaries but no signal was detected in shrunken follicle cells surrounding oocytes in the yolk granule stage. Electron microscopic examination indicated that egg yolk protein was transferred to the surface of yolk granule stage oocytes from the spaces between neighboring follicle cells and incorporated into the ooplasm through the microvilli (pinocytosis) and subsequently aggregate to form yolk bodies.

There is no evidence indicating RNA transfer from the follicle cells to the oocytes in penaeid species. Yolk protein mRNAs have a high turnover rate and a concomitant short life time (Cardoen et al., 1986). Accordingly, it is likely that the synthesis of *PmVtg1* mRNA in the follicle cells sustains translational activity and the follicular PmVtg1 protein may have processed to vitellin and translocated to developing *P. monodon* oocytes by pinocytosis as previously proposed in *M. japonicus*. The disappearance of immunological signals of *P. monodon* vitellin in ovarian follicular cells may be due to rapid translocation of processed Pm-vitellin in follicular cells per se. In addition, the sensitivity of immunohistochemistry is also limited and may not be sufficient to detect the newly translated PmVtg1 protein in follicular cells. In *M. japonicus*, positive signals of *Vtg* mRNA and vitellin protein were found in follicular epithelium using *in situ* hybridization (Tsutsui et al., 2000) and an immunofluorescence with anti-vitellin IgG (Yano and Chinzei, 1987).

It has also been proposed that the *Vtg* mRNA is translated in the ovaries, secreted to the hemolymph and subsequently sequestered by a specific receptor (i.e. vitellogenin receptor) to oocytes (Avarre et al., 2003). The presence of a signal peptide in the deduced PmVtg1 protein seems to support its translocation as an extracellular protein from oocytes (Phiriyangkul and Utarabhand, 2006; Raviv et al., 2006; Tiu et al., 2009).

In this study, the expression profiles and localization of *PmVtg1* mRNA and Pm-vitellin protein in ovaries of wild *P. monodon* were examined. Although the transcriptional site of *PmVtg1* mRNA was found in oocytes, the process for modification of translated PmVtg protein is still unknown. Nevertheless, we speculate that the translated protein is further cleaved to Pm-vitellin within the oocytes. The information in this study suggested that *PmVtg1* mRNA and Pm-vitellin plays the main role in development of oocytes and ovaries in *P. monodon*. The basic knowledge obtained allows further characterization of mechanisms of vitellogenesis in this economically important species.

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