Molecular Characterization of a cDNA Encoding Vitellogenin and Its Expression in the Hepatopancreas and Ovary during Vitellogenesis in the Kuruma Prawn, Penaeus japonicus

Authors: Tsutsui, Naoaki, Kawazoe, Ichiro, Ohira, Tsuyoshi, Jasmani, Safiah, Yang, Wei-Jun, et al.

Source: Zoological Science, 17(5) : 651-660

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.17.651

ZOOLOGICAL SCIENCE **17**: 651–660 (2000)

Molecular Characterization of a cDNA Encoding Vitellogenin and Its Expression in the Hepatopancreas and Ovary during Vitellogenesis in the Kuruma Prawn, *Penaeus japonicus*

Naoaki Tsutsui[1], Ichiro Kawazoe[1], Tsuyoshi Ohira[1], Safiah Jasmani[1], Wei-Jun Yang[2], Marcy N. Wilder[2] and Katsumi Aida[1]*

[1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan*
[2]*Japan International Research Center for Agricultural Sciences, Ministry of Agriculture, Forestry and Fisheries, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan*

ABSTRACT—In Crustacea, reproductive function and mechanisms regulating vitellogenesis have not been fully elucidated. This is due in great part to a lack of information concerning the biochemical nature of the vitellogenin molecule, the hemolymph precursor of yolk protein, vitellin, as well as the functional expression of the vitellogenin-encoding gene. We have therefore cloned a cDNA encoding vitellogenin in the kuruma prawn, *Penaeus japonicus* based on the N-terminal amino acid sequence of the 91 kDa subunit of vitellin. The open reading frame of this cDNA encoded 2,587 amino acid residues. This is the first investigation reporting a full-length cDNA and its corresponding amino acid sequence for vitellogenin in any crustacean species.

Northern blot analysis and *in situ* hybridization have revealed that mRNA encoding vitellogenin was expressed in both the follicle cells in the ovary and the parenchymal cells in the hepatopancreas. In non-vitellogenic females, vitellogenin mRNA levels were negligible in both the ovary and hepatopancreas, but in vitellogenic females, levels were dramatically increased in both tissues. In the ovary, highest levels were observed during the early exogenous vitellogenic stage, and thereafter rapidly decreased, whereas in the hepatopancreas, high levels were maintained until the onset of the late vitellogenic stage. Differing profiles of vitellogenin mRNA levels in the ovary and hepatopancreas suggest that the contribution of these tissues to vitellogenin synthesis harbor separate and complementary roles during vitellogenesis.

INTRODUCTION

In Crustacea as in other oviparous animals, a large amount of vitellin (Vt), the major yolk protein appearing in eggs and consumed as an important source of nutrients during embryogenesis, is firstly accumulated in oocytes as an important step during ovarian maturation. This physiological process is known as vitellogenesis, and its regulatory mechanisms have been well-defined in oviparous vertebrates and insects. However, in Crustacea, much less is known concerning the regulation of vitellogenesis and associated reproductive function, although a great many species are important targets of fisheries and aquaculture development.

The Vt molecule is derived from vitellogenin (Vg), a yolk protein precursor which is secreted into the circulation from the ovary itself or from extraovarian tissue(s). Vt and Vg are both lipoproteins of high molecular weight which are indistinguishable from one another immunologically and electrophoretically. It has been shown that Vg is produced in the liver in vertebrates, and in the fat body in insects (Raikhel and Dhadialla, 1992). In isopods and amphipods, investigations using immunological techniques have shown that Vg is present in significant quantities in the fat body (Charniaux-Cotton and Payen, 1988; Meusy and Payen, 1988). In decapod crustaceans, three tissues have been reported as possible sites of Vg synthesis: the adipose tissue in *Parapenaeus longirostris* (Tom *et al*., 1987) and *Scylla serrata* (Rani and Subramoniam, 1997); the hepatopancreas in *Carcinus maenas* and *Libinia emarginata* (Paulus and Laufer, 1987), *Penaeus vannamei* (Quackenbush, 1989a, b), *Penaeus semisulcatus* (Fainzilber *et al*., 1992), *Macrobrachium nipponense* (Han *et al*., 1994), and *S. serrata* (Rani and Subramoniam, 1997); and the ovary in *Pachygrapsus crassipes* (Lui and O'Connor, 1977), *Penaeus japonicus* (Yano and Chinzei, 1987), and *Callinectes sapidus*

* Corresponding author: Tel. +81-3-5841-5287;
FAX. +81-3-5841-8165.
E-mail: aida@uf.a.u-tokyo.ac.jp

(Lee and Watson, 1995). However, investigations up until present have not demonstrated conclusively that the above tissues are the actual sites of Vg synthesis in crustaceans. In order to fully resolve this question, it is necessary to obtain direct evidence concerning the precise site of Vg synthesis by demonstrating the expression of Vg mRNA in those tissues. However, such studies are just now emerging in decapod crustaceans.

We have selected the kuruma prawn, *Penaeus japonicus* as a model animal, because of the commercial importance of species of the *Penaeus* genus in fisheries and the potential to apply findings concerning this animal to the elucidation of reproductive mechanisms in a wide range of saltwater prawn species. In a previous paper, we have purified and characterized Vt from mature ovary of the kuruma prawn as a first step in elucidating mechanisms of vitellogenesis in crustaceans (Kawazoe *et al.*, 2000). It was found that Vt consisted of three subunits of molecular weights of 91, 128, and 186 kDa. We subsequently determined the N-terminal amino acid sequences of the 91 kDa subunit as well as those of 4 peptide fragments obtained by enzymatic digestion. The objective of the present study was to clone a full-length cDNA encoding Vg in the kuruma prawn based on the amino acid sequences of the N-terminus as well as internal portions of the Vg molecule, to deduce the full primary amino acid sequence of Vg, and finally, to determine the exact site of Vg synthesis by examining the expression of its mRNA.

MATERIALS AND METHODS

Animals

Mature female kuruma prawns were obtained from the Momoshima Station of the Japan Sea-Farming Association in Hiroshima Prefecture, Japan. Immature prawns were purchased from a local dealer. Mature prawns ranged from 52 to 86 g in body weight and from 1.2 to 8.9% in gonadosomatic index (GSI). Immature prawns ranged from 18 to 20 g in body weight and from 0.2 to 0.5% in GSI. The developmental stages of oocytes were classified into 9 stages (oogonium, bouquet, chromatin nucleolus, perinucleolus, fused nucleolus (PAS-positive granule), early yolk globule, late yolk globule, early cortical alveoli, and late cortical alveoli stage) mainly according to Hong (1977). In this study, prawns were grouped into the previtellogenic stage (oogonium, bouquet, chromatin nucleolus, and perinucleolus stage), early exogenous vitellogenic stage (early yolk globule stage), and late exogenous vitellogenic stage (late yolk globule stage). Prawns at the endogenous vitellogenic stage (PAS-positive granule) and maturation stage (early and late cortical alveoli stage) were not present in this study.

Isolation of a genomic DNA fragment encoding the N-terminal amino acid sequence of the 91 kDa subunit protein

Genomic DNA was isolated from the hepatopancreas of female prawns according to a standard protocol (Sambrook *et al.*, 1989). Two degenerate oligonucleotide primers, VtF and VtR (Fig. 1), were designed based on the N-terminal amino acid sequence of the 91 kDa subunit (Kawazoe *et al.*, 2000), being derived from the sequences of amino acid positions 1–5 and 25–29, respectively. Polymerase chain reaction (PCR) was carried out in a 50 μl solution containing 1.5 mM $MgCl_2$, 200 μM dNTP, 1×PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 2 μM of each primer, 10 ng of genomic DNA, and 2 U of *Taq* DNA polymerase (TaKaRa, Tokyo, Japan). After an initial denaturation at 94°C for 4 min, 37 cycles of amplification were carried out using the following program: denaturation at 94°C for 0.5 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. Final extension was done at 72°C for 7 min. The PCR product was subcloned into the pBluescript SK^- plasmid vector (Stratagene, CA, USA). Dideoxy sequencing reactions were carried out using a Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) and electrophoresis and signal detection were done on an automatic sequencer SQ-5500 (HITACHI, Tokyo, Japan).

Isolation of RNA

The abdominal muscle containing adipose tissue and fan blade containing epidermal tissue, as well as the hepatopancreas, intestine and ovary were dissected out from mature and immature prawns. Total RNA was isolated from these tissues using ISOGEN (Nippongene, Toyama, Japan) according to instructions of the manufacturer. A part of the ovary was fixed in Bouin's solution for determination of its developmental stage (see above).

N-terminal amino acid sequence of the 91 kDa subunit of vitellin.

```
                    10                  20                  29
A P W G A D L P R ? S T E ? P I S G S P K L A Y Q P E K K
  VtF                                         VtR
                                                VtR/2
```

Primers	(Directions)	Sequences
VtF	(forward)	5'-GCN CCN TGG GGN GCN GA-3'
VtR	(reverse)	5'-TTY TTY TCN GGY TGR TA-3'
VtR/2	(reverse)	5'-CTC GGG YTG GTA GGC CA-3'
adapter		5'-GAG TCG ACT CGA GAA TTC-3'
T17-adapter		5'-GAG TCG ACT CGA GAA TTC $(T)_{17}$-3'

Fig. 1. Positions and nucleotide sequences of oligonucleotide primers used to clone Vg cDNA. Two degenerate primers, VtF and VtR, were used for PCR, the products of which were used as a probe for Northern blot analysis. Other primers were used for 5' RACE PCR. The N-terminal sequence of the 91 kDa subunit of vitellin was obtained by protein sequencing (Kawazoe *et al.*, 2000).

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was carried out in a 10 μl solution containing 1 μg of total RNA from the hepatopancreas or ovary from prawns in the early exogenous vitellogenic stage, 100 pM dT_{17} primer, 250 μM dNTP, 10 mM dithiothreitol, 15 U of RNase inhibitor (GIBCO BRL, MD, USA), and 100 U of Superscript II™ reverse transcriptase (GIBCO BRL) at 50°C for 1 hr. Using 1 μl of the cDNA solution, PCR was carried out in the same manner as described above. The PCR product was subcloned and sequenced.

Rapid amplifications of 5' cDNA end (5' RACE)

Using Oligotex-dT30 super (Roche, Tokyo, Japan), poly(A)$^+$ RNA was prepared from total RNA of the ovary of prawn at the early exogenous vitellogenic stage. First-strand cDNA was synthesized in a 20 μl solution containing 1 μg of poly(A)$^+$ RNA, 100 pM of primer VtR, 500 μM dNTP, 10 mM dithiothreitol, 25 U of RNase inhibitor (GIBCO BRL), and 200 U of Superscript II™ reverse transcriptase (GIBCO BRL) at 50°C for 1 hr. The reaction was stopped by the addition of 80 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The resulting first-strand cDNA was tailed with poly(dA) by a 3'-end labeling kit (Amersham Pharmacia Biotech). A degenerate primer, VtR/2, was designed based on the nucleotide sequence of cDNA encoding the N-terminal sequence (Fig. 1). PCR was carried out in a 50 μl solution containing 500 nM of each primer VtR/2, adaptor, T17-adaptor (Fig. 1), 200 μM dNTP, 1×PCR buffer, and 2 μl of the poly(dA)-tailed cDNA as a template. Initial denaturation at 94°C for 4 min, 5 amplification cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 3 min was followed by 35 cycles of 94°C for 0.5 min, 60°C for 0.5 min and 72°C for 2 min. Final extension was done at 72°C for 7 min. The PCR product was subcloned and sequenced as described above.

Construction of the cDNA library

The poly(A)$^+$ RNA was subjected to cDNA library construction using a TimeSaver® cDNA synthesis kit (Amersham Pharmacia Biotech) using 370 ng of $Pd(N)_6$ primer for first-strand cDNA synthesis. The synthesized cDNA was ligated to the λ ZAP-II vector (Stratagene). The ligation product was packaged using Gigapack® III Gold (Stratagene).

Screening of the cDNA library

The cDNA fragment (corresponding to nucleotides 1–168, Fig. 3) which had been obtained by 5' RACE was labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using the Megaprime™ DNA labeling system (Amersham Pharmacia Biotech) and used as a probe to screen the cDNA library by plaque hybridization. Positive plaques were isolated in the second round of screening and the recombinant pBluescript SK$^-$ phagemids were rescued from the bacteriophage clones according to instructions of the manufacturer (Stratagene). After sequencing analysis of a number of clones, a DNA fragment of 398 bp in length which was located at the 3' end of the obtained DNA sequence was used as a probe to screen the same library. Positive plaques were isolated and the recombinant pBluescript SK$^-$ phagemids were rescued from the bacteriophage clones and sequenced in the same manner as above. From the newly-obtained DNA sequence, a DNA fragment was selected as a probe to screen the same library. We named such a method library walking, i.e., repeating the procedure of selecting new probes from the most recently obtained cDNA clones, screening the same library with the new probe, and sequencing the obtained clones. Library walking was carried out five times (Fig. 2).

Extraction and purification of vitellin

We obtained further amino acid sequences of vitellin in addition to those of the 91 kDa subunit in order to confirm the obtained deduced amino acid sequence. The ovary was homogenized on ice in 0.2 M Tris-HCl (pH 7.5) containing 1.5 M NaCl and 6 M urea using a Polytron homogenizer (Switzerland). After centrifugation at 10,000 g for 10 min, the supernatant was collected and subjected to a Microconcentrators 3 filter (Amicon, MA, USA) to cut off low molecular weight proteins. The retentate was then subjected to reversed-phase HPLC using a TSKgel Phenyl-5PW RP column (4.6×75 mm, Tosoh, Tokyo, Japan). Fractionation was performed with a 40-min linear gradient of 0–80% methanol containing 5% acetonitrile in 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. Elution was monitored by measuring the absorption at 225 and 280 nm. Two major peak materials (fractions 1 and 2) were collected manually.

Enzymatic digestion and separation of the digests

Fraction 1 in 30 μl of 0.05% TFA was added with 30 μl of 1 M Tris-HCl buffer (pH 9.0) containing 2 M urea and incubated with lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan) at an enzyme to substrate ratio of 1:40 (w/w) at 37°C for 6 hr. Subsequently, 40 μl of 1 M HCl were added to stop the reaction. The digests were then separated by reversed-phase HPLC using an Asahi Pak ODP-50 column (4.6×150 mm, Showa Denko, Tokyo, Japan) with a 60 min liner gradient of 0–60% acetonitrile in 0.05% TFA at a flow rate of 1 ml/min. The fragments were collected manually by monitoring the absorbance at 225 nm.

Amino acid sequence analysis

N-terminal amino acid sequences of the two proteins (fraction 1 and 2) and fragment peptides digested with lysyl endopeptidase for fraction 1 were analyzed on a LF-3000 protein sequencer (Beckman, CA, USA) in the pulsed-liquid mode.

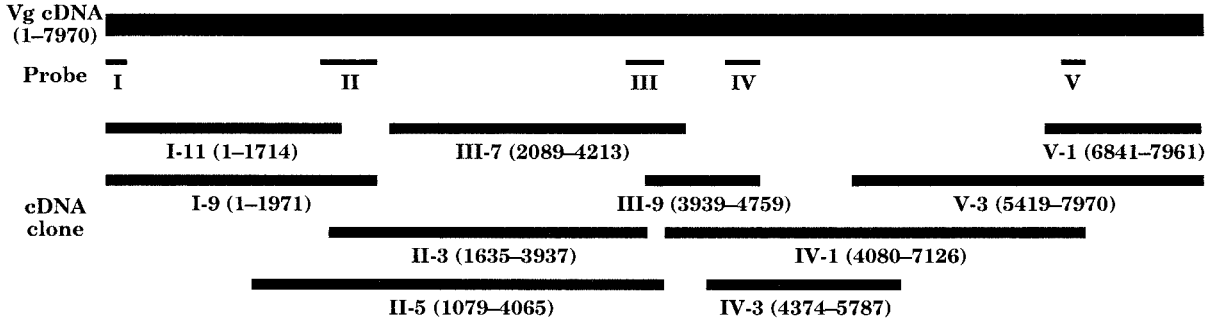

Fig. 2. Cloning strategy and schematic view of Vg cDNA. The merged Vg cDNA, probes, and clones sequenced are indicated by their base pair positions. Selected clones from cDNA library screening which were analyzed are designated as I-9, I-11, II-3, II-5, III-7, III-9, IV-1, IV-3, V-1, and V-3. Roman numerals indicate which probe was used to obtain a particular clone. Probes I, II, III, IV, and V were derived from 5' RACE, I-9, II-5, III-5, and IV-1, respectively.

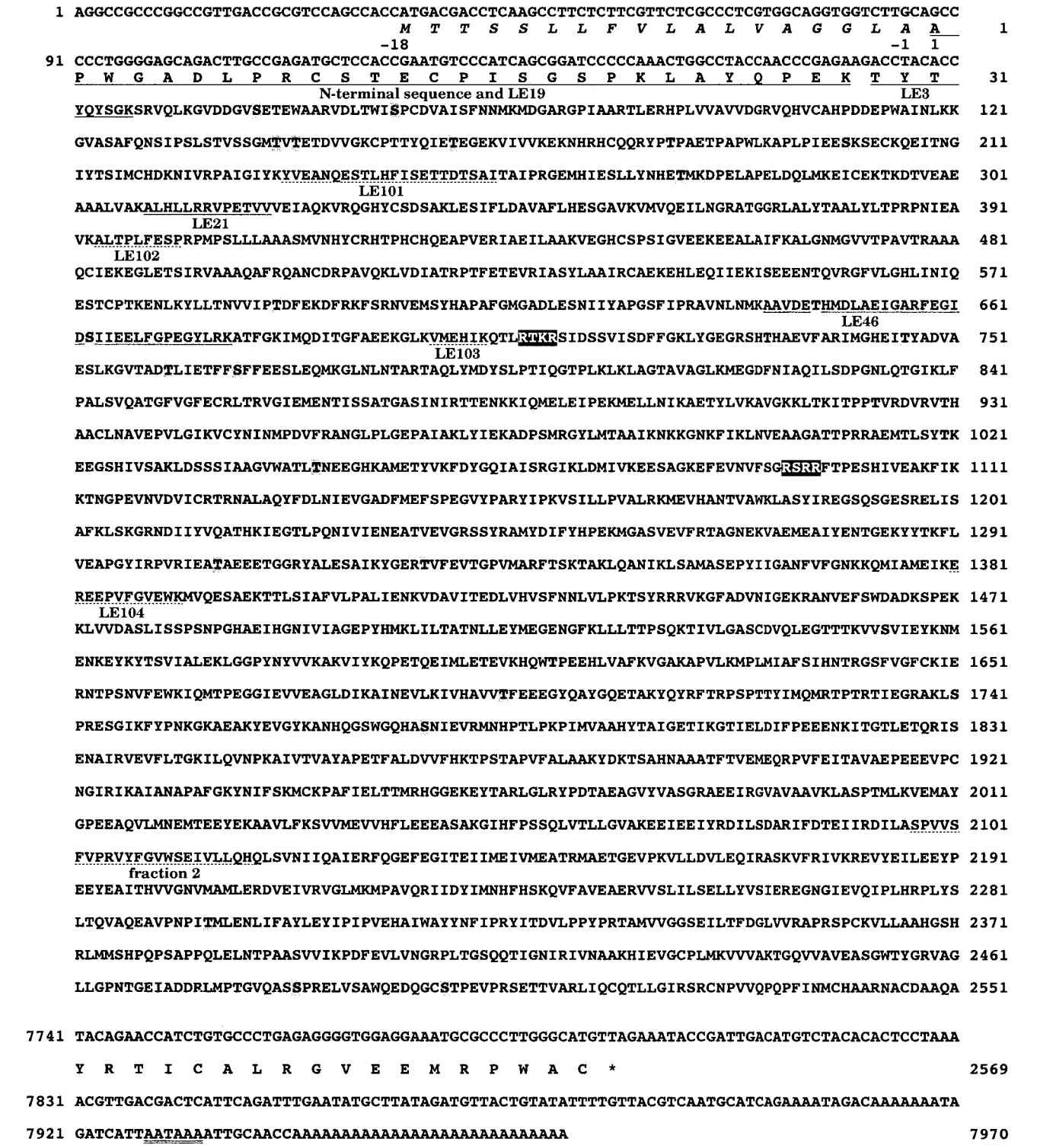

Fig. 3. Deduced amino acid sequence and 5' and 3' UTRs of Vg cDNA. The putative signal sequence is shown in *italics* (-18 – -1). Previously reported amino acid sequences are underlined (N-terminal sequence, LE3, LE19, LE21, and LE46). Amino acid sequences obtained in this study are underlined with a dotted line (LE101–104 and fraction 2). Ser or Thr residues that are possibly phosphorylated by casein kinase II are shaded. Two consensus cleavage sequences for processing by endoproteases of the subtilisin family are shown in white lettering with black background. Positions of nucleotides and amino acids are indicated by numbers on the left and right sides the figure, respectively. The polyadenylation signal is doubly underlined. The nucleotide sequence has been submitted to the GenBank™/EMBL Data Bank with accession number AB033719.

Northern blot analysis

For Northern blot analysis, 10 μg of total RNA isolated from the abdominal muscle, fan blade, hepatopancreas, intestine, and ovary were run on a 1% formaldehyde-agarose gel. The gel was stained with ethidium bromide, and ribosomal RNA contained in total RNA was measured using the Gel Doc 1000 Gel Documentation System and Molecular Analyst software (Bio-Rad, CA, USA). Subsequently, total RNA was transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech). The radiolabeled cDNA fragment encoding the N-terminal amino acid sequence of the 91 kDa subunit (corresponding to nucleotides 88–173, Fig. 3) was generated by PCR. The reaction was carried out in a 50 μl solution containing 1.5 mM $MgCl_2$, 200 μM of each dATP, dGTP, dTTP, 0.67 μM (100 μCi) of [α-^{32}P]dCTP (Amersham Pharmacia Biotech), 2 μM of each primer VtF and VtR, 5 ng of template DNA, 1×PCR buffer, and 2 U of *Taq* DNA polymerase (TaKaRa). After an initial denaturation at 94°C for 4 min, 40 cycles of amplification were carried out using the program described above. The membrane was then hybridized with the radiolabeled probe (1×10^5 cpm/ml) in 50% formamide, 6×SSC, 1×Denhardt's, 0.4% SDS and 100 μg/ml calf thymus DNA at 42°C for 16 hr and was washed with 0.2×SSC and 0.1% SDS at 55°C. RNA ladder (0.24–9.4 kb, GIBCO BRL) was used as the molecular weight marker. Hybridization signals were quantified using a Fujix Bio-imaging analyzer BAS 1000 (Fuji Film, Tokyo, Japan). An arbitrary value of 100 was applied to 4.5 ng of the pBluescript SK^- plasmid DNA into which was inserted the cDNA fragment encoding the N-terminal amino acid sequence of the 91 kDa subunit protein (corresponding to nucleotides 88–173, Fig. 3).

***In situ* hybridization**

The hepatopancreas and ovary from prawns in the early exogenous vitellogenic stage were excised and fixed overnight with 4% paraformaldehyde (PFA, TAAB Laboratories, UK) and 1% picric acid in 0.1 M phosphate buffer, pH 7.4 (PB) at 4°C. Tissues were dehydrated in ethanol and embedded in paraffin. Sections of 5 μm were cut onto APS (silane)-coated slides (Matsunami, Japan).

To generate anti-sense and sense RNA probes, DIG RNA Labeling Kit (Boehringer Mannheim, Germany) was used. The pBluescript SK^- plasmid DNA into which was inserted the 5' RACE PCR product (corresponding to nucleotides 1–168, Fig. 3) was linearized with *Xba* I (TaKaRa), and T7 polymerase was used to generate anti-sense probe. The sense probe was obtained after digestion with *Hin*d III (TaKaRa) and transcription with T3 polymerase.

In situ hybridization was carried out according to Naito *et al.* (1991) with some modifications. Sections were digested with 20 μg/ml proteinase K (GIBCO BRL) in TE buffer for 30 min, refixing with 4% PFA in PB for 10 min then were washed in PB for 5 min. Denatured probe (10 μg/ml) was mixed with hybridization buffer (25.8% formamide, 4× SSC, 1×Denhardt's, 0.5 mg/ml calf thymus DNA, 10.3% dextran sulfate). This buffer (90 μl) was applied to each slide and covered with a parafilm. Overnight hybridization was performed in a moist chamber at 37°C. On the following day, slides were dipped in 2×SSC to remove the parafilms, followed by one wash with 2×SSC for 20 min, two washes with 1×SSC for 30 min, and another wash with 2×SSC for 20 min. Slides were washed in TNE (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 100 mM NaCl) containing 20 μg/ml RNase A for 30 min, followed by one wash in TNE for 10 min, then in 2×SSC for 20 min and finally two washes in 0.2×SSC for 20 min. The hybridized probes were immunodetected with anti-digoxigenin-AP (Boehringer Mannheim) and were visualized with the colorimetric substrates NBT/BCIP (Boehringer Mannheim) according to the instructions attached to DIG DNA Labeling and Detection Kit (Boehringer Mannheim). Slides were mounted in 90% glycerol, sealed with nail polish, and observed under a light microscope.

RESULTS

Isolation of a cDNA fragment encoding the 91 kDa subunit of Vt

Two degenerate primers, VtF and VtR, were designed based on the N-terminal amino acid sequence of the 91 kDa subunit (Fig. 1). A PCR product of the expected size (86 bp) was obtained using these primers and genomic DNA as a template (data not shown). Results of sequence analysis showed that the product was derived from a genomic DNA encoding the 91 kDa subunit, as the deduced amino acid sequence was identical to the N-terminal sequence (corresponding to nucleotides 88–173, Fig. 3). We also confirmed using nucleotide sequence analysis that the two unidentified residues in the N-terminal amino acid sequence were cysteine. The residue Lys^{29} was threonine in the nucleotide sequence. The same PCR product was obtained by RT-PCR using cDNA from the hepatopancreas or ovary as a template (data not shown).

Isolation and characterization of cDNA clones encoding the 91 kDa subunit protein

Results of RT-PCR analysis showed that mRNA encoding the 91 kDa subunit was present in the hepatopancreas and ovary. In order to isolate the cDNA clones encoding the 91 kDa subunit, a cDNA library was prepared from the ovary of kuruma prawn at the early exogenous vitellogenic stage. A cDNA fragment obtained by 5' RACE (corresponding to nucleotides 1–168, Fig. 3) was used as a probe to screen the cDNA library. Among the isolated clones, two clones with long inserts were analyzed for their nucleotide sequences. Subsequently, a DNA fragment of 398 bp which was included in the 3' downstream region of the obtained nucleotide sequence was used as a probe to screen the same library. Newly-isolated clones were analyzed, and a new probe was selected from the obtained nucleotide sequence. As a result of five rounds of this screening, referred to as library walking (see materials and methods), 10 cDNA clones were obtained (Fig. 2), whose sequences were merged into the sole cDNA sequence containing 33 bp of a 5' UTR, 7,761 bp of an ORF, and 176 bp of 3' UTR (Fig. 3). The cDNA sequence contained a polyadenylation signal AATAAA at 10 nucleotides upstream of the site of poly(A) addition. The 4 partial amino acid sequences of the 91 kDa subunit reported in a previous paper (LE3, 19, 21, and LE46, Kawazoe *et al.*, 2000) were included in the deduced amino acid sequence (Fig. 3). In addition, microsequencing of two proteins obtained in this study (fraction 1 and 2) revealed that the N-terminus of fraction 1 was identical to that of the 91 kDa subunit, and N-terminal amino acid sequence of the fraction 2 was SPVVSFVPRVYFGVWSEIVLLQHQ (amino acid position 2097–2120, Fig. 3). N-terminal amino acid sequences of the 4 peptide fragments obtained by lysyl endopeptidase digestion of the fraction 1 were located at amino acid position 233–254 (LE101), 394–402 (LE102), 698–703 (LE103), and 1381–1392 (LE104, Fig. 3). Sequences for LE104 and fraction 2 were confirmed to exist in the C-

terminal side of the deduced amino acid sequence. This provided conclusive proof that the obtained cDNA clones encode Vg in the kuruma prawn.

Characterization of the deduced amino acid sequence

The cDNA had an ORF that encoded 2,587 amino acid residues with a predicted molecular mass of 286,702 Da. Based on the N-terminal sequence of the 91 kDa subunit, we were able to predict that a stretch of 18 highly hydrophobic residues (amino acid position -18–-1, Fig. 3) was a signal peptide. This deduced amino acid sequence contained two consensus cleavage sites, R–X–K/R–R (Arg^{707} to Arg^{710} and Arg^{1094} to Arg^{1097}, Fig. 3) capable of undergoing processing by endoproteases of the subtilisin family (Barr, 1991). In contrast to known invertebrate and vertebrate Vg's, this protein possessed no polyserine domain or no potential *N*-linked glycosylation sites. However, 11 serine residues and 18 threonine residues which were included in the consensus sequence for phosphorylation by casein kinase II were found in the deduced amino acid sequence. The BLAST algorithm (Altschul *et al.*, 1997) indicated that the deduced sequence had some similarity to apolipophorin of the tobacco hornworm (Sundermeyer *et al.*, 1996), retinoid- and fatty-acid binding glycoprotein (RFABG) of the fruit fly (Kutty *et al.*, 1996),

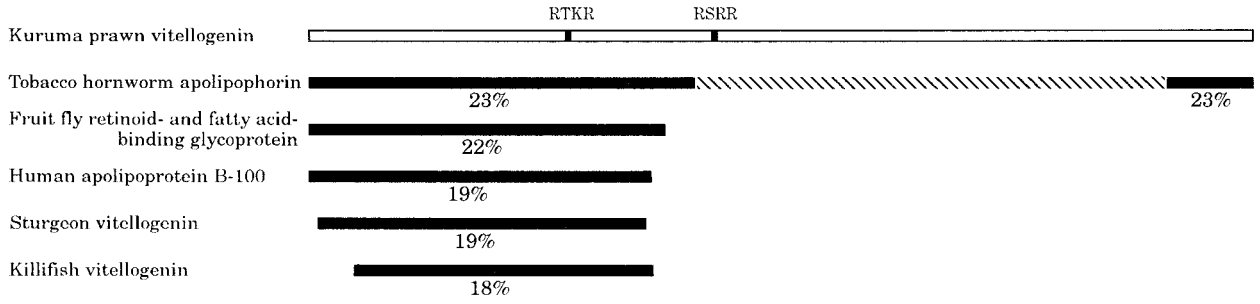

Fig. 4. Homology of kuruma prawn Vg with other structurally-related proteins. The open bar represents the deduced amino acid sequence of kuruma prawn Vg. The solid bars indicate the regions of Vg which have homology to proteins in other animals. Percentage of homology is indicated as well.

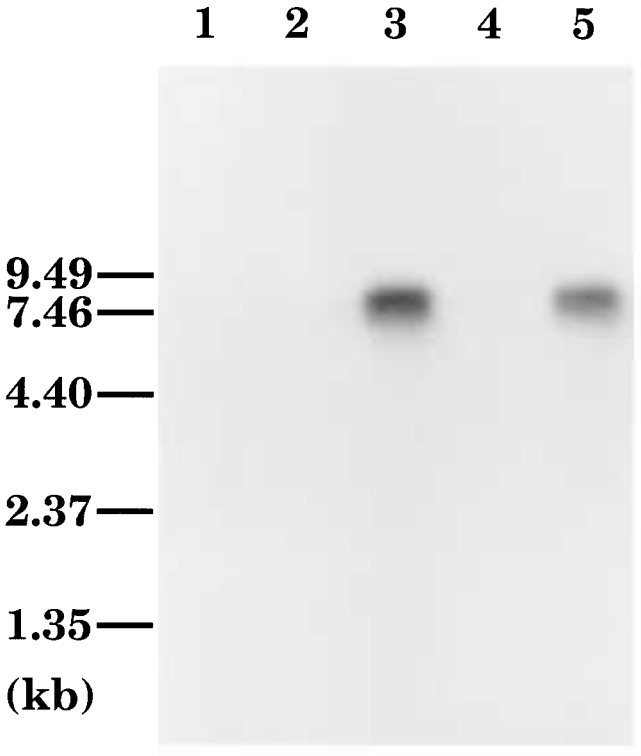

Fig. 5. Northern blot analysis of tissue specific expression of Vg mRNA. Results from a prawn at the early exogenous vitellogenic stage is shown as an example. Total RNA (10 μg/lane) from the abdominal muscle containing adipose tissue (lane 1), fan blade containing epidermal tissue (lane 2), hepatopancreas (lane 3), intestine (lane 4), and ovary (lane 5) were blotted onto a nylon membrane and probed with Vg cDNA which corresponded to nucleotides 88–173 (Fig. 3). Positions of the five molecular weight markers (9,490, 7,460, 4,400, 2,370 and 1,350 nucleotides) are indicated.

human apolipoprotein B-100 (Cladaras *et al*., 1986), and Vg in sturgeon (Bidwell and Carlson, 1995) and killifish (LaFleur *et al*., 1995). As shown in Fig. 4, the region of kuruma prawn Vg which showed similarity to other Vg's and lipoproteins was limited to the N-terminal half. The C-terminal regions of kuruma prawn Vg harbored some similarity to the C-terminal region of the aforementioned apolipophorin and D domains of von Willebrand factor (vWF) in mammals (Sadler, 1991; Ruggeri and Ware, 1993; data not shown), which is involved in the coagulation of blood (Voorberg *et al*., 1990; Mayadas and Wagner, 1992).

Distribution of mRNA encoding vitellogenin

Tissue-specific expression of Vg mRNA was analyzed by Northern hybridization. Total RNA was extracted from the abdominal muscle containing adipose tissue and fan blade containing epidermal tissue, as well as from the hepatopancreas, intestine, and ovary from female prawn. RNA blots were hybridized with the radiolabeled Vg cDNA and hybridized signals were thus detected in the hepatopancreas and ovary, but not in other tissues (Fig. 5). The probe hybridized to a single band of approximately 8 kb in each tissue. The size of the mRNA was in agreement with that of the Vg cDNA obtained in this study (7970 bp, Fig. 3).

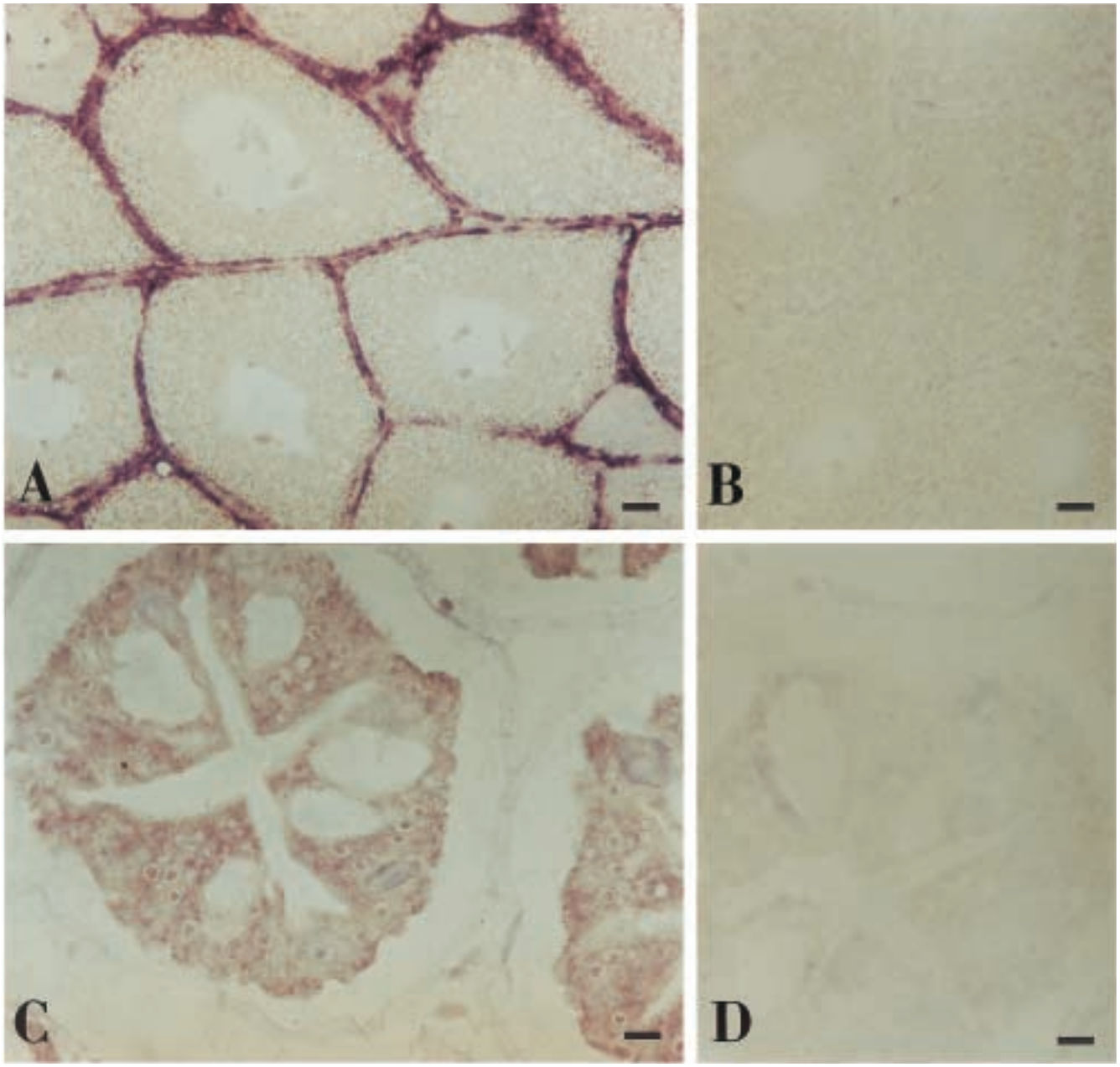

Fig. 6. Localization of Vg mRNA in the hepatopancreas and ovary by *in situ* hybridization. Strong expression of Vg was observed in the follicle cells (A) in the ovary, and in the parenchymal cells (C) in the hepatopancreas. Hybridization with a sense probe produced no significant signal in the ovary (B) and hepatopancreas (D). Bars=20 μm in A and B; 10 μm in C and D.

Fig. 6 shows the results of *in situ* hybridization using antisense and sense cRNA probes, indicating also the expression of Vg mRNA in the hepatopancreas and ovary. Vg mRNA signals were observed in the follicle cells that surrounded the vitellogenic oocytes (Fig. 6A). As for the hepatopancreas, signals were observed in the parenchymal cells (Fig. 6C). Hybridization with a sense control probe produced no significant signal in both tissues (Fig. 6B and D).

Changes in relative levels of mRNA encoding vitellogenin

Changes in Vg mRNA levels in the hepatopancreas and ovary during the non-vitellogenic and vitellogenic stages were examined by Northern hybridization. The same cDNA fragment was used to probe a Northern blot containing total RNA from immature and mature prawns. To ensure equal loading of RNA samples, gels were stained with ethidium bromide before blotting and levels of ribosomal RNA were measured for standardization. Results of changes in relative levels of Vg mRNA in the hepatopancreas and ovary are shown in Fig. 7. Sixteen ovaries were divided equally into four groups based on GSI and developmental stages of the oocytes as follows: GSI of 0.2–0.5% (previtellogenic stage), GSI of 1.2–3.7% (early exogenous vitellogenic stage), GSI of 4.4–7.2% (late exogenous vitellogenic stage), and GSI of 7.7–8.9% (late exogenous vitellogenic stage). In the hepatopancreas, the relative level of expression increased as vitellogenesis progressed, but decreased during later stages of maturation. In the ovary, highest levels were observed during the early exogenous vitellogenic stage and thereafter rapidly decreased.

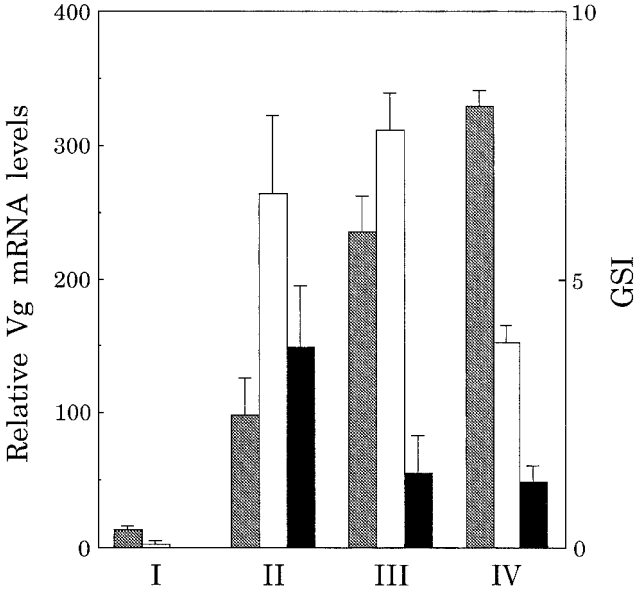

Fig. 7. Changes in relative expression levels of Vg mRNA in the hepatopancreas and ovary. Sixteen ovaries were divided into four groups based on GSI and developmental stages of the oocytes as follows: I, previtellogenic stage (GSI of 0.2–0.5%); II, early exogenous vitellogenic stage (GSI of 1.2–3.7%); III, late exogenous vitellogenic stage (GSI of 4.4–7.2%); IV, late exogenous vitellogenic stage (GSI of 7.7–8.9%). Four females were used in each group of this experiment. Results are presented as the mean±SEM. Shaded columns, GSI; open columns, Vg mRNA levels in the hepatopancreas; solid columns, Vg mRNA levels in the ovary.

DISCUSSION

In this investigation, we cloned a cDNA encoding Vg in the kuruma prawn based on the N-terminal amino acid sequence of the 91 kDa subunit of Vt determined in our previous paper (Kawazoe *et al.*, 2000). The ORF encoded 2,587 amino acid residues with a predicted molecular mass of 286,702 Da. This is the first investigation reporting a complete sequence for a Vg cDNA in a crustacean species. Its deduced amino acid sequence possessed two dibasic proteolytic processing sites R–X–K/R–R (Barr, 1991) that have also been found at subunit cleavage sites of insect Vg's, including *Anthonomus grandis* (Trewitt *et al.*, 1992), *Aedes aegypti* (Chen *et al.*, 1994), *Bombyx mori* (Yano *et al.*, 1994), *Lymantria dispar* (Hiremath *et al.*, 1997), and *Riptortus clavatus* (Hirai *et al.*, 1998). Four peptide fragments obtained by enzymatic digestion of the 91 kDa subunit of Vt were located on the N-terminal side of the first processing site, suggesting that the 91 kDa subunit would be produced as the result of cleavage at the first processing site. If this is the case, the resultant two subunits would have calculated molecular masses of 78,466 Da and 206,509 Da, corresponding to the 91 kDa and 186 kDa subunits reported previously (Kawazoe *et al.*, 2000). The discrepancy between the apparent molecular weight based on SDS-PAGE and the calculated one is not large and is considered to be within an acceptable error range. This can be confirmed by further elucidation of the N-terminal amino acid sequence of the 186 kDa subunit. Another subunit of 128 kDa was also found in the previous paper; however, in the present investigation no appropriate processing site which could produce a 128 kDa subunit was observed in the deduced sequence. Further investigation including isolation and sequence analysis will be necessitated in order to elucidate the 128 kDa protein. N-terminal amino acid sequence of the fraction 2 obtained in this study was located at amino acid positions 2097–2120 of kuruma prawn Vg (Fig. 3). This fraction would be produced artificially during purification because there were no dibasic proteolytic processing sites around the amino acid sequence of its N-terminus.

Kuruma prawn Vg showed low homology to several lipoproteins and Vg's thus far identified, including apolipophorin from the tobacco hornworm, RFABG from the fruit fly, human apolipoprotein B-100, and sturgeon and killifish Vg (Fig. 4). Biological similarities are seen between such lipoproteins and Vg from the point of view of binding of hydrophobic molecules, cell specific uptake, and the possibility that these proteins may have a common ancestor (Baker, 1988; Babin *et al.*, 1999). In this investigation, the region of kuruma prawn Vg which showed homology was limited to the N-terminal half of the molecule corresponding to the 91 kDa subunit region. The C-terminal half showed no significant homology to any proteins other than the C-terminal region of apolipophorin from the tobacco hornworm and vWF in mammals; this region thus

appears to possess unique properties compared with Vg of other known species. Kuruma prawn Vg did not show significant homology to Vg's of six insect species, i.e., *A. grandis*, *A. aegypti*, *B. mori*, *L. dispar*, *R. clavatus*, and *Pimpla nipponica* (Nose *et al*., 1997), and the plasma clotting protein of *Procambarus clarkii*, which is homologous to insect Vg's (Hall *et al*., 1998).

In Northern blot analysis, Vg mRNA of the same size was detected in the hepatopancreas and ovary of vitellogenic females and not detected in the adipose tissue, indicating that Vg is synthesized in the hepatopancreas and ovary in accordance with ovarian maturation (Fig. 5), which was reinforced by the results of *in situ* hybridization. Vg mRNA signals were detected in the follicle cells (Fig. 6A) and parenchymal cells (Fig. 6C). In *P. semisulcatus*, it has similarly been suggested through incubation studies that Vg is produced in both the hepatopancreas and ovary (Fainzilber *et al*., 1992). However, our investigation is one of the first in any oviparous animal to demonstrate based on mRNA expression that two differing tissues are simultaneously involved in vitellogenin synthesis. The detection of Vg mRNA expression in the follicle cells of the kuruma prawn is consistent with the results of a previous report on the same animal (Yano and Chinzei, 1987), in which it was concluded based on an immunofluorescence study that the follicle cells were responsible for ovarian Vg synthesis. Furthermore, the ovaries in several decapod crustaceans were found to synthesize Vg under *in vitro* tissue culture (Lui and O'Connor, 1977; Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Browdy *et al*., 1990; Lee and Watson, 1995). In most of these studies, levels of Vg synthesis were measured through the incorporation of radiolabeled amino acids into protein synthesized *de novo*.

As a result of detailed analysis, changes in Vg mRNA levels were determined to differ between these two tissues (Fig. 7). In the hepatopancreas, high mRNA levels were observed during the early and late exogenous vitellogenic stages (average GSI of 2.5% and 5.9%, respectively), and those levels were decreased following later stages of vitellogenesis (average GSI of 8.2%). On the other hand, highest mRNA levels were observed during the early exogenous vitellogenic stage in the ovary, and thereafter those levels were rapidly decreased and remained at low levels. In this investigation, we demonstrated changes in GSI and mRNA levels in the two tissues, hepatopancreas and ovary, and this would allow the examination of the dynamics of tissue-specific contribution to Vg synthesis.

In decapod crustaceans, vitellogenesis is negatively regulated by a neuropeptide, vitellogenesis-inhibiting hormone (VIH), produced by the X-organ sinus gland complex in the eyestalk, and vitellogenesis can be induced artificially via bilateral eyestalk ablation (Abramowitz and Abramowitz, 1940; Brown and Jones, 1948). Soyez *et al*. (1987) purified a VIH from the sinus glands of *Homarus americanus*, and this VIH inhibited the onset of vitellogenesis of *Palaemonetes varians*. Its amino acid sequence was subsequently determined (Soyez *et al*., 1991). More recently, VIH was purified from the terrestrial isopod *Armadillidium vulgare* (Gréve *et al*., 1999). Furthermore, seven crustacean hyperglycaemic hormones (CHH) were purified from kuruma prawn (Yang *et al*., 1996, 1997; Nagasawa *et al*., 1999), and were shown to be structurally similar to VIH. These *P. japonicus* peptides inhibited the synthesis of protein and mRNA in the ovary of *P. semisulcatus* in an *in vitro* tissue culture system (Khayat *et al*., 1998). In kuruma prawn, these neuropeptides are also expected to regulate the expression of mRNA encoding Vg in the same manner as VIH in the hepatopancreas and ovary. In consideration of the changes observed in Vg mRNA levels in the hepatopancreas and ovary, it is possible to assume that the expression of Vg mRNA is regulated in a tissue-specific manner. The results of this investigation will enable in further studies on the full elucidation of the hormonal regulation of vitellogenesis including the physiological functioning of VIH and putative vitellogenesis-stimulating hormone (VSH) and vitellogenesis-stimulating ovarian hormone (VSOH).

ACKNOWLEDGMENTS

We are grateful to the Momoshima Station, Japan Sea-Farming Association for providing the experimental animals. We also thank Prof. E. Lubzens of Israel Oceanographic and Limnological Research for valuable advice, and Dr. T.-W. Shih of the Department of Aquatic Bioscience at the University of Tokyo for encouragement and discussion. This work was supported partly by a Sasakawa Scientific Research Grant from The Japan Science Society (to N. T.), and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (to K. A.). This work in addition received support from the Japan International Research Center for Agricultural Sciences (JIRCAS), Ministry of Agricultural, Forestry, and Fisheries, where W.-J. Y. was a Science and Technology Agency (STA) Fellow.

REFERENCES

Abramowitz RK, Abramowitz AA (1940) Molting and growth after eyestalk removal in *Uca pugilator*. Biol Bull 78: 179–188

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Babin PJ, Bogerd J, Kooiman FP, Van Marrewijk WJ, Van der Horst DJ (1999) Apolipophorin II/I, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. J Mol Evol 49: 150–160

Baker ME (1988) Is vitellogenin an ancestor of apolipoprotein B-100 of human low-density lipoprotein and human lipoprotein lipase? Biochem J 255: 1057–1060

Barr PJ (1991) Mammalian subtilisins: the long-sought dibasic processing endoproteases. Cell 66: 1–3

Bidwell CA, Carlson DM (1995) Characterization of vitellogenin from white sturgeon, *Acipenser transmontanus*. J Mol Evol 41: 104–112

Browdy CL, Fainzilber M, Tom M, Loya Y, Lubzens E (1990) Vitellin synthesis in relation to oogenesis in in vitro incubated ovaries of *Penaeus semisulcatus* (Crustacea, Decapoda, Penaeidae). J Exp Zool 255: 205–215

Brown FA Jr, Jones GM (1948) Ovarian inhibition by a sinus-gland principle in the fiddler crab. Biol Bull 96: 228–232

Charniaux-Cotton H, Payen G (1988) Crustacean reproduction. In "Endocrinology of Selected Invertebrate Types Vol 2" Ed by Laufer, H and Downer, RGH, Alan R Liss, Inc, New York, pp

279–303
Chen JS, Cho WL, Raikhel AS (1994) Analysis of mosquito vitellogenin cDNA. Similarity with vertebrate phosvitins and arthropod serum proteins. J Mol Biol 237: 641–647
Cladaras C, Hadzopoulou-Cladaras M, Nolte RT, Atkinson D, Zannis VI (1986) The complete sequence and structural analysis of human apolipoprotein B-100: relationship between apoB-100 and apoB-48 forms. EMBO J 5: 3495–3507
Eastman-Reks S, Fingerman M (1985) In vitro synthesis of vitellin by the ovary of the fiddler crab, *Uca pugilator*. J Exp Zool 233: 111–116
Fainzilber M, Tom M, Shafir M, Applebaum SW, Lubzens E (1992) Is there extraovarian synthesis of vitellogenin in penaeid shrimp? Biol Bull 183: 233–241
Gréve P, Sorokine O, Berges T, Lacombe C, Van Dorsselaer A, Martin G (1999) Isolation and amino acid sequence of a peptide with vitellogenesis inhibiting activity from the terrestrial isopod *Armadillidium vulgare* (Crustacea). Gen Comp Endocrinol 115: 406–14
Hall M, Wang R, Van Antwerpen R, Sottrup-Jensen L, Söderhäll K (1999) The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood. Proc Natl Acad Sci USA 96: 1965–1970
Han CH, Okumura T, Suzuki Y, Aida K, Hanyu I, (1994) Immunocytochemical identification of the site of vitellogenin synthesis in the freshwater prawn *Macrobrachium nipponense*. Fisheries Sci 60: 149–154
Hirai M, Watanabe D, Kiyota A, Chinzei Y (1998) Nucleotide sequence of vitellogenin mRNA in the bean bug, *Riptortus clavatus*: analysis of processing in the fat body and ovary. Insect Biochem Mol Biol 28: 537–547
Hiremath S, Lehtoma K (1997) Structure of the gypsy moth vitellogenin gene. Arch Insect Biochem Physiol 36: 151–164
Hong DI (1977) Studies on sexual maturation and spawning in prawn *Penaeus japonicus*. PhD Thesis, Faculty of Agriculture, The University of Tokyo
Kawazoe I, Safiah J, Shih TW, Suzuki Y, Aida, K (2000) Purification and characterization of vitellin from the ovary of kuruma prawn, *Penaeus japonicus*. Fisheries Sci, in press
Khayat M, Yang WJ, Aida K, Nagasawa H, Tietz A, Funkenstein B, Lubzens E (1998) Hyperglycaemic hormones inhibit protein and mRNA synthesis in in vitro-incubated ovarian fragments of the marine shrimp *Penaeus semisulcatus*. Gen Comp Endocrinol 110: 307–318
Kutty RK, Kutty G, Kambadur R, Duncan T, Koonin EV, Rodriguez IR, Odenwald WF, Wiggert B (1996) Molecular characterization and developmental expression of a retinoid- and fatty acid-binding glycoprotein from *Drosophila*. J Biol Chem 271: 20641–20649
LaFleur GJ Jr, Byrne BM, Kanungo J, Nelson LD, Greenberg RM, Wallace RA (1995) *Fundulus heteroclitus* vitellogenin: the deduced primary structure of a piscine precursor to noncrystalline, liquid-phase yolk protein. J Mol Evol 41: 505–521
Lee CY, Watson RD (1995) In vitro study of vitellogenesis in the blue crab (*Callinectes sapidus*): Site and control of vitellin synthesis. J Exp Zool 271: 364–372
Lui CW, O'Connor JD (1977) Biosynthesis of crustacea lipovitellin. III. The incorporation of labeled amino acids into the purified lipovitellin of the crab *Pachygrapsus crassipes*. J Exp Zool 199: 105–108
Mayadas TN, Wagner DD (1992) Vicinal cysteines in the prosequence play a role in von Willebrand factor multimer assembly. Proc Natl Acad Sci USA 89: 3531–3535
Meusy JJ, Payen GG (1988) Female reproduction in malacostracan Crustacea. Zool Sci 5: 217–265
Nagasawa H, Yang WJ, Aida K, Sonobe H (1999) Chemical and biological characterization of neuropeptides in the sinus glands of the kuruma prawn, *Penaeus japonicus*. In "Peptide Science-Present and Future" Ed by Shimonishi Y, Kluwer Academic Publishers, Dordrecht, pp 453–454
Naito N, Hyodo S, Okumoto N, Urano A, Nakai Y (1991) Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. Cell and Tissue Res 266: 457–467
Nose Y, Lee JM, Ueno T, Hatakeyama M, Oishi K (1997) Cloning of cDNA for vitellogenin of the parasitoid wasp, *Pimpla nipponica* (Hymenoptera: Apocrita: Ichneumonidae): vitellogenin primary structure and evolutionary considerations. Insect Biochem Mol Biol 27: 1047–1056
Paulus JE, Laufer H (1987) Vitellogenocytes in the hepatopancreas of *Carcinus maenas* and *Libinia emarginata* (Decapoda, brachyura). Int J Invertebr Reprod Dev 11: 29–44
Quackenbush LS (1989a) Vitellogenesis in the shrimp, *Penaeus vannamei*: in vitro studies of the isolated hepatopancreas and ovary. Comp Biochem Physiol 94B: 253–261
Quackenbush LS (1989b) Yolk protein synthesis in the marine shrimp *Penaeus vannamei*. J Crustacean Biol 9: 509–516
Raikhel AS, Dhadialla TS (1992) Accumulation of yolk proteins in insect oocytes. Annu Rev Entomol 37: 217–251
Rani K, Subramoniam T (1997) Vitellogenesis in the mud crab *Scylla serrata* — an *in vivo* isotope study. J Crustacean Biol 17: 659–665
Ruggeri ZM, Ware J (1993) von Willebrand factor. FASEB J 7: 308–316
Sadler JE (1991) von Willebrand factor. J Biol Chem 266: 22777–22780
Sambrook J, Fritshi EF, Maniatis T (1989) Molecular Cloning: A Laboratory, Manual, 2nd ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Soyez D, Van Deijnen JE, Martin M (1987) Immunochemical and immunocytochemical studies of the crustacean vitellogenesis-inhibiting hormone (VIH). J Exp Zool 244: 479–484
Soyez D, Le Caer JP, Noel PY, Rosier J (1991) Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster *Homarus americanus*. Neuropeptides 20: 25–32
Sundermeyer K, Hendricks JK, Prasad SV, Wells MA (1996) The precursor protein of the structural apolipoproteins of lipophorin: cDNA and deduced amino acid sequence. Insect Biochem Mol Biol 26: 735–738
Tom M, Goren M, Ovadia M (1987) Localization of the vitellin and its possible precursors in various organs of *Parapenaeus longirostris* (Crustacea, Decapoda, Penaeidae). Int J Invertebr Reprod Dev 12: 1–12
Trewitt PM, Heilmann LJ, Degrugillier SS, Kumaran AK (1992) The boll weevil vitellogenin gene: nucleotide sequence, structure, and evolutionary relationship to nematode and vertebrate vitellogenin genes. J Mol Evol 34: 478–492
Voorberg J, Fontijn R, Van Mourik JA, Pannekoek H (1990) Domains involved in multimer assembly of von willebrand factor (vWF): multimerization is independent of dimerization. EMBO J 9: 797–803
Yang WJ, Aida K, Terauchi A, Sonobe H, Nagasawa H (1996) Amino acid sequence of a peptide with molt-inhibiting activity from the kuruma prawn *Penaeus japonicus*. Peptides 17: 197–202
Yang WJ, Aida K, Nagasawa H (1997) Amino acid sequences and activities of multiple hyperglycemic hormones from the kuruma prawn, *Penaeus japonicus*. Peptides 18: 479–485
Yano I, Chinzei Y (1987) Ovary is the site of vitellogenin synthesis in Kuruma prawn, *Penaeus japonicus*. Comp Biochem Physiol 86B: 213–218
Yano K, Toriyama-Sakurai M, Watabe S, Izumi S, Tomino S (1994) Structure and expression of mRNA for vitellogenin in *Bombyx mori*. Biochim Biophys Acta. Biochim Biophys Acta 1218: 1–10

(Received November 15, 1999 / Accepted January 14, 2000)