

The site of vitellogenin synthesis in Chinese mitten-handed crab *Eriocheir sinensis*

Kang Li¹, Liqiao Chen^{*}, Zhongliang Zhou, Erchao Li, Xiaoqin Zhao, Hui Guo

Department of Biology, East China Normal University, Shanghai 200062, PR China

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Abstract

The objective of this study was to investigate the synthesis site of vitellogenin in the Chinese mitten-handed crab, *Eriocheir sinensis*. Using the RT-PCR techniques, the cDNA fragments isolated from the ovaries of vitellogenic female crab, we found that its deduced amino acid sequence had a high identity with that from other decapods crustacean vitellogenin. This cDNA fragments were used as probes to examine the transcription of mRNAs encoding the Vg. The mRNA expression was observed in vitellogenic female hepatopancreas, which was not detected in any other tissues including muscle, heart, and subepidermal tissues. The positive immunocytological staining with antibody against vitellin were found in ovaries and hepatopancreas of vitellogenic female, which was determined by immunological and immunohistochemical techniques. These results suggest that both ovaries and hepatopancreas are capable of synthesizing vitellogenin. Therefore, it was concluded that hepatopancreas is the extraovarian site of vitellogenin synthesis in *E. sinensis*.

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Keywords: Crustacean; *Eriocheir sinensis*; Genomic DNA; Hepatopancreas; Synthesis site; Vitellin; Vitellogenesis; Vitellogenin gene

1. Introduction

Vitellogenesis is a decisive period in the female reproductive cycle of crustacean characterized by the accumulation of yolk and the formation of mature oocytes within the ovary. The main yolk protein, which is called vitellin (Vn), is composed of carbohydrate, phospholipid, carotenoid components and protein (Pateraki and Stratakis, 1997; Chen et al., 2004). It is well established that vitellogenin (Vg) is one of the precursors of Vn in crustaceans. It has been hypothesized that Vg is synthesized by extraovarian tissues, and then transported via hemolymph to developing oocytes where it is incorporated into yolk (Meusy, 1980).

Understanding vitellogenesis processes of crustacean is important because of the growing aquaculture industry and interests in new models for basic research. A large number of

methods, including biochemistry, immunology and molecular (Chen and Chen, 1994; Lee and Chang, 1999; Chen et al., 1999; Yang et al., 2000; Tseng et al., 2001; Garcia-Orozco et al., 2002; Okuno et al., 2002), have been used for understanding the vitellogenesis processes. In isopods, the biosynthesis of Vg occurs in the adipose tissue, and is then transported by hemolymph for deposit in oocytes (Picaud, 1980; Suzuki, 1987). The results of several studies on decapod crustaceans indicate that the Vn synthesis is endogenous or/and exogenous. Some species are capable of synthesizing Vg in ovaries (Lui and O'Connor, 1976; Browdy et al., 1990; Fainzilber et al., 1992; Lee and Watson, 1995), and in others, Vn is synthesized by the hepatopancreas (Paulus and Laufer, 1987; Lee and Chang, 1999; Chen et al., 1999; Soroka et al., 2000; Yang et al., 2000). Several crustaceans can produce Vg in both locations (Paulus and Laufer, 1982; Shafir et al., 1992; Sagi et al., 1995; Tsutsui et al., 2000). In *Eriocheir sinensis*, previous histology studies suggested that the oocytes produced endogenous Vg and also absorbed exogenous Vg (Du et al., 1999). However, few studies have been done on the vitellogenesis of *E. sinensis*, especially the site of Vn and Vg synthesis.

^{*} Corresponding author. Tel./fax: +86 21 62233637.

E-mail address: lqchen@bio.ecnu.edu.cn (L. Chen).

¹ Current address: Shanghai Academy of Public Measurement, Shanghai, 201206, PR China.

The objective of this study is to locate Vg mRNA in vivo by reverse transcription (RT) and polymerase chain reaction (PCR) and immunohistochemical staining the Vn-immunoreactive proteins in ovary and hepatopancreas to understand the site of Vg synthesis in *E. sinensis*.

2. Materials and methods

Adult Chinese hand-mitten crabs, *E. sinensis*, were collected from pond cultures in aquaculture farm in Shanghai. The vitellogenic females were determined by the ovarian development stages according to Xue et al. (1987). Tissues from both males and females were used for RNA isolation and subsequent verification of the Vg synthesis site in vivo. Hemolymph was collected by withdrawing from the sinuses at the base of the third walking legs with a syringe and mixed (1:2) with anticoagulant containing 1.4% Na₂HPO₄, 1.3% KH₂PO₄, 0.3% EDTA, 2% dextrose, and 0.25% sodium citrate, after centrifuged at 4000 ×g for 10 min at 4 °C and stored at –80 °C.

Total RNA was isolated from the ovary, hepatopancreas and other tissues (muscle, heart and subepidermal tissue) of vitellogenic female crab. The tissues were homogenized in Trizol reagent (GibcoBRL, USA) and purified according to the manufacturer's instructions. Purified RNA samples were diluted at about 1 mg/mL for RT-PCR or stored at –80 °C.

Genomic DNA was isolated from 100 mg testis tissue of the male crab by homogenization in 100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate. The homogenate was then treated with 0.1 mg/mL of proteinase K (Sigma-Aldrich, USA) at 65 °C for 2–4 h then transferred to 4 °C for 12 h. Successive phenol and chloroform–isoamyl alcohol extractions were performed followed by ethanol precipitation, and DNA pellets were resuspended in 100 μL of 10 mM Tris–1 mM EDTA (pH 8.0), and then stored at 4 °C until use for PCR.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with total RNA isolated from the crabs. First-strand cDNA synthesis was performed using a AMV reverse transcriptase kit (Promega, USA) to transcribe poly A RNA with oligo(d T) as primer. The reaction conditions recommended by the manufacturer were followed. The cDNA was used for the second-strand synthesis and subsequent amplifications. Both genomic DNA and cDNA from vitellogenic female crab were used as templates in the PCR.

Degenerate primers for the RT-PCR were designed according to two highly conserved domain sequences of crustacean Vg: forward, 5'-AAG GC (T/A) CT (G/T) GG (T/C) AAC ATG GG-3' (VF) and reverse, 5'-GGA GC (A/G) TAG ATG ATG TT (G/C) GA-3' (VR). The specific primers, sVP (5'-GAT GTG GCT GAG GTG CTG AT-3') and sVR (5'-AAA GGT CAA TGT TGC GGG AG-3'), which were designed on the basis of the nucleotide sequences of putative Vg fragment. PCR reactions contained 10 mM Tris–HCl buffer (pH 9.0), containing 10 mM KCl, 8 mM (NH₄)₂SO₄ and 0.5% NP-40, 2.0 mM MgCl₂, 1 U Taq polymerase (Bioasia, China), 0.25 mM dNTPs, and 1 μM primers. PCR conditions used for cDNA amplification with degenerate primers (VF and VR) were: denaturation at 95 °C

for 5 min; 30 cycles of denaturation at 94 °C for 1 min; annealing at 42 °C for 1 min; elongation at 72 °C for 2 min; and then followed by a 10 min extension at 72 °C and cooling to 4 °C. The PCR with specific primers (sVF and sVR) was accomplished by the following program: denaturation at 95 °C for 5 min; 30 cycles of denaturation at 94 °C for 45 s; annealing at 60 °C for 30 s; and elongation at 72 °C for 30 s; followed by 72 °C extension for 10 min and cooling to 4 °C.

The housekeeping gene beta actin of crab was used as an internal standard. The primers for RT-PCR in the present study, which were designed according to a highly conserved domain sequences of crustacean beta actin (Genbank accession no: AY910691), were as follows: actF 5'-CCT CCG GTC GTA CCA CTG GTA T-3' and actR 5'-CCA CGG AAG GTC TCA TTG CCG ATC GTG-3'.

PCR products were excised from the 1.0% agarose gels, followed by purification with a DNA gel extraction kit (Beyotime, China). The purified fragments were sequenced in both directions on a DNA sequencer. Sequence confirmation and amino acid translations were performed using the Vector NTI suite 8.0.

Vn purified from mature ovarian extract of *E. sinensis* by salt precipitation and further with Sephacryl S-300 HR column, was used to prepare rabbit antiserum (Chen et al., 2004). The tissues were homogenized in phosphate buffer saline (PBS, 10 mM sodium phosphate, pH 7.7, 0.01% EDTA, 0.1 M NaCl, 0.1 mM PMSF), and centrifuged at 12,000 ×g for 30 min at 4 °C. The supernatants were used for immunodiffusion in a 1% agar gel plate (2 mm thickness) developed according to the methods of Uchterlony and Nilsson (1978).

Ovary and hepatopancreas were fixed with Bouin's fixative, embedded in paraffin, and sectioned at 6 μm. The sections were stained with hematoxylin and eosin. For immunohistochemical examination, sections were rehydrated through xylene, absolute ethanol, 70% ethanol, 30% ethanol and distilled water. After inactivation of endogenous peroxidase activity with 3% H₂O₂ solution and then blocked in 3% BSA dissolved in PBS, tissue sections were incubated first with antibody to vitellin (1:2000 dilution) for 60 min, while negative control with PBS and then washed with PBS containing 0.5% Tween-20 (PBST) and PBS alone. Horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma-Aldrich) and substrate solution containing 0.5 mg/mL 3,3'-diaminobenzidine (DAB), 0.3% hydrogen peroxide in

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| C qu (475) | A | T | T | L | K | A | L | G | N | M | G | V | M | P | P | A | V | A | T | S | V | L | R | C | M | G | T | E | G |
| M en (476) | E | I | V | A | L | K | A | L | G | N | M | G | V | V | P | P | A | V | I | S | A | A | T | C | T | E | E | H | E |
| P se (476) | V | A | L | I | F | K | A | I | G | N | M | G | V | T | P | A | V | T | R | A | A | V | H | C | T | E | E | E | E |
| L va (476) | V | P | L | A | F | K | A | I | G | N | M | G | V | T | P | A | V | T | R | A | A | V | Q | C | T | E | E | E | E |
| C qu (631) | A | V | G | A | S | L | E | S | N | I | I | Y | A | P | G | F | P | R | S | I | D | F | N | L | T | A | A | L | |
| M en (632) | E | G | A | D | V | E | S | N | I | I | Y | A | P | G | A | F | V | P | R | L | T | M | N | L | A | I | I | I | |
| P se (632) | F | G | G | A | S | L | E | S | N | I | I | Y | A | P | G | F | P | R | A | V | N | I | N | M | A | D | V | I | |
| L va (632) | F | G | G | A | S | L | E | S | N | I | I | Y | A | P | G | F | P | R | A | V | N | I | N | M | R | A | T | V | |

Fig. 1. Amino acid sequence alignments of two vitellogenin fragments from various animal species: *Cherax quadricarinatus* (C qu) (AAG17936.1, Abdu et al., 2002), *Metapenaeus ensis* (M en) (AAM48287.1, Chan, S.M. and Tseng, W. Z.), green tiger shrimp *Penaeus semisulcatus* (P se) (AAL12620.3, Avarre et al., 2003), Pacific white shrimp *Litopenaeus vannamei* (L va) (AAP76571.2, Parnes et al., 2004). Forward and reverse primers employed were designed, based on the sequences indicated by the solid and broken lines.

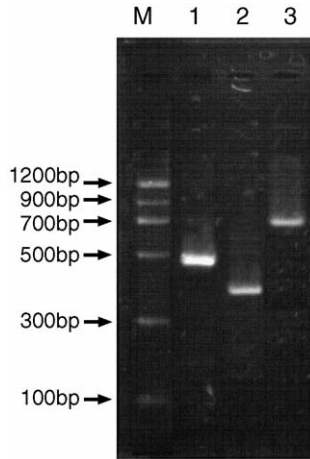


Fig. 2. PCR products obtained from genomic DNA and cDNA of *E. sinensis*. M: marker, Lane 1: PCR product of female ovarian cDNA with primer VF and VR, Lane 2: PCR product of female ovarian cDNA with primer sVF and sVR, Lane 3: PCR product of genomic DNA with primer sVF and sVR.

60 mM Tris buffer (pH 7.7) were applied on sections in sequence. Eventually, counter-staining with hematoxylin was performed after immunostaining. Proteins, which were immunologically identical to Vn, were stained as brownish precipitates and examined microscopically.

3. Results

3.1. PCR degenerate primer design and cDNA amplification

Two sets of degenerate primers for the RT-PCR were designed by comparing the conserved amino acid and nucleotide sequence of known crustacean Vg sequences. The alignment revealed two specific regions with a high degree of amino

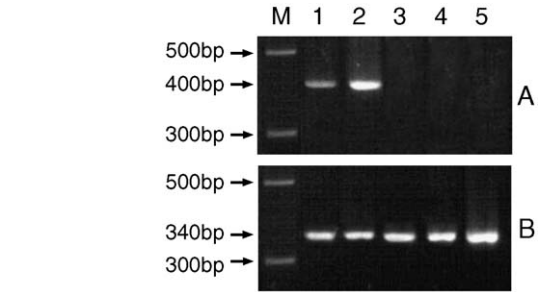


Fig. 4. Vitellogenin gene expression in the tissue of vitellogenic female *E. sinensis*. Amplified cDNA fragments of vitellogenin (A) and beta actin (B) were produced by RT-PCR using 30 cycles. M: marker, 1: ovary, 2: hepatopancreas, 3: subepidermis, 4: muscle, 5: heart.

acid sequence conservation (Fig. 1). Degenerate primers were designed according to two highly conserved domain sequences of crustacean Vg, KSLGNMG and SNIYYAP.

Total RNA isolated from the ovary of vitellogenic crab was used as the template for the first-strand cDNA synthesis in RT and then used for PCR, and the DNA was electrophoresed on 1.2% agarose gel (Fig. 2). The fragment was estimated to be 500 bp from the mobility on agarose gel compared with that of a ladder marker. The nucleotide sequences of the products were determined to be 477 bp. BLAST of the deduced amino acids from *E. sinensis* on EMBL and the identity between these sequences was calculated. The deduced amino acid sequence of the *E. sinensis* Vg (Genbank accession no: AY910692) is the closest to that of crab *Charybdis feriatus* (69% identity, AY724676), followed by the deduced amino acid sequences of the crayfish *Cherax quadricarinatus* (60% identity, AF306784), green tiger prawn *Penaeus semisulcatus* (56% identity, AY051318), Kuruma prawn *Penaeus japonicus* (55% identity, AB033719), coonstriped shrimp *Pandalus hypsinotus* (55% identity, AB117524), Pacific

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      D V A E V L I R Y M H S E Q K K V N N R
1  GATGTGGCTGAGGTGCTGATCCGCTACATGCACAGCGAGCAAAAAGTGAACAACCGT
      V A A V Q A F R L S K C N R E
61 GTGGCAGCTGTACAAGCCTTTAGGTTGTCCAAGTGTAACCGCGAGGTAAGTGTGTCTTTC
      ACCTTCTTCGCTACCACAGGACAACCATGACTTAGTCAGGAAAGCCTTCATCATTTCTCT
      V T Q K L V D F
181 CAGTCATATTTGTCCCTATATTTGTGTTTGAACAGGTGACGACAGAACTCGTCGACTTTG
      A V R P G K N T E V R I A A Y L T A L R
241 CCGTCCGACCCGGGAAAAACACGGAAGTTAGAATTGCTGCCTATCTGACAGCACTTCGCT
      C V D Y E D L Q T I V T K I S V E E N T
301 GTGTAGACTATGAGGACCTGCAGACCATTTGTGACAAAAGATCTCCGTTGAGGAAAAACGCG
      Q
361 AGGGTGAGTCGTATAGCGCACTGCCGTGGGAGAGAGGTGACTAACACTGAGTGGCCTAAT
      ATTCGTAATTTGTCTTTTTTCCCTTGGTGGGTTTGTTTGAGTCACCCATGTGTTCTA
421
      TCGTGCTCCAGCACTGAAGGTGTTCCGGGGCTGGTCAGCATAATTTGTTATTTGTTTTCAT
      V R G F I L S H L L N L Q E T N A P E
541 TTCA GTTCGTGGGTTTATCCTGAGCCATTTGCTGAACCTGCAAGAGACCAACGCCCCGA
      K E R L R S L L T N I L I P D N F E R D
601 AAAGGAAAGGCTGCGCTCCCTGCTGACAAACATTTCTCATCCCGATAATTTTGTAGCGTGA
      L R K Y S R N I D L
661 TCTAAGGAAGTACTCCCGCAACATTGACCTTT
    
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Fig. 3. The nucleotide and deduced amino acid sequences of cDNA encoding a vitellogenin fragment of Chinese mitten-handed crab, *Eriocheir sinensis*. Locations of the forward and reverse primers are denoted, respectively, with solid and broken lines. Introns are in black reverse print.

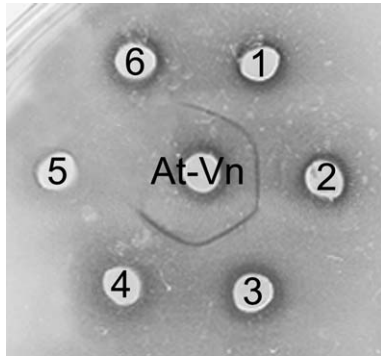


Fig. 5. Immunoprecipitation of hemolymph from vitellogenic female (1) and male (5), hepatopancreas extract of vitellogenic female (2) and male (6), ovarian extract (3), and purified vitellin (4) with an antiserum against vitellin (At-Vn).

white shrimp *Penaeus vannamei* (55% identity, AY321153), *Fenneropenaeus merguensis* (56% identity, AY499620) and sand shrimp *Metapenaeus ensis* (55% identity, AY530205). Subsequently the specific primers, designed based on the nucleotide sequences of this putative Vg fragment, were used for the successive RT-PCR.

The cDNA from the ovary of vitellogenic crab and the genomic DNA were used as templates and vSF and vSR as primers in the PCR. The lengths of the PCR products from the cDNA and genomic DNA were found around 400 and 700 bp, respectively (Fig. 2), and were determined finally to be 400 and 692 bp by

nucleotide sequence, respectively (Fig. 3). Two introns of 110 and 182 bp were found in this sequenced region of genomic DNA. Subsequently, using the first-strand cDNA from other tissues as templates in PCR, the product of 400 bp was found exclusively in hepatopancreas (Fig. 4).

3.2. Purification of vitellin and preparation of polyclonal antibodies

We purified and characterized vitellin of *E. sinensis* using gel filtration and polyacrylamide gel electrophoresis with different staining. Polyclonal antibodies were produced and western blotting demonstrated that these were specific against vitellin of *E. sinensis*. Details are giving in Chen et al. (2004).

3.3. Histology and Immunohistochemistry

The immunoprecipitation analysis revealed the presence of proteins recognized by the antibody against vitellin in the hemolymph, ovary, and hepatopancreas extracts from the females, which were immunologically identical with each other, but not in the hemolymph and hepatopancreas extract from the males (Fig. 5). The immunohistochemical examination of ovaries and hepatopancreas showed that the Vn-immunoreactivity was localized in vitellogenic oocytes present in the ovaries and the epithelial cells of the female hepatopancreas, but not found in the male hepatopancreas (Fig. 6).

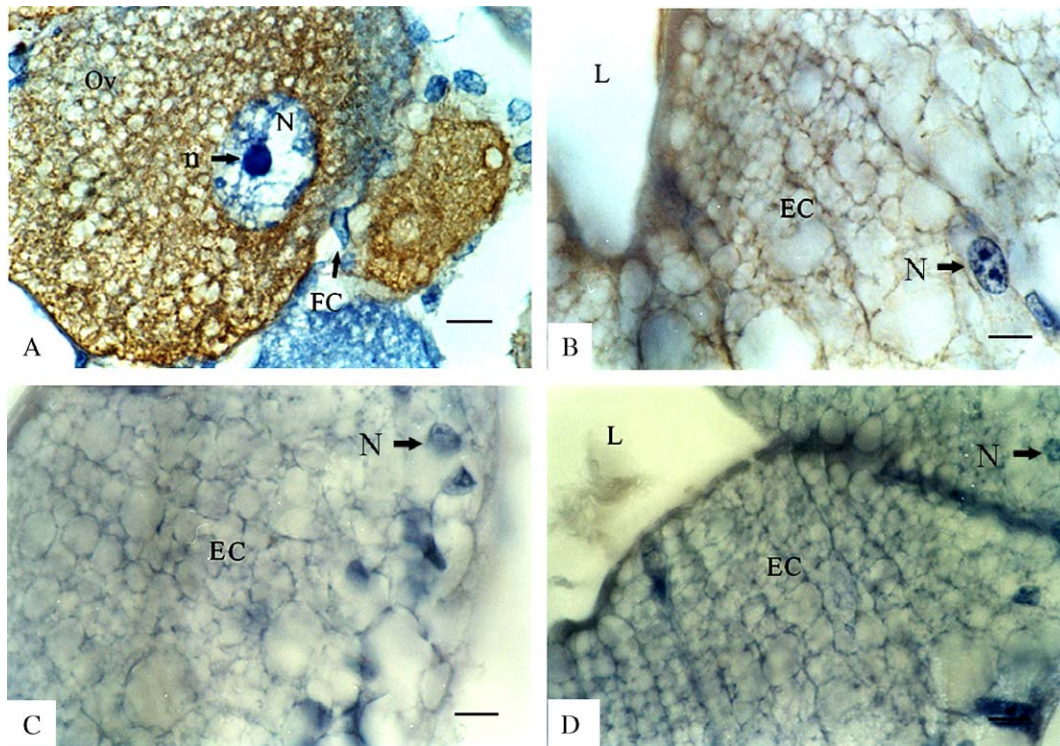


Fig. 6. Vn-immunoreactivity in ovaries (B) and hepatopancreas (D and F) of *E. sinensis*. Male (D), female (F) and negative control (E) of hepatopancreas and ovaries (B) were counter stained with hematoxylin. Ovaries (A) and female hepatopancreas (C) were also stained with hematoxylin and eosin. Abbreviations: follicle cell (FC), nucleus (N), nucleolus (n), vitellogenic oocyte (Ov), lumen of the digestive tubule (L), epithelial cells of the digestive tubule tract (EC). Bars indicate 50 μ m in A and 10 μ m in the others, respectively.

4. Discussion

Several Vg gene structures have been reported for crustaceans (Abdu et al., 2002; Avarre et al., 2003; Parnes et al., 2004). Generally, crustacean vitellogenins tend to be different from fish vitellogenins, although the amino acid sequence of Vg is highly conserved across decapods species. When designing our Vg primers, a region close to the C-terminus of the protein was selected where the most apparent conservation was present among sequences for shrimp and crayfish. The degenerate primers amplify a 477 bp fragment from *E. sinensis* ovarian RNA (Fig. 1). The size of the amplified *E. sinensis* fragment corresponds to the expected size based on decapods Vg sequences in the database. A BLAST search result shows that the amino acid sequence of the crab *E. sinensis* is highly similar to that of the other decapods crustacean. Two introns were found in the correspondent region of the genomic DNA, where the partial Vg gene structures from *Macrobrachium rosenbergii* (Chen et al., 1999), *Penaeus monodon* (Tseng et al., 2001) and MeVg1 from *M. ensis* (Tsang et al., 2003) possess identical exon–intron patterns. Although the fragment was too small to contain the entire coding sequence for Vg, it could be used in the studies of site of Vg synthesis.

To develop an effective tool to understand the site of Vg and Vn by measuring Vg mRNA expression in female crab, RT-PCR technique was used in this study. A potential problem of RT-PCR is genomic DNA contamination in the RNA preparation. This is particularly relevant when the target mRNA is expressed at a low level, which thus requires the amplified cDNA and amplified genomic DNA products to be differentiated. Therefore, the specific primers were synthesized based on the previously known segment sequences of *E. sinensis* Vg. Because the primers are located on the separate exons separated by two introns in Vg, RT-PCR products are obtained with introns spliced out. Thus, PCR products generated from contaminating genomic DNA are larger than products from cDNA separated by the agarose gel. In this study, using the same pair of primers vSF and vSR, the length of amplified product of the cDNA from the ovary of vitellogenic female is determined to be 400 bp, differing from that of the genomic DNA 692 bp. To avoid the interference from the RNA broken during the RNA preparation, the housekeeping gene beta actin of the crab was introduced as the internal standard. The single cDNA band could be obtained from sample without nonspecific bands using beta actin primers actF and actR.

Besides hemocytes, fat body adipose tissues, ovaries and hepatopancreas have been suggested as sites of Vg synthesis in crustaceans (Kerr, 1968; Tom et al., 1987; Browdy et al., 1990; Han et al., 1994; Rani and Subramoniam, 1997). However, discrepancy in the site determination was often encountered even in the same species, due to experimental protocols and animal conditions (Han et al., 1994; Chen et al., 1999; Yang et al., 2000). Immunohistochemical approaches and incorporating radiolabeled amino acid into proteins precipitable by antibodies raised against Vn in vitro or in vivo have often been employed to determine the synthesis site of Vg in crustaceans (Yano and Chinzei, 1987; Browdy et al., 1990; Shafir et al., 1992; Lee et al., 1996; Rani and Subramoniam,

1997). In *P. japonicus*, the ovarian tissues were reported to be capable of Vg synthesis as revealed by in vitro incubation of ovaries in the presence of labeled amino acids, but not the hepatopancreas (Yano and Chinzei, 1987). Immunohistochemical studies suggest that adipose tissue is a site for Vg synthesis (Vazquez-Boucard, 1985). However, Recent molecular studies show that the mRNA-encoding Vg is expressed in both ovary and hepatopancreas in the penaeid shrimp (Tsutsui et al., 2000).

Vg synthesis in vivo in the ovary and hepatopancreas is apparently a common phenomenon among penaeid shrimp (Browdy et al., 1990; Fainzilber et al., 1992; Shafir et al., 1992; Tseng et al., 2001). The mRNA expression of vitellins was detected in female hepatopancreas from *Macrobrachium rosenbergii*, but not in ovary, subepidermal tissue, gill, or muscle (Yang et al., 2000). In Brachyura decapods, the synthesis sites reported in previous studies include hemocytes in *Callinectes sapidus* (Kerr, 1968); ovarian tissues in *Pachygrapsus crassipes* (Lui and O'Connor, 1977), *Uca pugnator* (Eastman-Reks and Fingerman, 1985) and *C. sapidus* (Lee et al., 1996); hepatopancreas in *Scylla serrata* (Rani and Subramoniam, 1997), *Carcinus maenas* and *Libinia emarginata* (Paulus and Laufer, 1987); ovary and hepatopancreas in *Potamon potamios* (Pateraki and Stratakis, 2000).

In *E. sinensis*, the Vg synthetic site was suggested previously to be the ovary based on the use of electron microscope techniques (Du et al., 1999). However, these authors did not confirm that the expression of Vn in hepatopancreas or other relevant tissues. Multiple separate Vg genes were found in shrimp (Tsang et al., 2003), *Xenopus* (Wahli et al., 1981), chicken (Evans et al., 1988) and fish (Bowman et al., 2000). It is possible that different tissues express different Vg genes. The primers used in the RT-PCR analysis derived from one gene cannot amplify other Vg genes in other tissues. However, no information is available regarding whether similar gene organization for Vg occurs in the crab *E. sinensis*. In this study, RT-PCR results showed that mRNA fragments encoding the Vg were detected in the vitellogenic female ovary and the product of 400 bp was found in hepatopancreas, the sequence of which was identical with that found in ovary (Liqiao Chen, ECNU, personal communication). However, the expression of Vg gene could not be detected in other tissues including heart, muscle, or subepidermal adipose tissues (Fig. 4). Using the immunoprecipitation methods, the proteins, which were presumably considered to be Vg, could be recognized by antibody against vitellin in the ovary and hepatopancreas extracts from the vitellogenic females. These findings coincide with those from immunohistochemical examination of ovaries and hepatopancreas. Subsequently we conclude that both the ovary and hepatopancreas of *E. sinensis* are the sites of Vg gene expression. In other words, the hepatopancreas is an extraovarian Vg synthesis site in vivo in the crab targeted in this study.

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