Characterization of a *Vasa*-Like Gene From the Pacific White Shrimp *Litopenaeus Vannamei* and Its Expression During Oogenesis

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ABSTRACT The *vasa* gene encodes an ATP-dependent RNA helicase belonging to the DEAD-box family that, in many organisms, is specifically expressed in germline cells throughout the life cycle. In this study we first cloned Pacific white shrimp (*Litopenaeus vannamei*) partial cDNAs of two members of the DEAD-box family, one belonging to the *vasa* subfamily (*Lv-Vasa*) and the other to the *PL10* subfamily (*Lv-PL10*). Examination of their spatial expression pattern in adult tissues revealed that *Lv-Vasa* is restricted to the gonads, whereas *Lv-PL10* is found in gonads as well as in somatic tissues. Next, we cloned the full-length shrimp *vasa* cDNA and found that *Lv-Vasa* encoded a protein with a DEAD-like helicase domain followed by a helicase superfamily C-terminal domain. In addition, *Lv-Vasa* encoded N-terminal three repeats of the C2HC-type zinc finger domain, a motif encoded by *vasa* genes of several crustaceans and several other invertebrate organisms. In situ hybridization of ovarian sections showed that the *Lv-Vasa* transcript is localized to the cytoplasm of the oocyte throughout oogenesis. The abundance of *Lv-Vasa* mRNA in mature oocytes suggests a maternal contribution for the developing embryo. It is demonstrated that the *vasa* homolog from *L. vannamei* is a gonad specific germline cell marker that could be exploited to enhance our understanding of developmental and reproductive processes in the germline of this economically important shrimp. *Mol. Reprod. Dev.* © 2006 Wiley-Liss, Inc.

Key Words: crustacea; decapoda; stem cells; germ cells

INTRODUCTION

In most animals, germ cells specialized to produce haploid gametes are specified during embryogenesis (Dixon, 1994; Mahowald and Wei, 1994; Wylie, 1999). In many, the germ cells are specified by germplasm deposited in the fertilized egg, which contains germ cell-specific cytoplasmatic determinants (reviewed in Eddy, 1975). This germplasm is inherited by only one or a few cells, namely the primordial germ cells (PGCs), which constitute the unique founder population for the germline (Extavour and Akam, 2003). In some animals, germ cell specification is achieved during embryogenesis: undifferentiated cells receive an inductive signal from neighboring tissues and respond by differentiating as germ cells (Tam and Zhou, 1996; Lawson et al., 1999; Ying et al., 2000; Ying and Zhao, 2001).

The *vasa* gene encodes a protein belonging to the DEAD-like helicase superfamily (Hay et al., 1988) (Interpro Accession number IPR011545). DEAD-box proteins are putative ATP-dependent RNA-helicases present in many investigated organisms, ranging from viruses to mammals (Schmid and Linder, 1992). Expression of Vasa-like DEAD-box proteins has been shown in PGCs of metazoans (reviewed in Mochizuki et al., 2001). *Vasa* transcript or proteins are localized in germ granules not only in species whose germline formation is driven by germplasm (Hay et al., 1988; Gruidl et al., 1996; Olsen et al., 1997; Yoon et al., 1997), but also in germ cells from other animals, such as mouse, in which they are specified by signal induction (Tanaka et al., 2000; Toyooka et al., 2000). Moreover, *vasa* genes were shown to be expressed in adult germline stem cells in *Drosophila* (Hay et al., 1988), *Hydra* (Mochizuki et al., 2001), and *Crassostrea gigas* (Fabioux et al., 2004a). Thus *vasa* is an excellent candidate for serving as general marker of PGCs and germline stem cells.

In crustaceans, *vasa* cDNAs have been cloned from three branchiopod species: water fleas *Daphnia magna*, *Moina macrocopa*, and brine shrimp *Artemia franciscana* (Sagawa et al., 2005). The use of specific antibodies against *Daphnia* Vasa protein revealed that the PGCs in this organism segregate at a very early cleavage stage of embryogenesis in parthenogenetic and sexual eggs (as early as the 8-cell stage of development). In another study on the amphipod crustacean *Parhyale hawaiensis* using Vasa as a marker, it was shown that localized germ cell determinants are found early at the two-cell stage (Extavour et al., 2005).

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The Pacific white shrimp *Litopenaeus vannamei* belongs to the eumalacostracan crustacean family and is the most important shrimp species in global aquaculture. However, there are no data available so far regarding the identity of germline stem cells or about the mechanism of germline development in shrimps. Gaining a better understanding of this process would have basic scientific and applied importance and would facilitate greater control of the reproduction process in *L. vannamei*.

In this study, we first cloned partial cDNAs of two types of DEAD-box gene homologs from *L. vannamei*. Based on sequence homology, these proteins were classified into two different DEAD-box subfamilies, namely *vasa* and *PL10*. The expression patterns of the proteins, termed *Lv-Vasa* and *Lv-PL10*, respectively, were analyzed in adult tissues by RT-PCR. *Lv-Vasa* was found to be restricted to the gonads whereas *Lv-PL10* occurred in all tissues tested. Next, we cloned the full length of *Lv-Vasa* mRNA and found that the deduced protein sequence contains a DEAD-like helicase domain and N-terminal three repeats of the C2HC-type zinc finger domain. Analysis of the localization pattern of *Lv-Vasa* mRNA during oogenesis revealed that the mRNA is detected in oogonia and is highly abundant in late vitellogenic oocytes.

MATERIALS AND METHODS

Animals

L. vannamei broodstock (about 30 and 43 g per male and female shrimp, respectively) were purchased from SIS-Shrimp Improvement Systems, Inc., Islamorada, FL. The shrimps were acclimated and grown in artificial ponds at Ben-Gurion University of the Negev. The shrimps were fed with an enriched diet at about 25% of the wet body weight. Water temperature was maintained at $29 \pm 1^{\circ}$C. Photoperiod was 14 hr light and 10 hr dark. During the experiment, the water was recirculated through a biofilter to maintain quality. Randomly chosen females and males were dissected on ice, and tissues were processed immediately in order to prevent RNA degradation.

Sequencing and Analysis of *Vasa* and *PL10* cDNA

Total RNA was isolated from several tissues of adult *L. vannamei* females using TRIZOL Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was generated with oligo$(d(T))_{18}$ as primers using M-MLV reverse transcriptase H minus (Promega, Madison, WI), according to the manufacturer's instructions. Degenerated primers Vasa-F (5′-ATGGCNTGYGCN-CARACNGG-3′) and Vasa-R (5′-GGCATRTCRTARTT-DATNACRTG-3′) were designed based on conserved Vasa protein sequences from *Drosophila*, *Caenorhabditis*, *Xenopus*, zebrafish, and mouse as described in Fabioux et al. (2004b). Amplifications were performed by the polymerase chain reaction (PCR) with 25 μl of reaction mixture containing 0.5 μM of each primer, 0.2 mM dNTPs, 1× PCR buffer, and 0.2 U of *Taq* DNA Polymerase (Sigma-Aldrich, Rehovot, Israel), under the following conditions: initial denaturation at 94°C for 3 min; followed by 40 cycles at 94°C for 1 min, 55°C annealing temperature for 1 min and 72°C for 1 min; ending with a final extension at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, and the observed DNA band was excised, purified (QIAquick Gel Extraction kit, Qiagen, Hilden, Germany) and cloned into a pGEM-T Easy Vector (Promega). Clones containing the inserts were isolated and allowed to grow overnight; plasmid DNA was purified (Qiagen Miniprep kit) and the inserts were sequenced by the dideoxynucleotide chain termination method.

5′- and 3′-Rapid Amplification of cDNA Ends (RACE) was carried out using the SMART RACE kit (BD Bioscience, San Jose, CA). The RT reaction for completing the 5′-end was done using an *Lv-Vasa* specific primer (5′-TACCAGGATATTGTGAAC-3′). Primers used for 5′- and 3′-end amplifications were: 5′-GGAATCCTGCAGCTC-CACCGTAAGCCAC-3′ and 5′-CGTGTGCACAAACGGG-GAGTG GGAAGAC-3′, respectively. PCR products were analyzed by electrophoresis as described above.

Tissue Distribution of Presumptive Shrimp *Vasa* mRNA

Tissue distribution of the shrimp *vasa* and *PL10* mRNA was investigated by RT-PCR using the following specific primers, for *vasa*-like gene; Vasa F1 (5′-CATAC TTGACAATAACTGCCCAAGC-3′) and Vasa R2 (5′-CAATTTTGCCCTTCTCAAGGAAGTCC-3′). For *PL10*-like gene the following primers were used; PL10F (5′-CCCCATCCTTAACCAGATATATG-3′) and PL10R (5′-GGGTGGCAACTAATAGGTGGCAGC-3′).

Sequence Comparisons and Domain Analysis

Pairwise sequence comparisons of the cloned sequences were done mainly by performing BLAST-based analyses. Domain analysis was performed using the Simple Modular Research Tool (SMART) database (Schultz et al., 1998; Ponting et al., 1999).

Phylogenetic Analysis

Full-length protein sequences were aligned using ClustalX (version 1.81, default parameters) followed by a minimum evolution analysis, with the generation of a bootsrapped consensus tree (N = 1,000). The analysis was performed using Mega 3.1 software (Kumar et al., 2004).

Histological Sample Preparation

Acetone freeze substitution of ovary, testis, and muscle tissue samples was performed at −70°C for 5 days. The acetone from each sample was replaced with fresh cold acetone every day. Tissue samples were fixed in modified Carnoy's II for 24 hr and then dehydrated gradually through a series of increasing alcohol concentration solutions. Tissues were cleared and embedded in paraplast (Paraplast Plus, Kendall, Tyco Healthcare, Mansfield, MA) according to conventional procedures. Sections of 5 μm were cut on to

silane-coated slides (Superfrost® Plus, Menzel-Gläser, Braunschweig, Germany). Sections were stained with hematoxylin and eosin for morphological examination.

In Situ Hybridization (ISH)

Digoxygenin (DIG)-labeled oligonucleotides for antisense and sense probes corresponding to 903–1,275 nt of the cloned *vasa* cDNA were synthesized using in vitro RNA transcription SP6 and T7 RNA polymerases. Slides containing sections of the ovary and muscle were deparaffinized, rehydrated and rinsed in diethyl pyrocarbonate (DEPC)-treated doubly distilled water. Slides were fixed in 4% paraformaldehyde in PBS (10 mM phosphate buffer Na_2HPO_4, 150 mM NaCl, pH 7.4) for 4 min and washed twice in fresh PBS, then incubated in PBS containing 0.1% DEPC for 20 min. Samples were digested with 5 μg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) in 100 mM Tris-HCl 50 mM EDTA, pH 7.6, for 10 min at 37°C, incubated in PBS containing 2 mg/ml glycine for 10 min, and washed twice in PBS for 3 min. Slides were prehybridized for 2 hr at 42°C in hybridization buffer (50% formamide, 2.25 × saline sodium citrate (SSE), 10% dextran sulfate, 2.5 × Denhardt solution, 5 mM dithiothreitol, 40 U/ml RNAse inhibitor, and 0.1 mg/ml sheared and denatured salmon sperm DNA). Overnight hybridization was performed with 0.2 μg/ml antisense and sense probes in hybridization buffer (identical to prehybridization buffer). The slides were washed with 3 × SSC at room temperature for 5 min, and then washed in NTE buffer (500 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The slides were then incubated in NTE buffer containing 50 μg/ml RNAse-A for 30 min at 37°C, washed in 2 × SSC at room temperature, and incubated for 1 hr in 0.1 × SSC at 57°C. Thereafter the slides were washed with Tris-HCl buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 5 min at room temperature and blocked with blocking buffer (1% blocking reagent in Tris-HCl buffer; Roche Diagnostics) for 30 min at room temperature. Slides were finally incubated with a diluted (1:2,000) anti-DIG-AP conjugate (Roche Diagnostics) for 2 hr at room temperature. DIG was visualized with colorimetric substrates NBT/BCIP (Roche Diagnostics) according to the manufacturer's instructions. Slides were mounted and observed under a light microscope.

RESULTS

Isolation of *L. vannamei vasa* and *PL10* Ortholog Partial cDNA

A single fragment of ~400 bp was amplified from vitellogenetic female ovaries by RT-PCR with the degenerated primers Vasa-F and Vasa-R. This fragment was cloned and analyzed by DNA sequencing. Two transcripts were found and their amino acid sequences as predicated from the DNA sequences were compared with those of various known DEAD-box proteins. The comparison revealed that one of the transcripts (termed *Lv-Vasa*) showed 60% identity and 75% similarity to the *vasa* subfamily while the second (termed *Lv-PL10*, GenBank accession number: DQ095773) showed 75% identity and 85% similarity to the *PL10* subfamily.

Spatial Expression of Shrimp *Vasa* and *PL10* Orthologs

The expression patterns of the presumptive shrimp *vasa* and *PL10* genes were analyzed in several tissues by RT-PCR (Fig. 1) using specific primers for each gene. Our results show that whereas the *PL10* gene was detected in all tissues tested (muscle, hepatopancreas, eyestalk, ovary, and testis), a high level of *vasa* transcript was detected only in ovaries and testis.

Isolation of Full-Length Shrimp *Vasa* Ortholog cDNA

Full-length Shrimp *vasa* cDNA (2,762 nucleotides) was generated by 5′- and 3′-RACE reactions; the cDNA deduced protein sequence has been submitted to GeneBank (GenBank accession number: DQ095772). The full-length gene product is shown in Figure 2. Lv-Vasa harbors a DEAD-like helix superfamily domain and helicase superfamily C-terminal domain, as is typical of all known interspecies *vasa* sequences. Additionally, *Lv-Vasa* encodes within its amino-terminal region three CCHC-zinc finger domains, typically involved in RNA or single stranded DNA binding (Interpro accession: IPR001878). Such Zn-finger motifs have recently been discovered in other crustacean species (Sagawa et al., 2005).

Phylogenetic analysis demonstrates that Lv-Vasa does indeed segregate within the Vasa family of orthologs and does not cluster with the related proteins P68 or PL10 (Fig. 3). Within the Vasa clade of sequences, Lv-Vasa clearly clusters most closely to that of the gene products encoded by other crustacean species. Thus the phylogenetic data analysis provides additional evidence regarding the identity of the gene in question as *vasa* gene in *L. vannamei*.

Analysis of *Vasa* mRNA Localization in Ovaries by In Situ Hybridization

Overview of an ovary from mature (30 g) *L. vannamei* female (Fig. 4A) shows germinal layers of oogonia (Oog) in the center of the ovarian lobes and developed previtellogenic (Ooc), and secondary vitellogenic oocytes

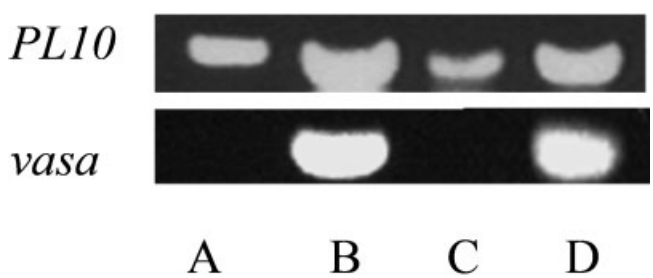

Fig. 1. RT-PCR analysis of *Lv-vasa* and *Lv-PL10* expression in tissues of adult female and male *Litopenaeus vannamei*. **A**, muscle; **B**, ovary; **C**, hepatopancreas; **D**, testis.

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  1 MSDDWDETDA APASDWNIES FGLPTSFGST KKTCNTGNAF NDGEGGFDEG
                                --- ZnF-C2HC-I  ---
 51 SQSNFDDPFR SGGGGFGGRG RGGPRACFKC GDEGHMARDC PSASDSRGNR

101 TNNRRQDNWG GGSSSKPANG EPFGFGSAFG DNQESDPFGA TESSGFGFGS
                  --- ZnF-C2HC-ii ---       --- ZnF-C2HC-iii
151 GSGSRGGRRN DGGRGCFKCG EEGHMSRDCP SGGGRNKGCF KCGQEGHNAR
     ---
201 DCPNPGEGSE EKKPRAPLYI PADVNEDELF VMGIEAGSNF DAYANVPANV

251 SGAEPIQPAA ESFQSMNLRP LLLENIVKAG YGCPTPVQKY TIPNVMNGRD

301 IMACAQTGSG KTAAFLLPML HYILDNNCPS NAFEEPAQPT GLVICPTREL

351 AIQIMREARK FSHSSVAKCC VAYGGAAGFH QLKTIHSGCH ILVATPGRLL

401 DFLEKGKIVF SSLKYLVLDE ADRMLDMGFL SSIKTVINHK TMTPTAERIT

451 LMFSATFPHE IQELASAFLN NYLFVVVGTV GAANTDVKQE VLCVPKFEKK

501 AKLVEMCEEI LISADDEKIL VFVEQKRVAD FVGTYLCEKK FRATTMHGDR

551 YQAQREQALS EFRTGVHNIL VATAVTARGL DIKGIGVVVN YDLPKDIDEY

601 VHRIGRTGRL GNRGLSISFY DDETDACLTK DLVKVLSEAN QTIPDWLTQK

651 ANASGHAQTY HGSGLFASSD IRSKNGGGRG WEKNQASSFL GGPSESNVDE

701 EWD
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Fig. 2. Deduced amino acid sequence of *Litopenaeus vannamei* Vasa. The *Lv-vasa* gene product of 703 amino acids length was deduced from the full-length cDNA sequence. Three ZnF-C2HC domain motifs, presumably involved in RNA binding, are indicated (bold and underlined). The DEAD-box motif (shaded in gray) and the helicase family C-terminal domain consensus (bold and dotted underline) are also indicated.

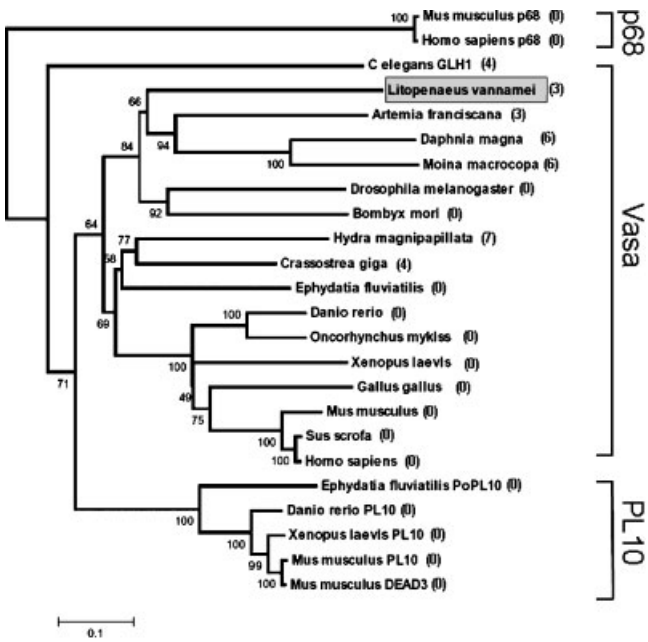

Fig. 3. Phylogenetic and zinc finger domain analysis of *vasa*-related proteins. Phylogenetic analysis was performed for a number of Lv-Vasa homologous protein sequences identified from different species. Lv-Vasa clearly co-segregates within the Vasa protein family, and clusters within a clade of crustacean *vasa* sequences including those from *Artemia franciscana*, *Daphnia magna* and *Moina macrocopa*. The C2HC-Zn finger domain is not universally encoded for all species. When encoded, a heterogeneous number of domain repeats can be observed (bracketed numbers). This phylogram was generated by a bootstrapped minimum evolution analysis and was rooted at its mid-point. The bootstrap values (from 1,000 trials) are represented as percentage scores (to the left of each node).

(Soc) located in the margins of the ovarian lobes. Single or grouped oocytes (Fig. 4F) arrayed in cysts are surrounded by follicle cells (Fcl), with the cysts separated by hemal sinuses (Sin). The nucleoli (Nuu) or aggregations of chromatin are located in the ovum nuclear border (Bell and Lightner, 1988). The cellular distribution of *vasa* transcript expression in the above ovary was determined by RNA ISH. Adjacent sections were probed with *vasa* sense and antisense probes. No signal was observed with the negative control sense-strand probe (Fig. 4B). Nor was any signal observed in peripheral tissues such as muscle when hybridized with antisense *vasa* probe (data not shown). The antisense probe gave a signal in the oogonia and in the cytoplasm of small oocytes and in the nucleoli located in the periphery of the nucleus (Fig. 4C–E). In the large oocytes (Soc) the antisense probe gave a strong signal both in the nucleoli and in the cytoplasm (Fig. 4F).

DISCUSSION

The *vasa* family of DEAD-box helicases is a conserved gene family whose expression is generally restricted to germ cells in all metazoans for which data are available (Mochizuki et al., 2001; Extavour and Akam, 2003). They are thought to have originated from a group of helicases constituting the *PL10* family, whose expression is found in both germ cells and pluripotent somatic stem cell types (Mochizuki et al., 2001). The *vasa* genes may have acquired a germ cell-specific role after their divergence from the *PL10* founder family (Mochizuki et al., 2001). In this study we first cloned partial cDNAs of two members of the DEAD-box family from the Pacific white shrimp (*L. vannamei*). Sequence comparison and spatial expression pattern analysis by RT-PCR implied that one of the cDNAs is the *vasa* homolog, whereas the other cDNA is the *PL10* homolog. Having decided to focus our analysis on the putative *vasa* homolog, we proceeded to extract its full-length sequence. Phylogenetic analysis confirmed the identity of the gene in question as *Lv-vasa* (Fig. 3). Besides the DEAD-like helix superfamily domain and helicase superfamily C-terminal domain found in all Vasa sequences, we detected an Lv-Vasa Zn-finger motif. The latter is not found in all species, but its probable function in RNA binding is in keeping with the general concept of the protein acting as an RNA helicase. The CCHC-Zn finger domain is encoded in other classes of organisms besides crustaceans, including *Caenorhabditis elegans* (Roussell and Bennett, 1993), oyster (Fabioux et al., 2004a), and *Hydra* (Mochizuki et al., 2001). The number of repeats of the CCHC-Zn finger also differs from species to species (enumerated in Fig. 4). Vertebrate and insect *vasa* sequences lack such Zn-finger domains. These data suggest that *vasa* may require the presence of such Zn-finger motifs for all species, but that in higher organisms the Zn-finger module has evolved and is encoded by an independent gene product.

Examination of the expression pattern of the *Lv-Vasa* gene by ISH revealed that it is specifically expressed in the adult gonad of female shrimp. No expression was

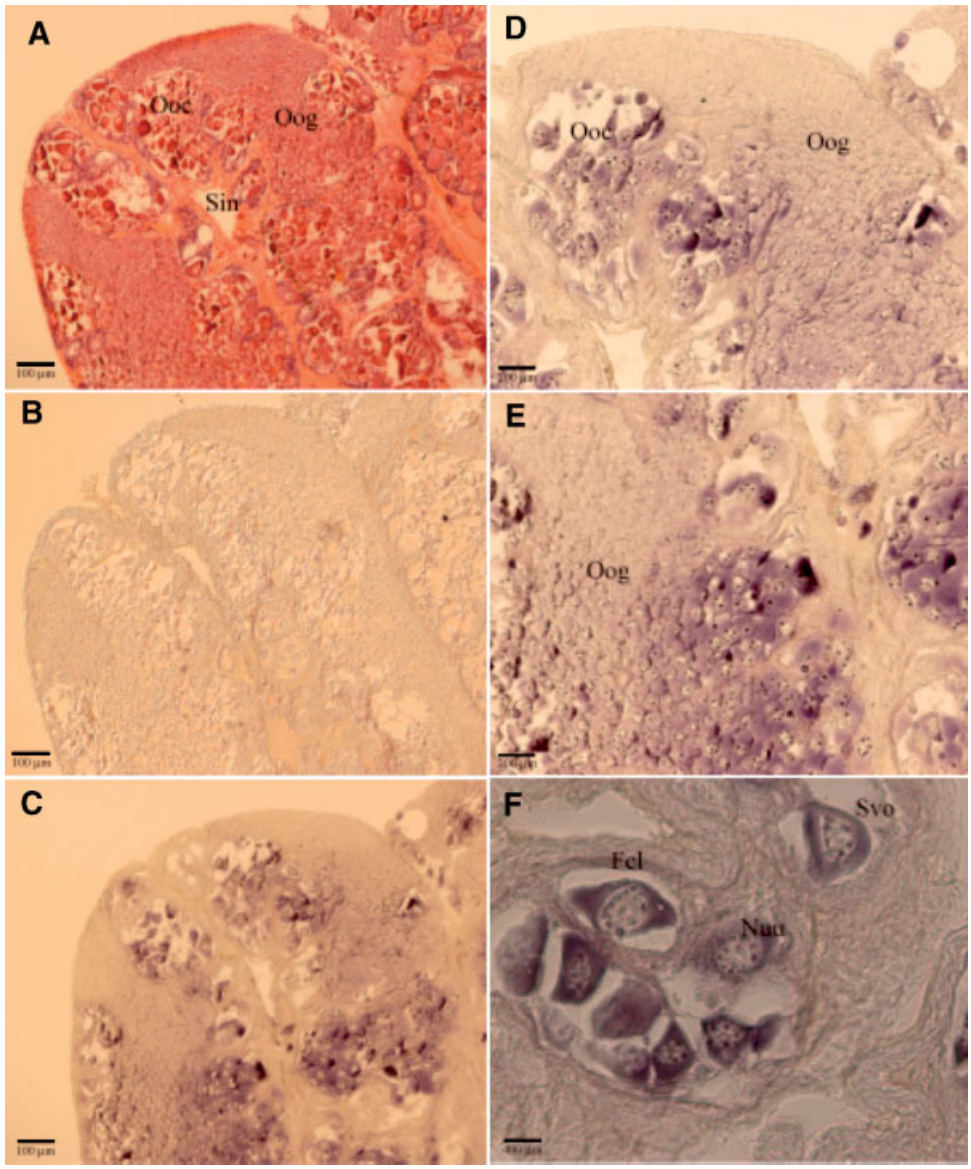

Fig. 4. Localization of *Vasa* expression by means of in situ hybridization. *Vasa* gene expression was examined in mature female ovaries during vitellogenesis. Hematoxylin and eosin staining (**A**), sense-*Vasa* probe (**B**), hybridization with antisense-*Vasa* probe (**C**–**F**). Oogonia (Oog), oocytes (Ooc), follicle cells (Fcl), sinuses (Sin), nucleoli (Nuu), secondary vitellogenic oocyte (Svo). Bar scale as indicated in the lower left side of the panels.

detected in somatic tissue within the ovaries (e.g., follicle cells, muscle, and connective tissues). These experiments confirm that in shrimp, as in other organisms examined to date, *vasa*-related genes are specifically expressed in germline cells (Hay et al., 1988; Lasko and Ashburner, 1988; Fujiwara et al., 1994; Komiya et al., 1994; Olsen et al., 1997; Nakao, 1999; Castrillon et al., 2000; Kobayashi et al., 2000; Mochizuki et al., 2001; Fabioux et al., 2004a). In the ovaries, *vasa* transcript is first detected in oogonia cells. The localization of *vasa* transcript in oogonia cells suggest that the *vasa* gene is expressed in adult germline stem cells of *L. vannamei*, as was shown also in *Hydra* (Mochizuki et al., 2001), *Drosophila* (Hay et al., 1988), and oyster (Fabioux et al., 2004a).

It has been shown that in *C. elegans*, *Drosophila*, and *Xenopus* the eggs contain maternally inherited cytoplasmic machinery for the determination of germ lineages involving Vasa-family proteins. In mouse, however, although the Vasa protein homolog MVH was uniformly detected in the primary oocyte cytoplasm, a marked decrease was observed as follicle maturation proceeded, reaching an undetectable level in mature oocytes (Toyooka et al., 2000). In the present study, ISH analysis revealed the presence of *Lv-Vasa* transcript in the cytoplasm of developing oocytes throughout shrimp

oogenesis. Therefore, it is possible that Lv-Vasa plays a role as a maternal factor for the determination of germ lineages.

In conclusion, our results demonstrate that the *Lv-Vasa* gene is specifically expressed in male and female gonads of *L. vannamei*. In females, this gene is expressed during late oogenesis, suggesting a possible maternal contribution. All together, we believe that *Lv-Vasa* can serve as a useful marker for analyzing germ cells development in shrimp.

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