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Cloning of cDNA encoding vitellogenin and its expression in red sea urchin, *Pseudocentrotus depressus*

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ABSTRACT—Both male and female red sea urchins, *Pseudocentrotus depressus*, accumulate a large quantity of the major yolk protein (MYP) in the nutritive phagocytes of immature gonads before the initiation of gametogenesis. To examine the accumulation mechanism of this protein in the gonad, we cloned full-length cDNA encoding vitellogenin (Vg; the MYP precursor in the coelomic fluid), and investigated its expression in various tissues of immature adults. The nucleotide sequence of Vg contains an open reading frame of 4050 bp encoding 1349 amino acids. The deduced amino acid sequence near the N-terminal showed 25% homology to the vertebrate transferrin family. Vitellogenin mRNA was detected in the ovary, testis, stomach, intestine and rectum by Northern blot analysis, with the highest level of mRNA expression in the gonad. Weak expression was also detected in the esophagus and coelomocytes by RT-PCR. *In situ* hybridization demonstrated that nutritive phagocytes, which exclusively fill the lumina of the immature gonad, contained Vg mRNA. These results suggested that the MYP stored in the immature gonads is synthesized and accumulated mainly within the nutritive phagocytes.

INTRODUCTION

The most abundant yolk protein found in sea urchin eggs, a glycoprotein with a molecular weight of 160–180 kDa, has been termed the major yolk protein or major yolk glycoprotein (MYP; Harrington and Easton, 1982; Kari and Rottmann, 1985; Yokota and Kato, 1988; Scott and Lennarz, 1989). Unlike other oviparous animals where the yolk protein is female-specific, sea urchins store a large amount of MYP in nutritive phagocytes of both the ovary and testis before gametogenesis (Ozaki *et al*., 1986; Unuma *et al*., 1998), whereafter MYP is utilized as a nutrient source for spermatogenesis as well as for oogenesis (Unuma *et al*., 1998). A precursor of MYP, vitellogenin (Vg), has a slightly higher molecular weight (180–200 kDa) than MYP and is contained in the coelomic fluid of both sexes (Harrington and Easton, 1982; Shyu *et al*., 1986). Shyu *et al.* (1986) have cloned a partial cDNA encoding Vg and have demonstrated that Vg mRNA is expressed in the intestines and gonads of both sexes in the mature *Strongylocentrotus purpuratus*. The sequence of full-length Vg cDNA is still unknown, however, and its expression pattern before gametogenesis has not yet been clarified.

To understand the accumulation mechanism of MYP in the gonad, determining the site of the production of this protein before gametogenesis is essential. In the present study, we cloned and characterized the full-length Vg cDNA of *Pseudocentrotus depressus* and examined its expression in immature adults.

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MATERIALS AND METHODS

Animals

Six-month-old juveniles of *P. depressus*, hatched and reared at the Fukuoka Prefectural Fish Farming Center, were transferred to the Coastal Station of the National Research Institute of Aquaculture, maintained in 1,000-liter tanks supplied with sand-filtered sea water at 30 l/min, and reared on kelp, *Eisenia bicyclis*. Two- to three-year-old immature adults were used in the study.

Isolation of RNA

After removing the peristomial membrane, coelomic fluid was collected with Pasteur pipettes and centrifuged at 600 g for 5 min to obtain the coelomocytes. The esophagus, stomach, intestine, rectum and gonad were excised and their total RNA was isolated with the TRIZOL reagent (GIBCO BRL, USA) according to the manufacturer's instructions. Small specimens of gonads were fixed in Bouin's solution for histological observation. Paraffin sections 6 μm thick were prepared and stained with haematoxylin and eosin.

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was carried out by the Superscript II preamplification system for first strand cDNA (GIBCO BRL, USA) using the oligo(dT) primer according to the manufacturer's instructions. The primers used for PCR amplification are listed in Table 1. Primers F1 and R3 were used for fragment 2, F2 and R4 for fragment 3 (Fig. 1).

Table 1. Sequences of the primers

Primer	Sequence
AP (adaptor primer)	5'-GGCCACGCGTCGACTAGTAC-3'
F1	5'-ATGAGGGCTGCAATTCTNTTYTGY-3'
F1'	5'-ATGAGGGCTGCGATTCTCTTCTGC-3'
F2	5'-ATCCTGAAGACCCAGGACTTC-3'
F3	5'-GCAGAACCTCACCTCCCATGTCTTCAAC-3'
F4	5'-GGCGTTGAGGGAATTTCTGACCTTGTCA-3'
R1	5'-GAAGTCCTGGGTCTTCAGGATGGC-3'
R2	5'-AGGGTACAGGCCAACCTTGCGGAT-3'
R3	5'-CTCGAGACGCACCRTCNGAYTG-3'
R3'	5'-TCGAGACGCACCATCTGATGGTGT-3'
R4	5'-ACNGCRTCRAARTTNGGRTA-3'

Thirty cycles of amplification were carried out under the following conditions: 95°C for 0.5 min, 50°C for 0.5 min, and 72°C for 1 (fragment 2) and 3 (fragment 3) min. Fragment 2 was subcloned into the pCR II plasmid with the TA cloning kit (Invitrogen, USA) and used as a template to generate the digoxigenin-labeled DNA probe. The nucleotide sequence of the fragments was determined with the BigDye Terminator Cycle Sequencing FS Ready Reaction kit and the ABI PRISM 377 DNA sequencer (PE Biosystems, USA). For the RT-PCR analysis of Vg mRNA expression, primers F1' and R3' were used. Thirty cycles of amplification were carried out under the following conditions: 95°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min.

5'RACE and 3'RACE

Rapid amplification of the 5'cDNA and 3'cDNA ends was carried out by the 5' RACE and 3' RACE system for rapid amplification of cDNA ends (GIBCO BRL, USA) according to the manufacturer's instructions. In 5'RACE, primers R3, R2 and R1 were used for reverse transcription, the first PCR and the nested PCR, respectively. Thirty cycles of amplification were carried out under the following conditions: 95°C for 0.5 min, 60°C for 0.5 min and 72°C for 1 min. In 3'RACE, primers F3 and F4 were used for the first PCR and the nested PCR, respectively. Thirty cycles of amplification were carried out under the following conditions: 95°C for 0.5 min, 62°C for 0.5 min and 72°C for 3 min. The nucleotide sequence of the fragments was determined as described above.

Isolation and amino acid sequence analysis of MYP

MYP used for amino acid sequence analysis was purified from the immature gonad according to the purification method for MYP from the egg (Unuma *et al.*,1998) with some slight modifications. One gram of the immature gonad was homogenized with 15 ml of 10 mM Tris-HCl buffer containing 10 mM NaCl (pH 8.0; TBS) using Polytron (Kinematica, Switzerland). The homogenate was centrifuged at 25,000 g for 20 min at 4°C, and the supernatant was applied to a HiLoad 16/10 Q Sepharose Fast Flow column (Amersham Pharmacia Biotech, England) equilibrated with TBS. After washing with 60 ml of the same buffer, the retained proteins were eluted with a NaCl linear gradient from 10 mM to 1 M (300 ml in total) using FPLC (Amersham Pharmacia Biotech) at a flow rate of 3.0 ml/min. The protein eluted with 240 mM NaCl was collected, concentrated threefold using Molcut LGC (Millipore Corp., USA), and applied to a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia Biotech) equilibrated with 10 mM Tris-HCl buffer containing 150 mM NaCl (pH 8.0). The proteins were eluted with the same buffer at a flow rate of 1.0 ml/min. The MYP eluted at an elution volume of 50 ml was collected, incubated with 8M urea for 2 h at 37°C, and then digested with lysyl endopeptidase (Wako Pure Chemical Industries, Ltd., Japan) in 0.1M N-ethylmorpholine (pH 9.0) at 37°C overnight. The digests were then separated by reverse-phase HPLC using a Cosmosil 5C18 AR-II column (2.0×150 mm; Nacalai Tesque, Japan) with a 60 min linear gradient of 0–60% acetonitrile in 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.2 ml/min. The amino acid sequence of the peptide fragment eluted at 31.1 min was determined by the G1005A Protein-Sequencing system (Hewlett-Packard, USA) in the pulse-liquid mode.

Northern blot analysis

The digoxigenin-labeled cDNA probe, 415 bp, was prepared using the PCR DIG probe synthesis kit (Loche Diagnostic, Germany) with primers F1' and R3'. Thirty cycles of amplification were carried out under the following conditions: 95°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min. The total RNA (5 μg) extracted was electrophoresed on a 1% formaldehyde-agarose gel and blotted onto nylon membranes. The blotted membrane was hybridized with the probe dissolved in Easy Hyb (Loche Diagnostic) at 60°C for 20 hr and washed with 0.1×SSC/0.1%SDS at 65°C for 40 min. The hybridized probe was visualized with the DIG nucleic acid detection kit (Loche Diagnostic) according to the manufacturer's instructions.

***In situ* hybridization**

In situ hybridization of the immature gonad was done with the *In situ* hybridization starting kit (Nippon Gene Co., Ltd., Japan) and oligonucleotide probes. Four sense and four antisense oligonucleotide probes (60 bp each) were synthesized and labeled with digoxigenin using the DIG-tailing kit (Loche Diagnostic) according to the manufacturer's instructions. The positions of the oligonucleotide probes are shown in Fig. 2. The immature gonad was excised and fixed with 4% paraformaldehyde in PBS (10 mM phosphate buffer, 0.1M NaCl, pH 7.4). Tissues were dehydrated in ethanol, embedded in paraffin, and cut into 6-μm-thick sections. After removing the paraffin, the sections were digested with proteinase K (2 μg/ml) in PBS for 4 min, and then acetylated with 0.1 M triethanolamine (pH 8.0) containing 0.25% (v/v) acetic anhydride for 10 min. The four antisense or sense oligonucleotide probes (0.025 μg/ml each, 0.1 μg/ml total) were mixed with the hybridization buffer (50% formamide, 2×SSC, 1 μg/μl tRNA, 1 μg/μl salmon sperm DNA, 1 μg/μl BSA, and 10% dextran sulfate). After 30 min of prehybridization in 50% formamide/2×SSC at 42°C, hybridization was performed for 16 hr at 42°C. The slides were washed with 50% formamide/2×SSC for 60 min at 42°C and then with 0.1×SSC for 120 min at 42°C. The hybridized probes were visualized with the DIG nucleic acid detection kit (Loche Diagnostic) according to the manufacturer's instructions.

RESULTS

Isolation of the cDNA encoding Vg

In a preliminary Northern blot analysis with the *S. purpuratus* Vg probe (Shyu *et al.*, 1986) provided by Dr. Blumenthal, Vg mRNA expression was confirmed in the immature gonad of *P. depressus*. Thus the Vg cDNA was isolated from the immature gonad.

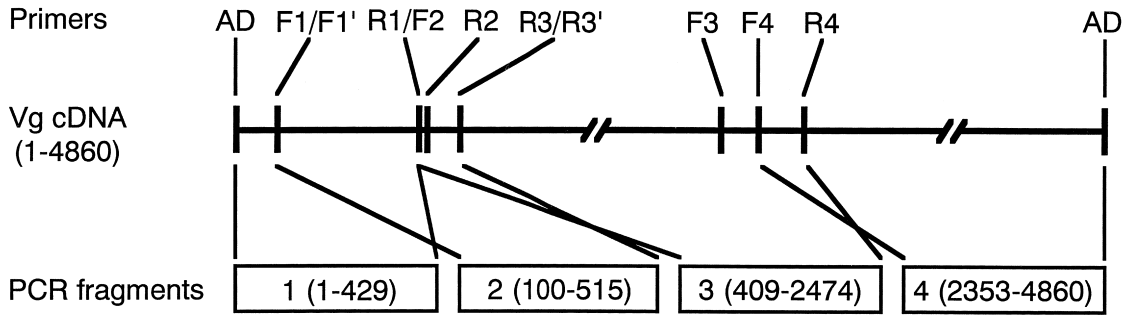


Fig. 1. Cloning and sequencing strategy for Vg cDNA of *P. depressus*. Primers used and PCR fragments obtained are shown. Numbers in the parentheses indicate their base pair positions.

The strategy for cloning Vg cDNA is shown in Fig. 1. As a first step, a set of degenerate primers (F1 and R3) was designed based on the *S. purpuratus* Vg cDNA (Shyu *et al*. 1987), then RT-PCR was conducted with these primers and the total RNA from the immature gonad as a template. A PCR fragment of the expected size (416bp; fragment 2) was isolated, subcloned and sequenced. The nucleotide sequence showed 85% homology to the corresponding region of *S. purpuratus* Vg.

To obtain information on the internal amino acid sequence, MYP was purified from the immature gonad, digested with lysyl endopeptidase, and the amino acid sequence of the peptide fragment was analysed. A degenerate primer (R4) was designed based on the sequence, LSNSYPNFDAVR (Fig. 2), then RT-PCR was conducted with this primer and another primer (F2) which was designed based on the nucleotide sequence of fragment 2. A PCR product of 2066 bp (fragment 3) was isolated and sequenced.

Primers F3, F4, R1, R2 and R3' were designed based on the nucleotide sequences of fragments 2 and 3 and used in conducting 5'RACE and 3'RACE. PCR products of 430 bp (fragment 1) and 2508 bp (fragment 4) were isolated and sequenced.

Characterization of cDNA encoding Vg

The nucleotide and deduced amino acid sequences of *P. depressus* Vg cDNA are shown in Fig. 2. The cDNA had an open reading frame that encodes a 1349-amino-acid protein with a predicted molecular mass of 153.6 kDa. The prediction of protein sorting signals (PSORT) program (http://psort.ims.u-tokyo.ac.jp) predicted the first 15 amino acids as a signal sequence of the protein. Twenty-one potential *N*-linked glycosylation sites were found in the deduced amino acid sequence (positions not shown). The amino acid sequence was compared with that of other proteins in the database by FASTA (http://fasta.genome.ad.jp), and *P. depressus* Vg was found to have little homology to Vg of other animals other than that to *S. purpuratus* Vg; instead, it showed about 25% homology in its 132- to 510- amino-acid region to the vertebrate transferrin family.

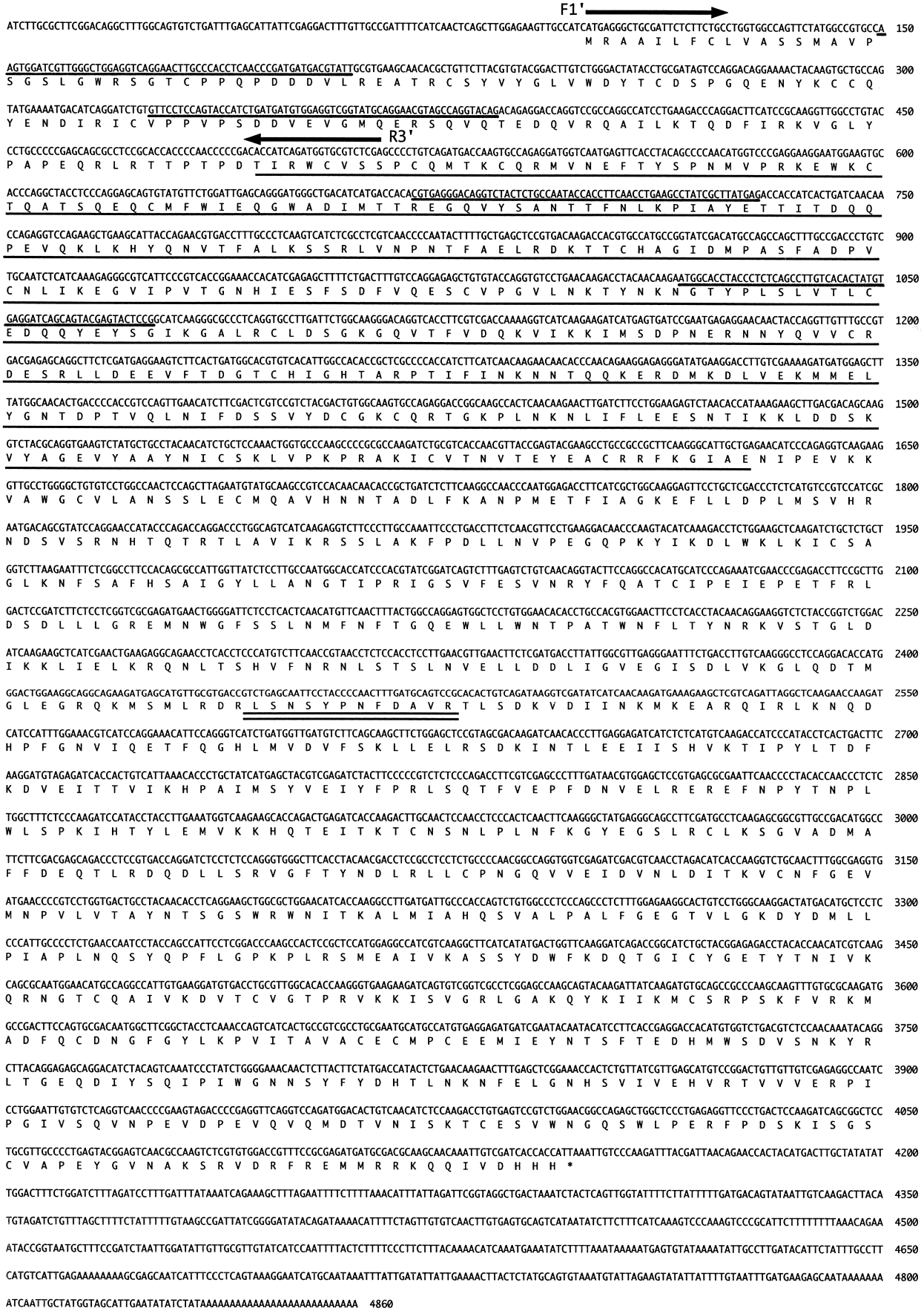
Distribution of Vg mRNA.

Total RNA was extracted from the esophagus, stomach, intestine, rectum, immature gonad and coelomocytes of the immature adult *P. depressus*, and the tissue specific expression of Vg mRNA in these organs was analysed by Northern blotting (Fig. 3). Vg mRNA was detected in the gonad, stomach, intestine and rectum, but not in the esophagus or coelomocytes. The immature gonad showed the most intense signal. The probe hybridized to a single band of approximately 5 kb in each tissue. The size of the mRNA was in agreement with that of the Vg cDNA obtained in this study.

The Vg mRNA expression not detected in the esophagus or coelomocytes by Northern blotting was revealed by RT-PCR using primers F1' and R3' (Fig. 4). The PCR products of the expected size (416 bp), detected in both the esophagus and coelomocytes as well as in the immature gonad, were not derived from genomic DNA contamination since no product was detected when reverse transcriptase was excluded from the RT-PCR protocol (- RT).

A comparison of the Vg mRNA expression in female and male gonads and the histological features of the immature ovary (Fig. 5A) and testis (Fig. 5B) are shown in Fig. 5. The gonadal lumina of both sexes were filled with nutritive phagocytes; in the ovary, small oocytes were observed at the periphery of the acini; in the testis, however, male germ cells were difficult to detect by light microscopy. Instead, testicular nutritive phagocytes often contained haematoxylin-stained dark-blue amorphous speckles derived from phagocytized residual spermatozoa. The immature gonads with these characteristics were sexed, and their Vg mRNA expression was compared by Northern blot analysis using two individuals of each sex (Fig. 5C). Similar amount of transcripts was detected in both the immature ovaries and testes.

In situ hybridization was carried out in the immature ovary and testis using antisense and sense oligonucleotide probes. Hybridization with antisense probes in the immature ovary revealed Vg mRNA signal in nutritive phgocytes (Fig. 6A); hybridization with a sense control probe produced no significant signal (Fig. 6B). In the immature testis, a Vg mRNA signal was also detected in nutritive phagocytes, as in the ovary (data not shown).



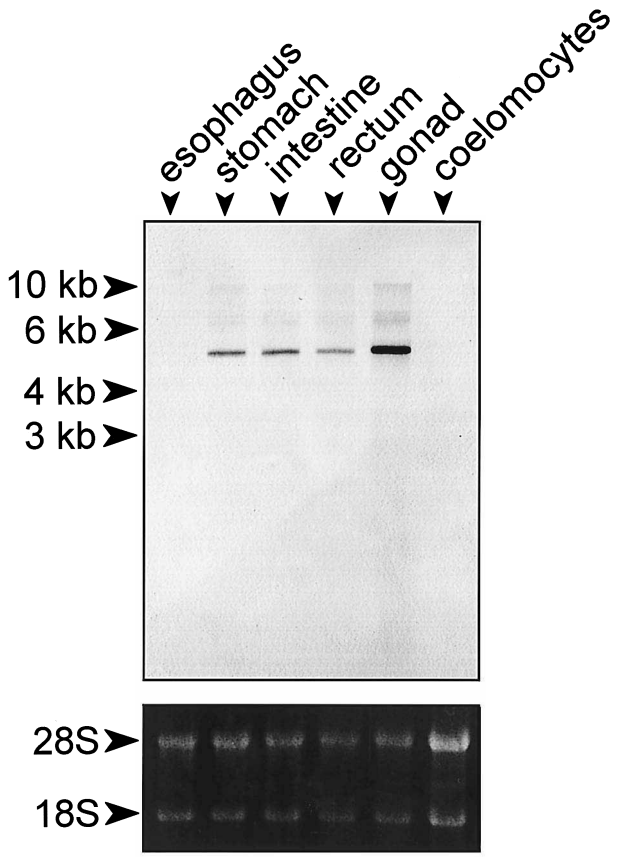


Fig. 3. Northern blot analysis of tissue-specific Vg mRNA expression. Total RNA (5 μg/lane) extracted from the esophagus, stomach, intestine, rectum, gonad, and coelomocytes of immature *P. depressus* was electrophoresed, blotted onto a nylon membrane, and probed with digoxigenin-labeled Vg cDNA which correspond to nucleotides 100–514. The positions of the four molecular weight markers (10, 6, 4, 3 kb) are indicated on the left margin. Ribosomal RNA visualized with ethidium bromide is shown as a loading control in the lower panel.

DISCUSSION

We sequenced the full-length Vg cDNA of *P. depressus* and investigated its expression in the immature adult. This is the first report on the complete nucleotide sequence of Vg cDNA in the echinoderm. The open reading frame encodes a 1349-amino-acid residue with an expected molecular mass of 153.6 kDa, which is consistent with the report showing that the initial translation product of *S. purpuratus* Vg mRNA is 155-kDa (Shyu *et al.*, 1986). Shyu *et al.* (1987) sequenced the 5'-end of the genomic DNA encoding Vg in *S. purpuratus*, which contains 511 bp of cDNA, approximately one-tenth of the full-length cDNA. Little homology has been found between this region of *S. purpuratus* Vg and other animals' Vg. We compared the full-length deduced amino acid sequence of *P. depressus* Vg with other proteins in the database, and little homology was found with Vg of other animals. Instead, we found that, in its 132- to 510- amino-acid region, Vg has approximately 25% homology to the vertebrate transferrin family (transferrin, serotransferrin, lactoferrin, etc.), an iron-binding glycoprotein that controls the level of free iron in biological fluid (Crichton and Charloteaux-Wauters, 1987). Sea urchin Vg may have binding affinity for iron or other metals and may play the role of a carrier protein, as postulated by Shyu *et al.* (1986).

Unuma *et al.* (1998) have reported that both male and female sea urchins accumulate abundant MYP in their nutritive phagocytes in the gonad before gametogenesis and then utilize it to produce gametes. The concentration of MYP in the immature gonad, which is at the same level in both sexes, is much higher than that in the mature ovary or testis (Unuma *et al.*, in preparation). We assume that most of the MYP is previously synthesized as a nutrient reserve in both sexes before gametogenesis begins. Therefore, it is important to determine the site of the production of MYP before gametogenesis in order to clarify the accumulation mechanism of this protein in the gonad. In this study, Northern blot analysis revealed Vg mRNA expression in the ovary, testis, stomach, intestine and rectum of immature *P. depressus*, and showed that the amount of transcripts was predominant in the gonads of both sexes. *In situ* hybridization demonstrated Vg mRNA expression in nutritive phagocytes. These results indicate that before gametogenesis MYP stored in the nutritive phagocytes is produced mainly within the nutritive phagocytes themselves. In contrast, Shyu *et al.* (1986) have shown that in mature *S. purpuratus*, the expression of Vg mRNA was much higher in the intestine than in the ovary or testis, concluding that Vg is synthesized mainly in the intestine in the mature *S. purpuratus*. Their report and our present study suggest that the main site of MYP production changes with gametogenesis. Probably the expression level of Vg mRNA decreases in the gonads as gametogenesis proceeds, and nutritive phagocytes become small. To verify this, it is necessary to investigate changes in the expression patterns of Vg mRNA during gametogenesis and its expression in germ cells.

Harrington and Ozaki (1986) have reported that Vg is produced mainly in coelomocytes. Shyu *et al.* (1986), however, have detected no Vg mRNA in coelomocytes by Northern blot analysis. Cervello *et al.* (1994) have reported that colorless spherule cells, one of the subpopulations of coelomocytes, contain Vg and discharge it under stress conditions. In our present study, the expression of Vg mRNA was detected in the coelomocytes by RT-PCR, although it was not detectable by Northern blot analysis. We consider the amount of Vg produced in coelomocytes to be very low compared to

Fig. 2. Nucleotide and deduced amino acid sequences of *P. depressus* Vg cDNA. Numbers on the right margin indicate nucleotide positions from the first nucleotide. Amino acid numbers from the first methionine are indicated on the left margin. The termination signal of translation is indicated by an asterisk. The underlined region of the deduced amino acid sequence has about 25% homology to the vertebrate transferrin family. Double underline represents the result of amino acid sequence analysis. Arrows represent primers F1' and R3', which were used for the synthesis of the DIG-labeled DNA probe and for the analysis of tissue expression by RT-PCR. The underlined nucleotide sequences represent the position of DIG-labeled oligonucleotide probes used for *in situ* hybridization.

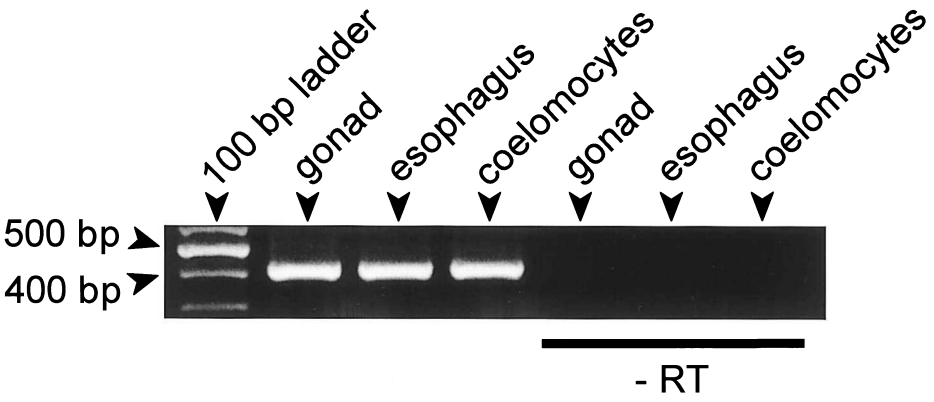


Fig. 4. RT-PCR analysis of Vg mRNA expression. Total RNA extracted from the esophagus, coelomocytes, and gonad was reverse transcribed and amplified by PCR using primer F1' and R3'. The products were not derived from the genomic DNA contamination since no product was detected when reverse transcriptase was excluded from the RT-PCR protocol (-RT).

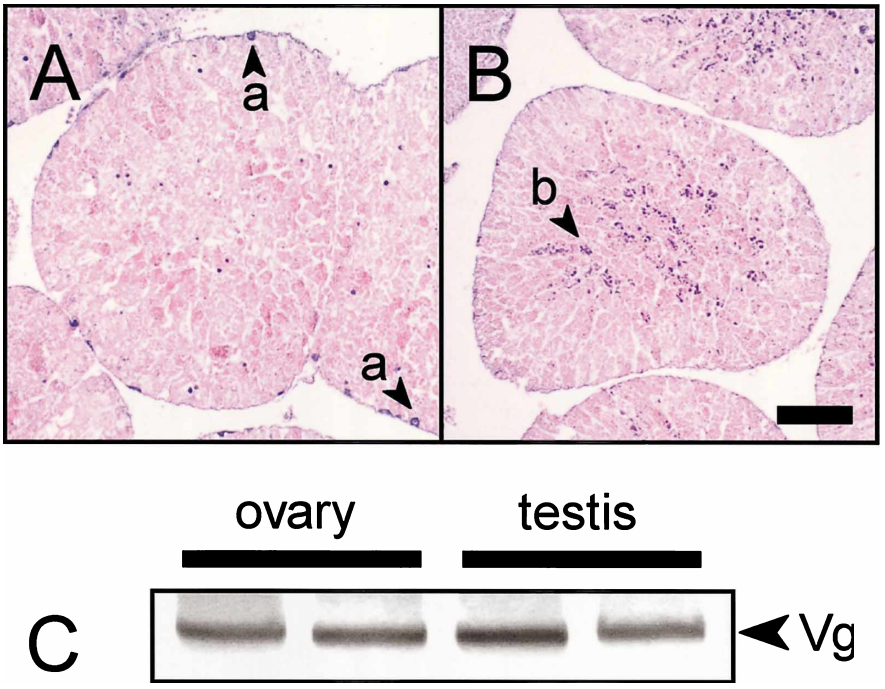


Fig. 5. Histological features of the immature ovary and testis, and their Vg mRNA expression by Northern blot analysis. (A) The immature ovary stained with haematoxylin and eosin. Gonadal lumina are filled with nutritive phagocytes. Small oocytes (a) are observed at the periphery of the acini. (B) The immature testis stained with haematoxylin and eosin. Gonadal lumina are filled with nutritive phagocytes, as in the ovary. Male germ cells are not observed. Nutritive phagocytes contain dark-blue amorphous speckles (b), derived from phagocytized residual spermatozoa. The bar represents 100 μm. (C) Total RNA (5 μg/lane) extracted from the immature gonads was subjected to Northern blot analysis as described in Fig. 3. Two individuals of each sex were analysed.

that in digestive tracts and gonads, at least under normal conditions. Cervello *et al.* (1994) have proposed that sea urchin Vg may be involved in the clotting phenomenon that occurs after host invasion, basing this hypothesis on the finding that a protein called toposome, which is derived from Vg and contained in the embryo, functions as a cell-adhesive molecule during embryogenesis (Noll *et al.*, 1985; Cervello and Matranga, 1989; Matranga *et al.*, 1991). Investigation of Vg mRNA expression in colorless spherule cells under stress conditions or after host invasion is needed to further elucidate this matter.

As described above, the accumulation mechanism of MYP in the gonad is complicated. Vg is produced at least in nutritive phagocytes of gonads, digestive tracts and coelomocytes. We assume that Vg produced in nutritive phagocytes is processed to MYP and accumulated there, and that Vg produced in the digestive tracts and coelomocytes is secreted into the coelomic fluid. To evaluate how the digestive tracts and coelomocytes contribute to MYP accumulation in the gonad, clarification of the roles of Vg in the coelomic fluid and estimation of the quantity of Vg taken up from the coelomic fluid into the gonad are needed. This study suggested that nutritive

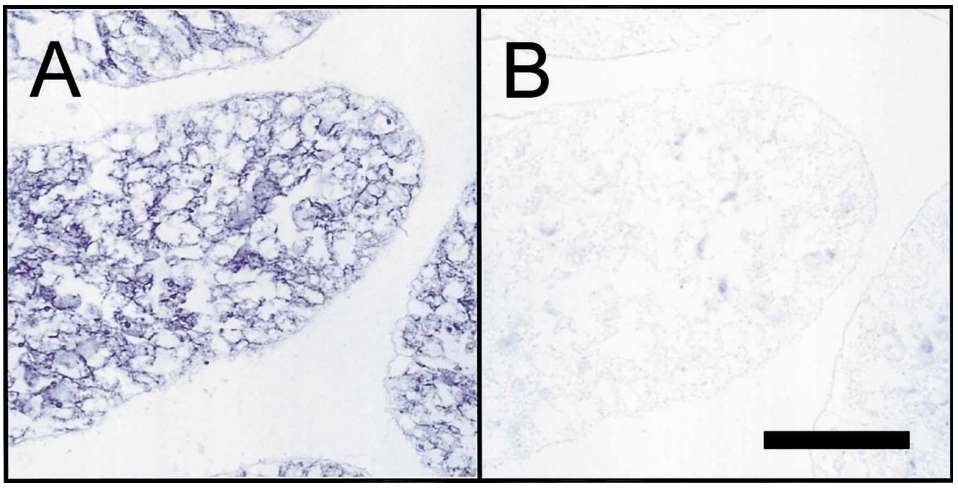


Fig. 6. *In situ* hybridization of Vg mRNA. (A) Antisense oligonucleotide probes were hybridized with a section of the immature ovary; nutritive phagocytes expressed Vg mRNA. (C) Sense oligonucleotide probes were hybridized with a section. No significant signal is observed. The bar represents 100 μm.

phagocytes make a greater contribution to MYP accumulation before gametogenesis than digestive tracts or coelomocytes do.

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