การแสดงออกสูง มีหลักฐานที่แสดงว่า RPL10a มีส่วนในการเจริญของเซลล์ เช่น embryogenesis และ organogenesis (Fisicaro et al. 1995; Kubiczek et al. 1997) ยับยั้งการเจริญของเซลล์ในไทมัสของหนู (Fisicaro et al. 1995) และเป็น tumor antigen (Koga et al. 2003). ในกุ้งพบ *RPL10a* mRNA จำนวนมากใน เนื้อเยื่อ ovaries และ lymphoid tissue ในระยะที่มีกุ้งมีการสร้าง vitellogenin (Wonglapsuwan et al. 2009) เราจึงสันนิษฐานว่า RPL10a อาจเกี่ยวข้องกับการเจริญและการพัฒนาของไข่ พบการมี RPL10a จำนวนมากทั้งในไซโตพลาสมและนิวเคลียสจึงคาดว่า RPL10a สังเคราะห์ในไซโตพลาสมและส่งไปสะสมในนิวเคลียสของเซลล์ไข่ระยะที่มีกาพัฒนา ทั้งนี้เนื่องจากพบการสะสมของโปรตีน RPL10a จำนวนมากทั้งในไซโตพลาสมและนิวเคลียสของเซลล์ไข่กุ้ง อีกทั้งใน RPL10a ยังพบ intranuclear localization signal (Wonglapsuwan et al., 2009) RPL10a ลูกผสมที่ผลิตจาก *E. coli* กระตุ้นการแสดงออกของยีน *TCTP*, *HSP70* และ *SOP* genes ใน explants culture ของเซลล์ไข่กุ้ง แต่ไม่พบการเร่งการแสดงออกของ Vn การใช้ explants culture ในการศึกษาหน้าที่ของhormone เช่นใช้ ศึกษาหน้าที่ของ gonad-stimulating hormone จาก *M. ensis* (Shirley and Siu 2007) และใช้ศึกษาการชักนำการเจริญของไข่กุ้งในปู *Chasmagnathus granulate* (Zapata et al., 2003) ยิ่งกว่านั้นRPL10a ลูกผสมไม่กระตุ้นการแสดงออกของ *TCTP*, *SOP* และ *HSP70* ในเนื้อเยื่อกล้ามเนื้อซึ่งชี้ว่าการกระตุ้นของ *RPL10a* อาจมีความจำเพาะกับเซลล์ไข่กุ้ง และไม่พบความเป็นพิษกับ ovarian explant cultures ซึ่งแสดงโดย LDH assay

โดยสรุปเราได้ศึกษาคุณลักษณะของยีน *RPL10a* ได้แสดงความสามารถของโปรตีนลูกผสม RPL10 ที่สามารถกระตุ้นการแสดงออกของยีนในระยะแรกของ ovarian maturation การค้นพบนี้มีความสำคัญในการเป็นจุดเริ่มต้นสำหรับการพัฒนารังไข่กุ้ง

## 5. เอกสารอ้างอิง


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ORIGINAL ARTICLE

# Characterization and Biological Activity of the Ribosomal Protein L10a of the White Shrimp: *Fenneropenaeus merguiensis* De Man During Vitellogenesis

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**Abstract** The molecular events in the ovaries of *Fenneropenaeus merguiensis* De Man during vitellogenesis were investigated. The ribosomal protein L10a (*RPL10a*) was characterized and cloned. It consisted of 669 bp and the deduced polypeptide had 217 amino acids (GeneBank™/EBI accession number FJ623402). The calculated molecular mass and p*I* were 25.7 kDa and 10.06, respectively. An immunohistochemical technique showed that RPL10a was located in the cytoplasm and nucleus of developing oocytes and follicle cells. Treatment of undeveloped ovarian explant cultures with recombinant histidine (His)-RPL10a stimulated the expression of translationally controlled tumor protein (*TCTP*), heat shock protein 70 (*HSP70*), and shrimp ovarian peritrophin (*SOP*) genes, previously shown to be involved in ovarian maturation. The transcripts of all three genes in the ovarian explants showed their highest expression after 4 h incubation with the His-RPL10a at 37°C. The *TCTP* and *HSP70* transcripts declined after 12 h, while the transcript of *SOP* remained high until 24 h. The His-RPL10a did not stimulate the expression of the *TCTP*, *SOP*, and *HSP70* genes in shrimp muscle tissue. The information on the molecular behavior of the RPL10a in this study may, in the future, lead to new methods to stimulate ovarian development in shrimp.

**Keywords** Expression · *Fenneropenaeus merguiensis* · Genes · Ovary · RPL10a · Shrimp

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## Introduction

Developing novel methods for controlling the maturation of shrimp gonads is challenging as the molecular mechanisms concerned with ovarian development are not well understood. Genes involved in the development of the ovary remain largely unidentified. Recent molecular biology studies have identified genes whose transcripts increase during ovarian development such as for shrimp vitellogenin (*Vn*; Tsukimura 2001), thrombospondin (*TSP*; Yamano et al. 2004), cyclin B, cathepsin C (Qiua et al. 2004), and shrimp ovarian peritrophin (*SOP*; Loongyai et al. 2007). In addition, Lo et al. (2007) showed that many genes in the ovaries of *Metapenaeus ensis* had increased expression during vitellogenesis including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), arginine kinase, translationally controlled tumor protein (*TCTP*), actin, keratin, and heat shock protein70 (*HSP70*). When differential expression was studied in *Fenneropenaeus merguiensis*, another set of genes such as *TCTP*, *SOP*, *TSP*, *Vn*, *HSP70*, and *RPL10a* were identified (Wonglapsuwan et al. 2009).

Ribosomes are the machines responsible for protein synthesis in every living cell (Ditlev and Poul 2005) and the translational activity of the cell is normally regulated, to

some extent, by the ribosomal proteins (Rhodes and Van 1997). However, it is still not clear how the ribosomal proteins control cell growth and proliferation (Thomas 2000). There are some evidences that ribosomal proteins are involved in ovarian functions, such as during oogenesis of *Drosophila melanogaster* where expression of an antisense ribosomal protein A1 (*rpA1*) severely disrupted oogenesis and produced a "small egg" female-sterile phenotype (Qian et al. 1988). Ovarian development in *D. melanogaster* was also destroyed by the presence of an antisense product of the putative ribosomal protein S3A (*rpS3A*) gene (Reynaud et al. 1997). High amounts of ribosomal protein S24 (*rpS24*) mRNA have been reported in the sea urchin, *Paracentrotus lividus*, during oogenesis (Sgroi et al. 1996). In the marine shrimp, *Marsupenaeus japonicus*, expression of ribosomal proteins was much higher in the ovary than in other organs (Zhang et al. 2007). These studies support our previous results showing that ribosomal protein L10a gene was upregulated during ovarian maturation of the banana prawn, *F. merguiensis* (Wonglapsuwan et al. 2009).

The *RPL10a* gene (cyclosporin A-19 or neural precursor cell expressed developmentally downregurated protein 6) encodes for ribosomal protein L10a, a component of the 60S subunit of the ribosome. The RPL10a protein belongs to the L1P family of ribosomal proteins. It is located in the cytoplasm along with the ribosomes. It has been suggested that RPL10a might play a role in embryogenesis and organogenesis (Fisicaro et al. 1995; Kubiczek et al. 1997). Interestingly, the expression of this gene was downregulated in the thymus of mice by cyclosporin A. Cyclosporin A is a highly specific immunosuppressive drug used to treat the rejection of allogeneic transplants (Fisicaro et al. 1995). In addition, an interaction between RPL10a and trichosanthin was reported (Xia et al. 2005). Trichosanthin is a type I single-chain ribosome-inactivating protein (RIP). It has been used to terminate early and midtrimester pregnancies. Pharmacological studies reported that trichosanthin was also able to suppress immune responses (Leung et al. 1986). Furthermore, *RPL10a* was identified as a new radiation-responsive gene which is downregulated in cultured human cells by two types of radiations that include X-rays and fission neutrons (Kubiczek et al. 1997). In hairless mice, this gene was also regulated by radiation (Lee et al. 2006). RPL10a has also been identified as a tumor antigen that is recognized by human leukocyte antigen-A26 (HLA-A26)-restricted cytotoxic T lymphocytes (Koga et al. 2003). All the above evidence indicates that RPL10a is most likely to be involved in cell proliferation.

Therefore, there is a possibility that RPL10a will be involved in the cell proliferation that occurs during the maturation of shrimp ovaries. For this reason, we decided to identify and characterize the *RPL10a* found in the ovaries of *F. merguiensis*. In addition, an in vitro assay was established to determine the effect of RPL10a on the genes that were previously shown to be highly expressed during ovarian maturation including *SOP* (Loongyai et al. 2007), *TCTP* (Loongyai et al. 2006), and *HSP70* (Wonglapsuwan et al. 2009).

## Materials and Methods

### Animals

Female banana prawns with matured and nonmatured ovaries were obtained from the Gulf of Thailand, Nakornsithammarat Province, Thailand. The gonadosomatic index (GSI) was calculated as a percentage of ovarian weight relative to the body weight. The maturation of female prawns was classified into three stages: stage 1, previtellogenic (GSI, 2.65±0.2); stage 2, endogenous vitellogenic (GSI, 3.60±0.28); stage 3, exogenous vitellogenic (GSI, 5.13±0.74).

### Histology

The ovaries of female shrimp were collected from each stage of ovarian maturation. These samples were fixed in neutral buffered formalin solution (10% formalin, 33 mM $NaH_2PO_4$, 45 mM $Na_2HPO_4$) for at least 72 h. The tissues were then dehydrated, embedded in paraffin, and cut into approximately 5 to 6 μm thick sections using an 820 Spencer rotary microtome (MedCon, Colorado, USA). The sections were stained with hematoxylin and eosin for histological evaluation. Serial sections from the same tissue blocks were retained and placed onto 2% silane pretreated slides for further immunohistochemistry (IHC).

### Immunohistochemistry

Tissue sections were dewaxed twice for 5 min each in xylene and rehydrated through a graded series of ethanol until finally into distilled water, incubation in 1 mM ethylenediaminetetraacetic acid pH 7.5 at 55°C for 1 h. The slides were twice washed in Tris-buffered saline–Tween 20 (TBST) pH 7.5 (0.1% Tween 20, 25 mM Tris–HCl, 2.7 mM KCl, and 140 mM NaCl, pH 7.5). The sections were treated with a blocking buffer (4% bovine serum albumin and 0.02% $NaN_3$ in phosphate-buffered saline [PBS]) at room temperature for 30 min and then rinsed briefly with TBST. The sections were blocked again in 5% skim milk at room temperature for 30 min. After that, the slides were washed with TBST. Sections were blotted with mouse monoclonal anti-RPL10a (Abcam, Cambridge, UK) and incubated overnight at room temperature. The slides were washed with TBST again and incubated with

rabbit antimouse–alkaline phosphatase (AP) for 2 h at room temperature. Developing solution containing 0.37 mM Nitro blue tetrazolium, 0.23 mM 5-bromo-4-cholro-3-indolyl phosphate, 0.1 M $NaHCO_3$, and 1 mM $MgCl_2$, pH 9.8, was used as substrates for detection. The images of histological sections were captured by a CH40 Olympus digital camera.

### *RPL10a* Sequence Analysis

Full-length *RPL10a* cDNA was obtained from the clone U33 isolated from the subtraction cDNA library of the banana prawn ovary (Wonglapsuwan et al. 2009). To further confirm these sequences, full-length cDNA was amplified using the RPL10a forward primer (5′ GGATC CATGTCGAGCAAGGTG 3′) and RPL10a reverse primer (5′ GAATTCTAGTGATTAGCGTGGTCG 3′). The obtained polymerase chain reaction (PCR) fragments were cloned into pGEM-T Easy (Promega, Madison, WI, USA) and sequence analysis was performed using the ABI prism 377 apparatus.

Nucleotide and predicted amino acid sequence data were analyzed and aligned with sequences in the GenBank databases using the BLASTx search from NCBI (http://www.ncbi.nlm.nih.gov) to determine gene identity. Prediction of the subcellular localization and any putative localization signals was computed by the PSORTII server through ExPASy molecular biology (http://psort.nibb.ac.jp/). The potential phosphorylation sites were predicted via http://www.cbs.dtu.dk/services/NetPhos/. A phylogenetic tree was produced using the neighbor-joining method (http://www.ebi.ac.uk/clustalw).

### Expression and Purification of a Recombinant Ribosomal Protein L10a in *Escherichia coli*

The PCR product of the full length of RPL10a was digested by *Bam*HI and *Eco*RI (Promega, Madison, WI, USA) and ligated to pET-28a(+) (Novagen, Madison, WI, USA). The constructs were transformed into *E. coli* BL21 (DE3) cells. The transformed cells were grown in Luria–Bertani or 2× yeast tryptone medium supplemented with 30 mg/ml of kanamycin until an OD of 0.5–0.6 at 600 nm was reached. Protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were further grown at 37°C for 3 h. The bacterial cells were harvested by centrifugation (4,000×*g* for 20 min), resuspended in lysis buffer (50 mM $NaH_2PO_4$, pH 8.0, 300 mM NaCl, and 10 mM Tris–HCl, pH 8.0), lysed by sonication (6×10 s, 200 to 300 W), and centrifuged at 10,000×*g* for 20 min at 4°C. Pellets containing inclusion bodies were resuspended in PBS (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, and 140 mM NaCl, pH7.4) then sonicated (6×10 s, 200 to 300 W), and centrifuged at 5,000×*g* for 15 min at 4°C. The supernatant was collected as a fraction while the pellet was subjected to the same procedure several times until a number of fractions of purified soluble His-RPL10a were obtained (Violand 2001). The presence of purified RPL10a protein was detected by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

### In Vitro Activity of the Recombinant His-RPL10a (Explant Cultures)

Nonmatured ovaries were carefully dissected, cut into small pieces, and each piece placed in a 24-well plate containing 1.5 mL of medium 199 (GIBCO, Grand Island, NY, USA) dissolved in crab saline (440 mM NaCl, 11 mM KCl, 13.3 mM $CaCl_2$, 26 mM $MgCl_2$, 26 mM $Na_2SO_4$, and 10 mM HEPES, pH 7.4; Duan and Cooke 1999) with or without 1 μM purified RPL10a protein under sterile condition. Two percent penicillin G–streptomycin was added to prevent bacterial growth. The cultures are incubated at 37°C and sampled at 0.5, 2, 4, 6, 12, and 24 h. At the end of the incubation, the tissues were collected for RNA extraction followed by real-time PCR. The culture supernatants were harvested for lactate dehydrogenase (LDH) assay. Muscle tissues from the same shrimp were treated in the same way and used as a negative control.

### Real-Time PCR

Total RNA was isolated from incubated ovaries using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Four hundred nanograms of total RNA was incubated with 200 ng of random primers at 70°C for 5 min and cooled on ice for 5 min. This mixture was added to 1× avian myeloblastosis virus (AMV) buffer, 1 mM deoxynucleotide triphosphate, and 10 U of AMV reverse transcriptase (Promega, Madison, WI, USA) in 25 μl of reaction mixtures and incubated at 48°C for 2 h. Individual 50 μl of reaction of real-time PCR included: 25 μl of iQ™SYBR®Green Supermix (Bio-Rad, Hercules, CA, USA), 20 pmol of each primer, and 300 ng of cDNA. The primers for the *TCTP*, *SOP*, *HSP70*, and *Vn* genes are shown in Table 1. *GAPDH* primers were used as an internal control. Thermal cycling and fluorescence detection were performed using the Mx3000P™ (Stratagene, USA). All samples were tested in triplicate. PCR was initiated with a first denaturation step of 5 min at 95°C, followed by 40 cycles of 94°C for 2 min, annealing at 50°C (*HSP70*), 55°C (*Vn*), or 60°C (*TCTP*, *SOP*) for 1 min, and extension at 72°C for 1 min. Negative controls consisted of reactions with no template cDNA. For quantifying all genes, a standard curve was

**Table 1** Primer sequences used for quantitative PCR

| Primer name | Primer sequences |
|---|---|
| Forward TCTP | 5′ TTATAGCTTCTCCTCGTTAGAC 3′ |
| Reverse TCTP | 5′ ATGAAGGTCTTCAAGGATATG 3′ |
| Forward SOP | 5′ GAGATGCCTGAGTTTGGTGGTGG 3′ |
| Reverse SOP | 5′ ACTGCAAGTGAAGGGCTTCTGGAGC 3′ |
| Forward HSP70 | 5′ CGATGCCAACGGTATCCTGC 3′ |
| Reverse HSP70 | 5′ CGTCTTCTGTTGGAGGAGGCG 3′ |
| Forward GAPDH | 5′ CAAGAAGGTCATCATCTCCGCT 3′ |
| Reverse GAPDH | 5′ TCCACGGTCTTCTGTGTGGC 3′ |
| Forward Vn | 5′ CGGTGTGTCTTCTGGCACCT 3′ |
| Reverse Vn | 5′ GCCACACAGAAGACCGTGGA 3′ |

*TCTP* translational tumor protein (GeneBank ™/EBI accession number AY700595), *SOP* shrimp ovarian peritrophin (GeneBank ™/EBI accession number AY775291), *HSP70* heat shock protein 70 (GeneBank ™/EBI accession number CO267931), *GAPDH* glyceraldehyde-3-phosphate dehydrogenase (GeneBank™/EBI accession numberAI770197), *Vn* vitellogenin (GeneBank ™/EBI accession number AY051318)

prepared using serial dilutions of the linearized purified PCR products of each gene. The detection range was determined by preparing tenfold serial dilutions of all genes in the range between $1\times10^7$ and $1\times10^3$ copies. The copy number of each reacted product was calculated according to its molecular weight and then converted into the copy number based on Avogadro's number by the formula: number of copies = (amount(ng) × 6.022× $10^{23}$)/(length(bp) × 1 × $10^9$ × 650). The copy number of the *each* gene relative to the *GAPDH* genes in the incubated sample was calculated.

## Cell Viability of the Ovarian Explants

LDH assay was performed for detecting cell viability. The LDH released from tissue culture cells was determined by the LDH detection kit (Roche, Mannheim, Germany). Briefly, culture medium from the explant cultures was collected in microfuge tubes and centrifuged (3,000×*g*, 5 min) to remove the cells. A 0.1-ml aliquot of supernatant was dispensed into several wells of a 96-well microtiter plate followed by 0.1 ml of LDH substrate (consisting of diaphorase NAD mixture, iodophenyl-nitrophenyl-phenyltetrazolium chloride, and sodium lactate), prepared according to the manufacturer's directions (Roche, Indianapolis, IN, USA). Plates were read after 30 min of incubation at 25°C using an automated microplate reader (BIO-TEK Instruments, Vermont, USA) at 490/630 nm.

## Statistical Analysis

Three separate aliquots of RNA from each sample were used for real-time PCR. The mean of each of the mRNA expression levels in each sample was calculated. The mRNA expression levels were compared statistically using the Independent-Samples *T* Test of SPSS version 15.0 software at a 95% confidence level ($p<0.05$).

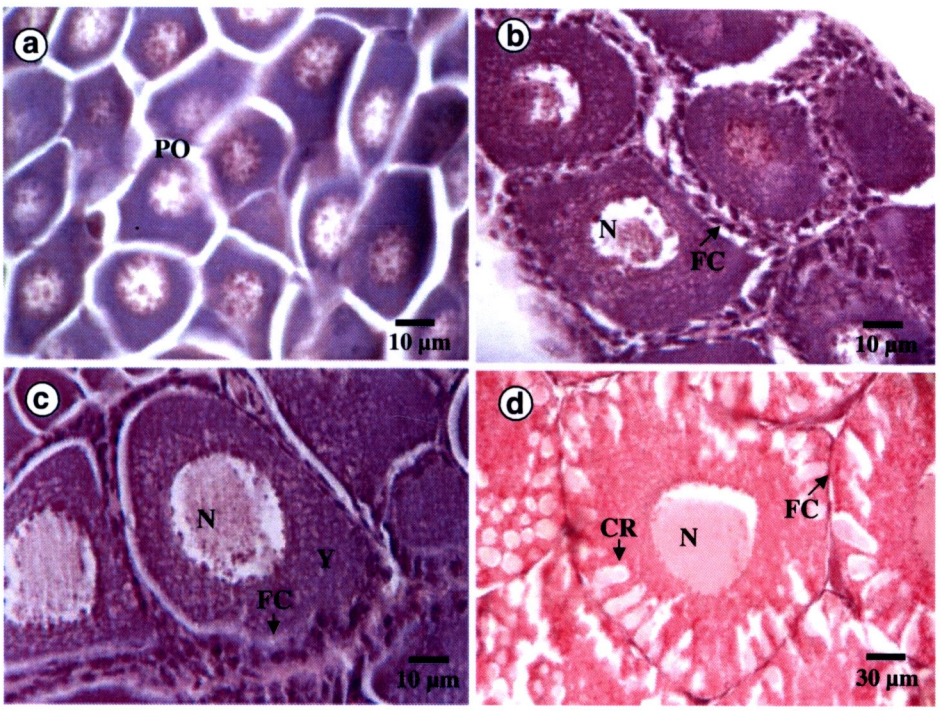


**Fig. 1** Histological sections of oocytes of shrimp at different stages of development stained with hematoxylin and eosin. **a** Previtellogenic oocytes, **b** stage 1 (early developing oocytes), **c** stage 2 (developing oocytes), **d** stage 3 (mature oocytes). *CR* cortical rod, *FC* follicle cell, *N* nucleus, *PO* perinucleolar oocyte, *Y* yolk. Bars=10 μm in **a**–**c** and 30 μm in **d**

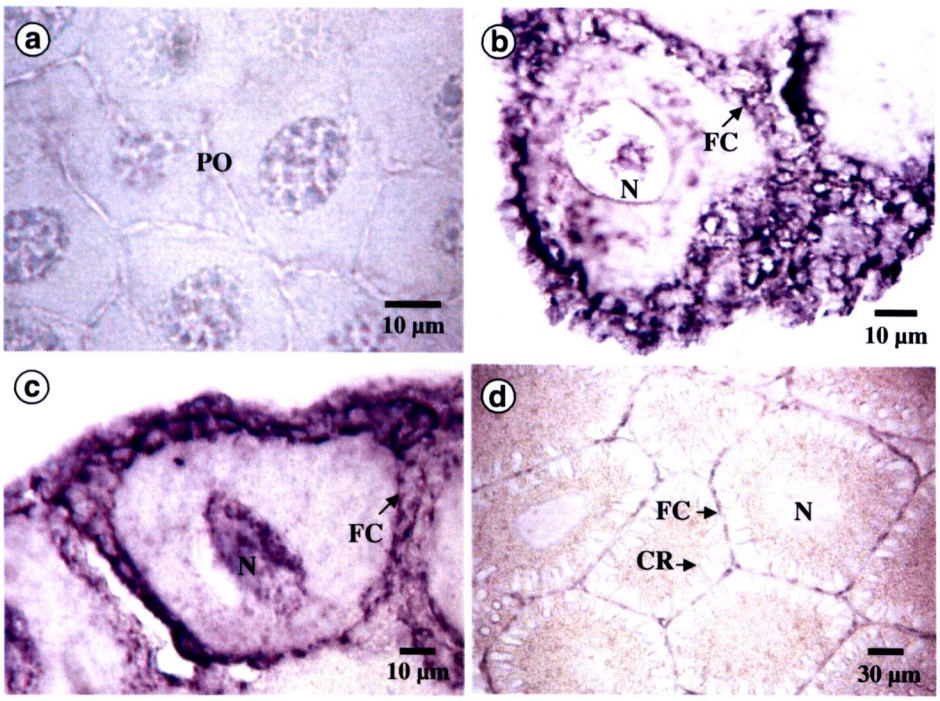


**Fig. 2** Immunohistochemical staining for RPL10a in oocytes of shrimp at different stages of development. **a** Previtellogenic oocytes, **b** stage 1 (early developing oocytes), **c** stage 2 (developing oocytes), **d** stage 3 (mature oocytes). *CR* cortical rod, *FC* follicle cell, *N* nucleus, *PO* perinucleolar oocyte, *Y* yolk. Bars=10 μm in **a**–**c** and 30 μm in **d**

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    CGGACAAGGAGCACCATGTCGAGCAAGGTGACGAGGGACACCCTCTACGAG  51
 1                M  S  S  K  V  T  R  D  T  L  Y  E
    TGCATCAACGGCGTGCTTCAGGGCGCCAAGGACAAGAAGCGCAACTTCCGC 102
13  C  I  N  G  V  L  Q  G  A  K  D  K  K  R  N  F  R
    GAGACGGTCGAGCTCCAGATCGGCCTCAAGAACTATGACCCCCAAAAGGAT 153
30  E  T  V  E  L  Q  I  G  L  K  N  Y  D  P  Q  K  D
    AAGCGTTTCAGTGGCACAGTGAAGTTGAAGCACATCCCCAAGCCCAACATG 204
47  K  R  F  S  G  T  V  K  L  K  H  I  P  K  P  N  M
    AAGATCTGTGTCCTTGGTGACCAGATGCACATTGATGAGGCTAAGGAGAAC 255
64  K  I  C  V  L  G  D  Q  M  H  I  D  E  A  K  E  N
    AACATTCCCTGCATGTCTGCGATGACCTCAAGAAGCTGAACAAGGACAAG 306
81  N  I  P  C  M  S  A  D  D  L  K  K  L  N  K  D  K
    AAGCTTGTCAAGAAGCTGGCAAAGAAGTATGATGCCTTCATTGCCTCTGAT 357
98  K  L  V  K  K  L  A  K  K  Y  D  A  F  I  A  S  D
    GCCCTTATCAAGCAGATTCCCCGTCTGTTGGGCCCTGGTCTCAACAAGGTT 408
115 A  L  I  K  Q  I  P  R  L  L  G  P  G  L  N  K  V
    GGCAAGTTCCCTACCATGTGCACTCACTCTGAGAAGTTGACAGACAAGTGC 459
132 G  K  F  P  T  M  C  T  H  S  E  K  L  T  D  K  C
    AATGAGATCAAGGCCACCATCAAGTTCCAGATGAAGAAGGTGTTGTGCCTG 510
149 N  E  I  K  A  T  I  K  F  Q  M  K  K  V  L  C  L
    TCTGTTGCCATTGGCCACGTTGAGATGGCTTCGGATGAACTAGTGCAGAAC 561
166 S  V  A  I  G  H  V  E  M  A  S  D  E  L  V  Q  N
    GTTTACCTGGCCATGAACTTCTTGGTTTCGCTGTTGAAGAAGCATTGGCAG 612
183 V  Y  L  A  M  N  F  L  V  S  L  L  K  K  H  W  Q
    AACGTGCGATCCCTGCATATTAAGTCTACCATGGGAAGGCCCCAACGCCTG 663
200 N  V  R  S  L  H  I  K  S  T  M  G  R  P  Q  R  L
    TACTAG 669
217 Y  *
```

**Fig. 3** Nucleotide and deduced amino acid sequences of the *RPL10a* gene. The nucleotide sequence is displayed in the 5′ to the 3′ directions and numbered from *left* to *right*. The deduced amino acid sequence is shown in the single-letter amino acid code. Codons are numbered at the *left* with the methionine initiation codon. An *asterisk* denotes the termination codon. The nuclear localization signal is *underlined*. The phosphorylation sites, which include four serine, three threonine, and one tyrosine residues, are marked with *circles*

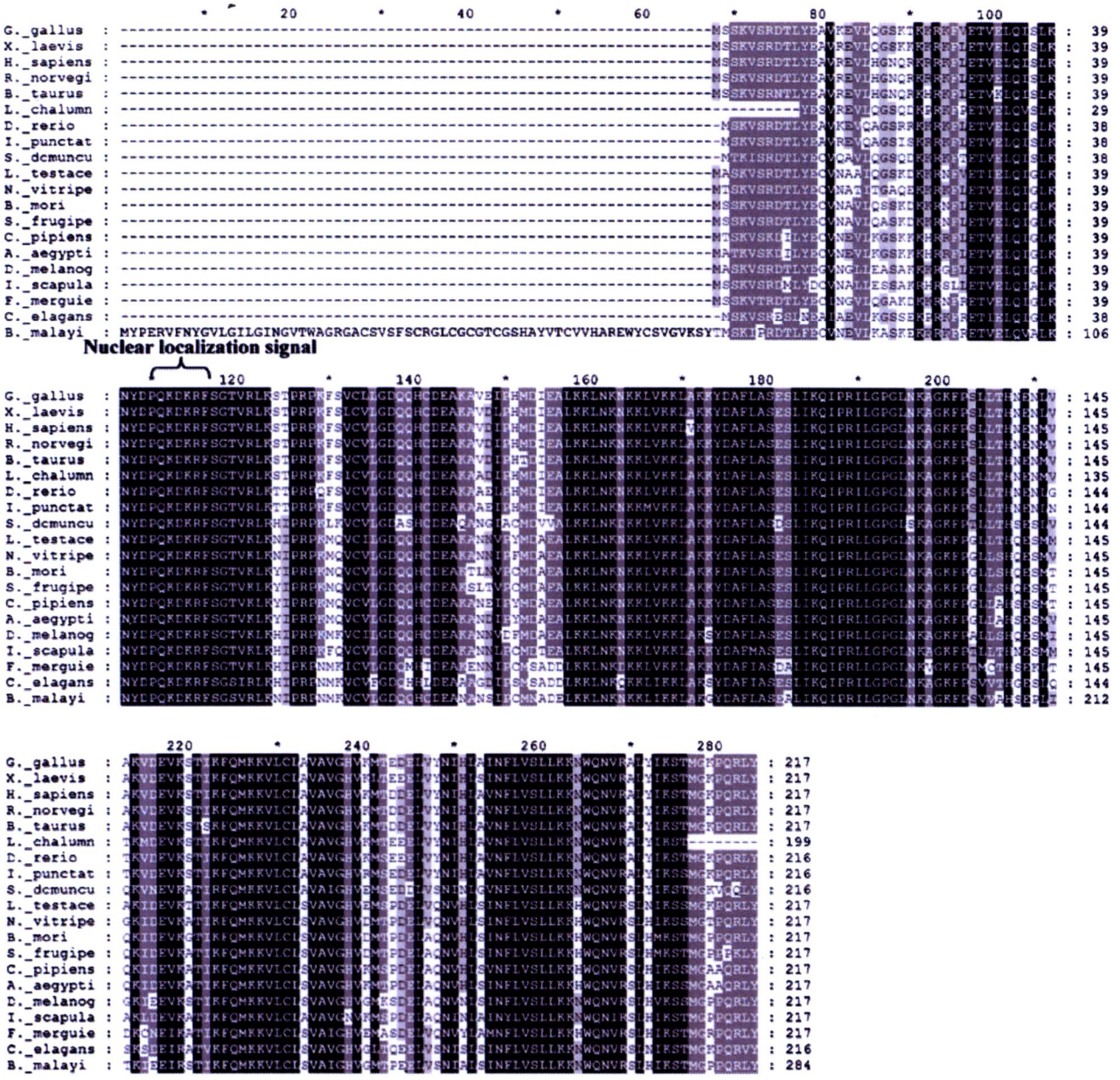


**Fig. 4** Alignment of the deduced amino acid sequence of RPL10a from *F. merguiensis*, GenBank accession number FJ623402 with those of other RPL10a homologues from other species. Species names are abbreviated at the *left* and shown as follows: *Bombyx mori*, GenBank accession number NP_001037147; *Spodoptera frugiperda*, GenBank accession number Q963B6; *Lysiphlebus testaceipes*, GenBank accession number AAX62464; *Nasonia vitripennis*, GenBank accession number XP_001605533; *Culex pipiens quinquefasciatus*, GenBank accession number XP_001842939; *Aedes aegypti*, GenBank accession number XP_001656504; *D. melanogaster*, GenBank accession number NM_140257; *Ixodes scapularis*, GenBank accession number AAY66960; *H. sapiens*, GenBank accession number AAA86463; *Rattus norvegicus*, GenBank accession number NP_112327; *Bos taurus*, GenBank accession number XP_001249633; *Latimeria chalumnae*, GenBank accession number AAS49547; *Gallus gallus*, GenBank accession number XP_418020; *Xenopus laevis*, GenBank accession number NP_001080205; *Danio rerio*, GenBank accession number AAH71510; *Ictalurus punctatus*, GenBank accession number Q90YV8; *Suberites domuncula*, GenBank accession number AAX48842; *C. elegans*, GenBank accession number Q9N4I4; *B. malayi*, GenBank accession number XP_001891868. Sequence alignment was performed using CLUSTAL X

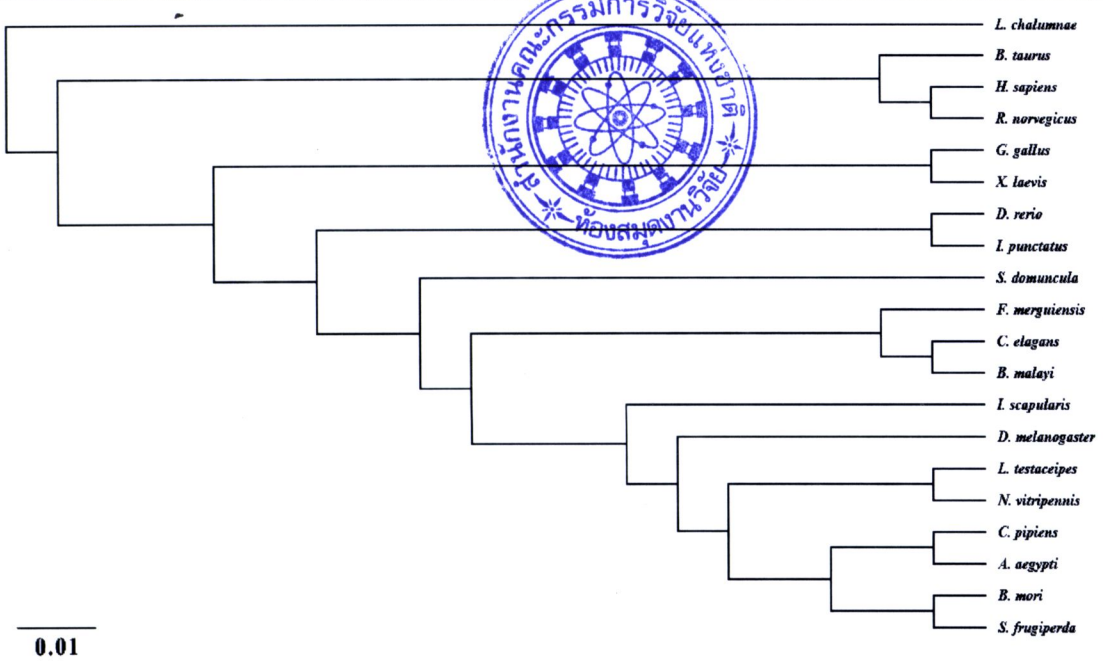


**Fig. 5** Phylogenetic tree of RPL10a from different organisms based on the amino acid sequence comparisons shown in Fig. 4

## Results

### Histology and Immunohistochemistry of the RPL10a in Ovaries of Shrimp

Histological observation of the previtellogenesis oocytes revealed the presence of a highly basophilic cytoplasm and the nucleus containing large granular nucleoli (Fig. 1a). In the early developing ovary, stage 1 (Fig. 1b), ovarian cells were surrounded by multiple ovarian follicle cells. Oocytes had the highly basophilic cytoplasm containing many small fatty droplets around the nucleus. Furthermore, in this stage, the nucleus was enlarged and had many chromatin and granular nucleoli locating on the inner surface of the nucleus. In stage 2 (Fig. 1c), oocytes had the highly basophilic cytoplasm producing granular yolk globules among many small fatty droplets around the nucleus. In stage 3 (Fig. 1d), all of cells were the mature oocytes. Their acidophilic cytoplasms contain many granular yolk globules around the nucleus and rod-shaped yolk globules (cortical

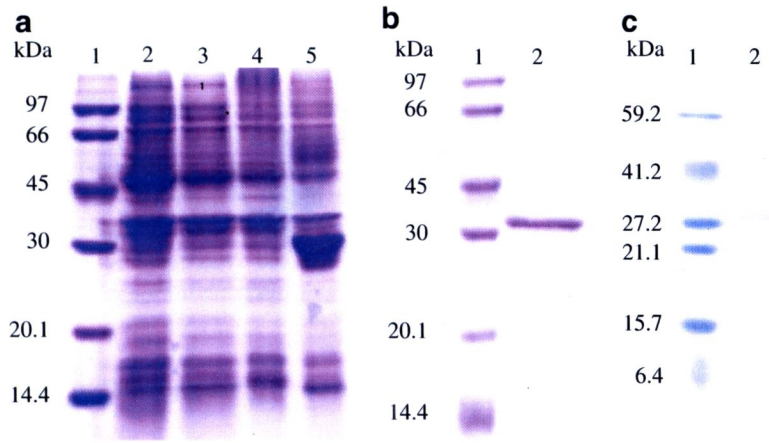


**Fig. 6** Expression and purification of the His-RPL10a from *E. coli* BL21 (DE3) cells harboring *pET-RPL10a*. The proteins were separated on 12% SDS-PAGE. **a** Coomassie blue staining of the cell-free extracts; *lane 1* molecular weight markers, *lane 2* noninduced pET cell lysate, *lane 3* induced pET cell lysate, *lane 4* noninduced His-RPL10a cell lysate, *lane 5* induced His-RPL10a cell lysate. **b** Coomassie blue staining of the purified RPL10a protein; *lane 1* molecular weight markers, *lane 2* purified RPL10a protein. **c** The His-RPL10a protein (*lane 2*) was detected by anti-His monoclonal antibody after Western blotting

rods) were at the periphery. Their nuclei have fine granular chromatin and granular nucleoli on the inner surface. The surrounding ovarian follicle cells become thin.

To investigate the location of the RPL10a protein within oocytes during ovarian development, IHC was performed using mouse monoclonal anti-RPL10a. The results indicated that no synthesis of RPL10a took place within immature oocytes (Fig. 2a). However, in stage 1, ovarian follicle cells showed strongly active synthesis of RPL10a protein in their cytoplasm. Developing oocytes showed active synthesis of RPL10a in the cytoplasm, which were observed to form masses in IHC. In addition, their nuclei showed evidence containing RPL10a protein (Fig. 2b). In stage 2, follicle cells still showed active production of RPL10a protein. The developing oocytes displayed active synthesis of RPL10a protein which was observed to form many granules in the entire cytoplasm of IHC. IHC also revealed increased RPL10a protein in the nucleus (Fig. 2c). In the mature ovary, stage 3, ovarian follicle cells have been flattened around mature oocytes and RPL10a protein disappeared in the cytoplasm. Moreover, this protein was markedly decreased in the cytoplasm and the nucleus of mature oocytes (Fig. 2d).

## *RPL10a* Sequence Analysis

Full-length *RPL10a* cDNA was obtained from the shrimp ovary subtraction cDNA library (Wonglapsuwan et al. 2009). The *RPL10a* cDNA clone was 669 bp, producing a deduced polypeptide of 217 amino acids (GeneBank ™/EBI accession number FJ623402). The calculated molecular mass and p*I* were 25.7 kDa and 10.06, respectively. In addition, eight potential phosphorylation sites were detected at $S^{2}$, $S^{50}$, $S^{86}$, $S^{141}$, $T^{9}$, $T^{52}$, $T^{139}$, and $Y^{11}$ (Fig. 3).

Comparison of the deduced RPL10a sequence from *F. merguiensis* with the RPL10a sequence from other organisms is shown in Fig. 4. RPL10a of *F. merguiensis* shares sequence similarities with other organisms, both invertebrates and vertebrates. For example, *F. merguiensis* RPL10a shares 78% and 70% identities with *Brugia malayi* and *Homo sapiens* RPL10a, respectively. Furthermore, the highly conserved sequence of the nuclear localization signal, PQKDKRF, was present in all organisms. The RPL10a of *F. merguiensis* was judged to have been derived from the same ancestor as *Caenorhabditis elegans* and *B. malayi* (Fig. 5).

## Expression and Purification of Recombinant *RPL10a*

The cDNA encoding *RPL10a* was amplified by PCR with the specific primers containing restriction sites for *Bam*HI and *Eco*RI to facilitate insertion into the pET-28a(+) expression vector. The recombinant plasmid (*pET-RPL10a*) was transformed into *E. coli* BL21 (DE3) and expressed after IPTG induction. A cell lysate was analyzed via SDS-PAGE and Western blotting using anti-His monoclonal antibody and showed the presence of an expressed protein of about 30 kDa, consistent with the expected molecular weight of the fusion protein, His-RPL10a (Fig. 6a–c).

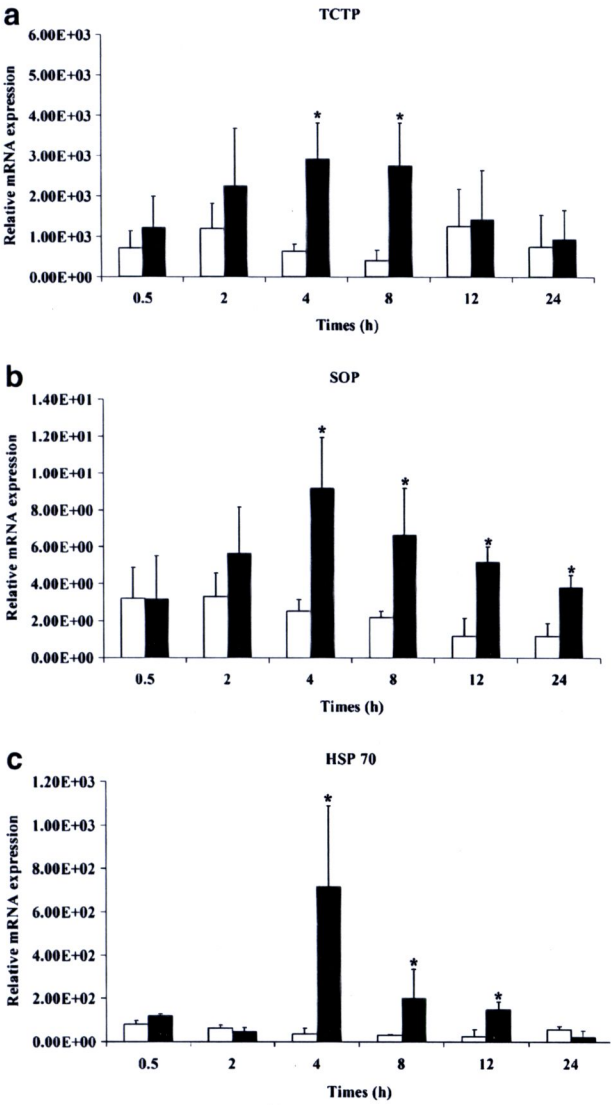


**Fig. 7** Effect of the recombinant RPL10a on the amounts of *TCTP*, *SOP*, and *HSP70* mRNA on shrimp's ovarian explants. The relative mRNA expression levels of **a** *TCTP*, **b** *SOP*, and **c** *HSP70* in the ovarian explants after incubating with the His-RPL10a at different periods of time. The expression of the genes were quantified by real-time PCR and the products were normalized to the expression level of the internal standard GAPDH (mean ± SD, $n=3$ and $p<0.05$). The *white bars* show the relative mRNA levels of genes in untreated ovarian explants. The *black bars* show the relative mRNA levels of the genes after treating the ovarian explants with recombinant RPL10a. The significant differences ($p<0.05$) of the relative mRNA levels of the genes are indicated by an *asterisk*

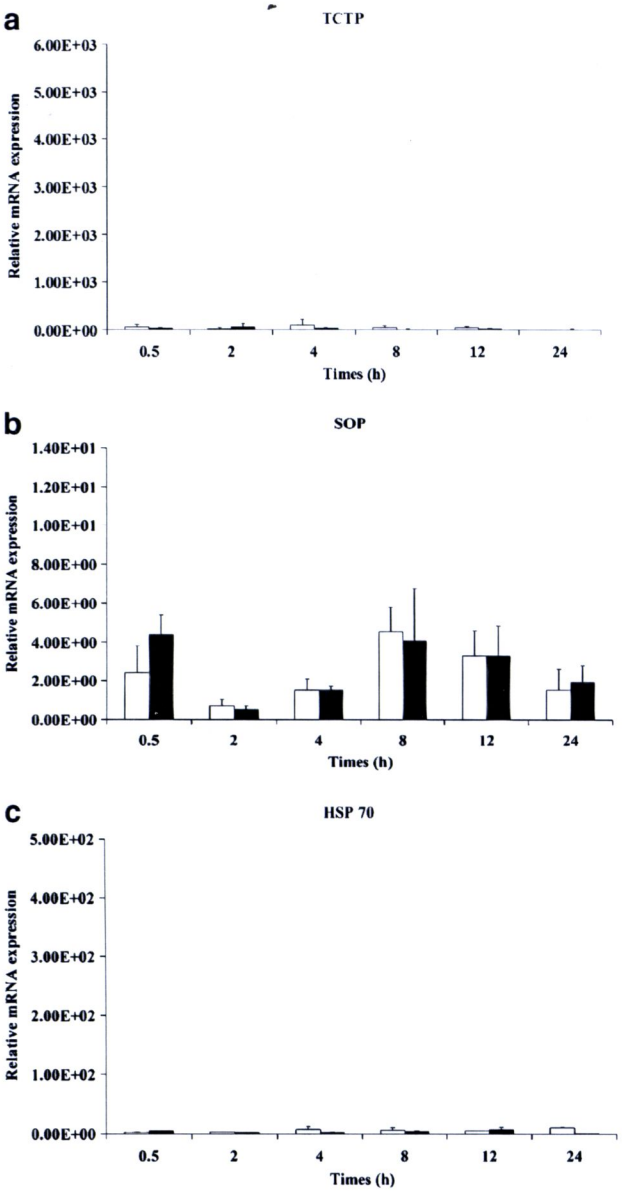


**Fig. 8** Effect of His-RPL10a on *TCTP*, *SOP*, and *HSP70* mRNA on shrimp skeletal muscle explants from the abdominal segment. The relative mRNA expression levels of **a** TCTP, **b** SOP, and **c** HSP70 in the muscle explant after incubating with His-RPL10a for different periods of time. The expressions were quantified by real-time PCR and the products were normalized to the expression level of the internal standard GAPDH (mean ± SD, $n=3$ and $p<0.05$). The *white bars* show the relative mRNA levels of the genes in the untreated muscle explants. The *black bars* show the relative mRNA levels of the genes after treating the muscle explants with His-RPL10a. Significant differences ($p<0.05$) of the relative mRNA levels of the genes are indicated by an *asterisk*

## Activity of the Recombinant His-RPL10a In Vitro

To elucidate the activity of His-RPL10a during the shrimp reproductive process, ovarian explants produced from females at an undeveloped stage of gonad maturation were used to study the molecular activity of His-RPL10a. His-RPL10a was incubated with explanted ovarian tissue at different periods of time and the mRNA level of various genes was determined. The expression of *TCTP*, *SOP*, and *HSP70* genes of the ovarian explants were determined by real-time PCR and no significant changes of *TCTP*, *SOP*, and *HSP70* transcript levels were detected after incubation with the His-RPL10a for 0.5–2 h. A significant increase of the *TCTP* transcript level was observed in the ovarian explants between 4 and 8 h after treating with His-RPL10a, then the expression levels decreased to about the same level as in the ovarian tissue without treatment with His-RPL10a after 12 h incubation (Fig. 7a). In addition, a significant increase of the *SOP* transcript level was found at 4 h to the end of the experiment (Fig. 7b), while an increase of *HSP70* was found at between 4 and 12 h after incubation (Fig. 7c). Each of the three transcripts was seen to be at its maximum level 4 h after treatment with His-RPL10a. To confirm the specificity of His-RPL10a as a gonad stimulatory effect, muscle tissue was also treated with His-RPL10a. In this case, the expressions of *TCTP*, *SOP*, and *HSP70* genes were determined by real-time PCR and there was very little change of *TCTP*, *SOP*, and *HSP70* transcript levels at any point after the incubation (Fig. 8).

## Cell Viability of the Ovarian Explants

The release of LDH from cells was used to evaluate the cell viability of the ovarian fragment during the ovarian explant assays. Both the supernatant from untreated and treated ovarian explants with His-RPL10a showed no significant differences in the LDH specific activity at any point after incubation compared to the specific activity at 4 h after incubation. Furthermore, these activities were much lower than the LDH activity released from the positive control (cell death) at any point of incubation (data not shown).

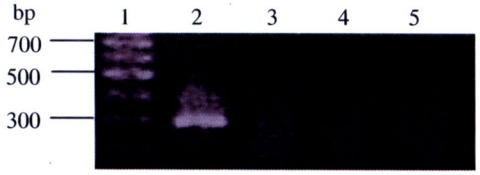


**Fig. 9** Quantitative PCR of vitellogenin gene expression in ovaries after incubated with RPL10a protein. The PCR products were separated on 1.2% agarose gel by electrophoresis and detected with ethidium bromide. *Lane 1* standard DNA marker, *lane 2* positive control, *lane 3* PCR products from incubated ovary in M199 medium without RPL10a protein, *lane 4* PCR products from incubated ovary in M199 medium with RPL10a protein, *lane 5* negative control

## Discussion

In the hope of developing a method for controlling the quality and quantity of shrimp and its eggs in aquaculture, many researchers have been attempting to clarify ambiguities concerning the molecular mechanisms involved in ovarian development. In our previous study, using suppressive subtractive hybridization, many genes associated with the ovary became highly expressed during ovarian maturation (Lo et al. 2007; Loongyai et al. 2007; Wonglapsuwan et al. 2009). *RPL10a* that encodes for ribosomal protein L10a is one of those genes. Several evidences have indicated that the ribosomal protein L10a may be involved in cell proliferation such as by playing a role in embryogenesis and organogenesis (Fisicaro et al. 1995; Kubiczek et al. 1997), downregulating in the thymus of mice after treating with an immunosuppressive drug (Fisicaro et al. 1995), and as a tumor antigen (Koga et al. 2003). In a previous study, we have shown that *RPL10a* mRNA was highly expressed only in the ovaries and lymphoid tissue during the vitellogenic state of the shrimp while its level was low in the brain, heart, intestine, and hepatopancreas of the undeveloped ovarian shrimp (Wonglapsuwan et al. 2009). We, therefore, suggest that RPL10a may be involved in the proliferation and development of the shrimp ovarian cells. Moreover, the highest *RPL10a* expression was in the early vitellogenic stage of the shrimp (Wonglapsuwan et al. 2009) and this was confirmed by the localization of the RPL10a protein using an immunohistochemical technique with anti-RPL10a antibody. Additionally, the RPL10a protein was actively synthesized in the cytoplasm and deposited in the nucleus in the developing stages. This result also suggested that the synthesized RPL10a protein translocated from the cytoplasm into the nucleus as it demonstrated the intranuclear localization signal.

Sequence analysis showed five phosphorylation sites in RPL10a. While the recombinant RPL10a produced in *E. coli* was able to activate the expression of the *TCTP*, *HSP70*, and *SOP* genes in the shrimp ovarian cells. So it is unknown whether phosphorylation of the recombinant RPL10a occurred inside the cells or not required to activate the expression of the *TCTP*, *HSP70*, and *SOP* genes in the shrimp ovarian cells. The high conservation among several species of both invertebrate and vertebrate as shown by the amino acid alignments indicated that the *RPL10a* gene is important in organisms and can be used for evolutionary studies in the future. Interestingly, the phylogenetic tree indicates that *F. merguiensis* is more closely related to worms such as *C. elegans* and *B. malayi* than to an insect such as *D. melanogaster*.

The explants culture method has been used by many research workers to study the effects of proteins or hormones on tissues. Examples include a study of the reproductive functions of a gonad-stimulating hormone from *M. ensis* (Shirley and Siu 2007) and observations on the hormonal induced ovarian growth in the crab *Chasmagnathus granulate* (Zapata et al. 2003). Therefore, we used the explant culture method to study the effects of the His-RPL10a protein on the ovarian maturation of shrimp. The results showed that recombinant His-RPL10a can stimulate the expression of *TCTP*, *SOP*, and *HSP70* genes which are those that were highly expressed during early vitellogenesis. *Vn*, however, was not stimulated in this experiment (Fig. 9). Thus, RPL10a may be involved in the early steps of shrimp ovarian maturation. Additionally, this study showed that the His-RPL10a did not stimulate the expression of *TCTP*, *SOP*, and *HSP70* in the muscle tissues and, therefore, indicated that the stimulation of *RPL10a* may be specific to the ovarian cells. Furthermore, addition of His-RPL10a to ovarian explant cultures showed no cytotoxicity to the cells during the experiment as indicated by the LDH assay.

In conclusion, we have characterized the *RPL10a* gene and showed the genetic-related species and the localization signal of the RPL10a. This report has also indicated that His-RPL10a was able to induce the expression of genes in the early step of ovarian maturation. This finding could be an important starting point for improvements in ovarian development in shrimp aquaculture.

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